In the name of God, the almighty

A Study of Neuronal Precursors Using Retrovirus-Mediated Gene Transfer

by

Mohammad Hajihosseini

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Laboratory of Developmental Neurobiology,

National Institute for Medical Research,

The Ridgeway, Mill Hill,

London.

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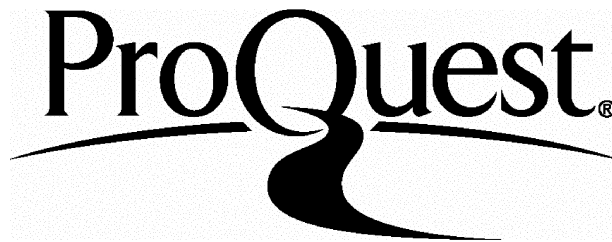
Abstract

This study concerns an investigation of factors that may influence the behaviour and development of neuronal precursors derived from embryonic rat cerebral cortex. In this study, single retrovirally-labelled E16 or E14 cortical precursors were cultured amongst unlabelled cells, on monolayers of cortical astrocytes in the presence or absence of basic fibroblast growth factor (bFGF). Several important observation were made when the fate of such cells was analysed after seven days in culture. Most virally-labelled E16 and E14 cortical cells were found to produce clones that were composed of only one of the cell types found in the adult brain; namely neurones, oligodendrocytes, or astrocytes, showing that cortical precursor cells are specified in the phenotypic fate at the time of their isolation from the embryonic cortex. bFGF did not override this specification. However, bFGF was found to act as a survival factor and possibly a mitogen exclusively for neuronal precursor derived from either of the embryonic ages; significantly more and larger neuronal clones were found in the presence of this factor. To test whether bFGF's effects were mediated by cortical astrocytes, on which the embryonic cells were routinely grown, cultures were grown on substrates other than astrocytes in the presence or absence of bFGF. It was discovered that survival of cultures grown in the absence of cortical astrocytes was poor and that bFGF could enhance the survival of neuronal precursors in such cultures, thus arguing for a direct effect by bFGF. However, despite the poor survival of cultures, absence of cortical astrocytes resulted in an increase in the size of neuronal clones even when bFGF was absent. This observation suggested that cortical astrocytes inhibit the proliferation of cortical neuronal precursors.

In a second line of pursuit, this study investigated whether retroviral DNA can integrate into post-replication DNA of its host. This was addressed by analysing the distribution of viral genes amongst the progeny of single retrovirally-labelled NIH-3T3 cells. This analysis showed that retroviruses integrate almost exclusively into post-replication DNA of host cells as only half the progeny of single infected NIH-3T3 cells were found to inherit the viral genes, a finding that could be used to explain the high frequency of single-cell neuronal clones generated by retrovirally-labelled neural precursors. Results of this study suggested that single-cell neuronal clones arise when only one daughter of an asymmetrically-dividing neuronal precursor inherits the viral genes.

Dedication

To my dearest parents

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Abbreviations

AER	Apical ectodermal ridge
AGF	Astroglial growth factor
ATP	Azidothymidine
bFGF	basic fibroblast growth factor
BDNF	Brain derived neurotrophic factor
bp	base pair
CAM	Cell adhesion molecule
CFSE	5-carboxyfluorescein diacetate, succinidimyl ester
cfu	colony forming units (measure of viral titre)
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
Coumarine	7- amino-4 methylcoumarin - 3 acetic acid
DAB	Diaminobenzedene
DMEM	Dulbecco's modified Eagle medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
E	Embryonic day (e.g. E16)
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FCS	Foetal calf serum
GABA	Gamma-amino-butyric acid
GAP-43	Growth associated protein-43
GFAP	Glial fibrillary acidic protein
gp	glycoprotein
HEPES	N-[2-Hydroxyethyl]poperazine-N'-[2-ethanesulfonic acid]
HIV	Human imunodeficiency virus
HRP	Horse radish peroxidase
HSPG	Heparan sulphate proteoglycans
HSV-1	Herpes Simplex Virus type-1
IGF-I	Insulin-like growth factor-I
kDa	Kilo Daltons
KGF	Keratinocyte growth factor
LIF	Leukemia inhibitory factor
LRD	Lysinated rhodamine dextrans
LTR	Long term repeat
MAP-2	Microtubule associated protein-2

MAP-5	Microtubule associated protein-5
min	Minutes
MLV	Murine leukemia virus
MoMLV	Moloney Murine leukemia virus
NCAM	Neural cell adhesion molecule
NCS	New born calf serum
NGF	Nerve growth factor
NIH	National Institutes of Health
NLS	Nuclear localisation sequence
NP-40	Nonident 40
NR	Neural retina
NT-3/4	Neurotrophic factors 3 and 4
PBS	Phosphate buffered saline
Pbs	Primer binding site
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDL	Poly-D-lysine
pI	Isoelectric point
PLC	Phospholipase C
PNMT	Phenylethanolamine-N-methyl transferase
PNS	Peripheral nervous system
R	Receptor
RNA	Ribonucleic acid
rpm	Rounds per minute
RSV	Rous Sarcoma Virus
sec	Seconds
SV-40	Simian virus-40
SVZ	Subventricular zone
TGF	Thyroid growth factor
TPA	12-O-tetradecanoyl phorbol-13-acetate
VSV-G	Vesicular somatitis virus-G
VZ	Ventricular zone
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
X-phos	5-Bromo-4-cholo-3-indolyl phosphate
ZPA	Zone of polarising activity

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Chapter I

General Introduction

1.1 Introduction

The mature mammalian central nervous system (CNS) contains a vast array of morphologically and functionally distinct cell types that fall into two broad categories; neurones and glial cells. Glial cells, which can be divided further into macroglia (astrocytes and oligodendrocytes) and microglial cells, have a supportive or homeostatic role in the mature CNS. Astrocytes maintain the integrity of the blood-brain barrier and regulate the level of ions and metabolites within the neuropil. Oligodendrocytes, by contrast, enhance axonal conduction by ensheathing neuronal processes in layers of myelin, thereby creating the so-called white matter of the CNS. In contrast to these roles, groups of neurones set up complex networks and produce physiologically distinct centres within the CNS. CNS neurones can be grouped into two major types; the projection neurones and inter-neurones, a subdivision that is based on morphology and function. Projection neurones of the cerebral cortex, for example, have a pyramidal appearance and use the excitatory neurotransmitter glutamate. Cortical interneurones, on the other hand, are non-pyramidal and utilise gamma aminobutyric acid (GABA) and contain a range of other neuropeptides, such as somatostatin, neuropeptide Y and cholecystokinin (see a comprehensive review by DeFelipe, 1993).

Neurones and glial cells can also be distinguished by virtue of their characteristic morphology and expression of cell type-specific proteins. Antibodies raised against components of such proteins can be used to identify each of these cell types in vivo and in vitro. Astrocytes express a characteristic intermediate filament protein, glial fibrillary acidic protein, GFAP (Bignami et al., 1972), while mature oligodendrocytes possess myelin associated proteins and lipids, such as the lipids recognised by monoclonal antibody O4 (Sommer and Schachner, 1981). Similarly, mature neurones can be recognised by a range of cytoskeletal proteins, such as the neurone-specific isoforms of tubulin (Geisert and Frankfurter, 1989), or the neurofilament proteins.

Despite this diversity, all CNS macroglia and neurones are derived from the neuroepithelial (or 'Ventricular', Boulder Committee, 1970) cells of the neural tube (Jacobson, 1978), an ectoderm-derived cylinderical structure which also serves as an early scaffold for the developing CNS. In a transverse section of this tube, ventricular cells appear as an homogeneous population of radially arranged bipolar cells.

An outstanding question in neurobiology is, therefore, how does an apparently uniform population of ventricular cells within the neural tube, give rise to the broad spectrum of morphologically, serologically and functionally distinct cell types found in the adult CNS? This complex question encompasses a series of other questions. The most immediate of these would relate to ventricular cells themselves: are ventricular cells of the neural tube as homogeneous in developmental potential as they are in appearance; i.e. does every ventricular cell have the potential of generating all CNS cell types (neurones and macroglial cells) throughout the entire period of CNS development, or do ventricular cells give rise to

subpopulations of developmentally-restricted precursor cells capable of generating only one cell type, or a particular combination of cell types? If so, when does this restriction take place? Furthermore, if different cell types are generated by multipotential ventricular cells, what factors govern the phenotypic choice of such cells? Alternatively, if different cell types are generated from restricted precursors, what factors regulate the rate and mode of such generation?

The aim of this chapter is to review the approaches used in addressing these questions. Initially, this review examines the early hypotheses about generation of cellular diversity in CNS, how such hypotheses were based on the appearance of ventricular cells and how cell lineage studies reshaped these hypotheses. I will also discuss the techniques used in studying cell lineage, in particular, retrovirus-mediated gene transfer. I then review factors that may influence the development of neural precursors and systems in which such factors have been characterised. In this section, the emphasis will be on fibroblast growth factors, for which no clear role in the development of the central nervous system has been described.

1.2 Appearance of ventricular cells within the neural tube and the ventricular zone

As already noted, the walls of the neural tube are made up of a single layer of bipolar, ventricular cells: cells that have one process in contact with the outer (pial), and the other with the luminal (ventricular) surface of the tube (figure.1.1). Initially, the entire thickness of neural tube wall corresponds to the length of a bipolar ventricular cell. During subsequent CNS development, however, additional layers are formed within the thickening neural tube and ventricular cells become confined to a layer lining the ventricles, the so-called ventricular zone (VZ) (figure. 1.1). Therefore, ventricular cells also called ventricular zone cells.

Whether at the neural tube stage or within the VZ, the nuclei of ventricular cells undergo a wave of intracytoplasmic migrations; during S phase, the DNA synthesising phase of the cell cycle, they are found close to the pial side, while during mitosis, they reside close to the luminal surface (Sauer, 1935). Although all ventricular cells are homogeneous in appearance, at any one moment, different ventricular cells are at different stages of the cell cycle (Sauer, 1935). As a result of this phenomenon and the interkinetic nuclear movements, the walls of the neural tube and the VZ appear falsely as a stratified structure.

1.2.1. Cell cycle times and pattern of VZ cell division

Kauffman (1968) and Hoshino et al. (1973) have shown that on embryonic day 10 (E10) in mouse, a day after neural tube's closure, VZ cells have a cell cycle time of approximately 7-8 hours (Hoshino et al., 1973; Kauffman, 1968). It is thought that VZ cells achieve this short cycle time by undergoing a rapid transition between S and M phases of the cell cycle. However, with progressive CNS development, there is marked increase in this cell cycle

Figure 1.1 Development of the neural tube and appearance of ventricular zone and ventricular zone cells. The neural tube forms by infolding of the neural plate. Closure of the neural tube closure, which occurs around E9 to E10 in mouse (approximately two days later in rat) results in the formation of a central canal that gives rise to the ventricular space. Neural tube cells adjacent to this space are thus called ventricular cells. Initially, the walls of the neural tube are only one cell thick with processes of ventricular cells contacting both the ventricular and the presumptive pial surface. With subsequent development, ventricular cells become confined to a layer lining the ventricles, thereby also being called ventricular zone cells. In the opposite figure, development of different cortical layers is also depicted inorder to outline the spatial relationship of cortical VZ.

Neurones of the mammalian cerebral cortex are generated from mitotic precursors found only in the VZ. Post-mitotic neurones leave the VZ by using the processes of a special class of glial cells, called radial glia (RG), and migrate to their relevant cortical lamina within the developing cortical plate (CP). A peculiar aspect of cortical development is the 'inside-out' formation of its laminae; older neurones populate laminae closest to the VZ, while younger neurones migrate past the previously formed lamina to populate areas more distal from the VZ.

Also shown in this figure, is a population of neural cells located at the crests of the infolding neural plate, the so called neural crest (NC) cells. During development, these cells migrate from their dorsal neural tube position to populate diverse areas of the embryo, giving rise to cells of the preipheral nervous system as well as a variety of mesenchymal cells.
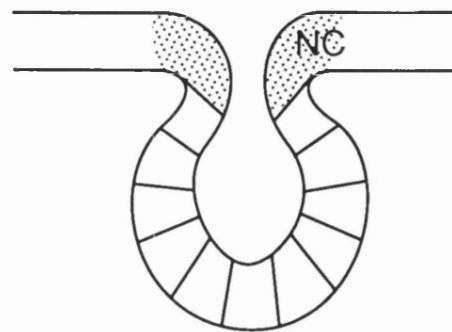
Developing Neural Tube

Neural Tube

VZ
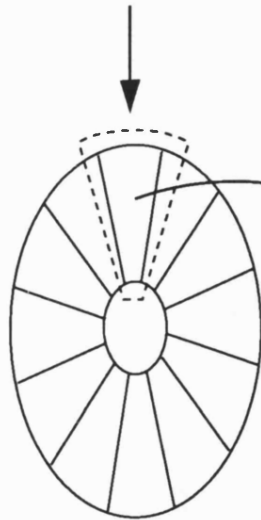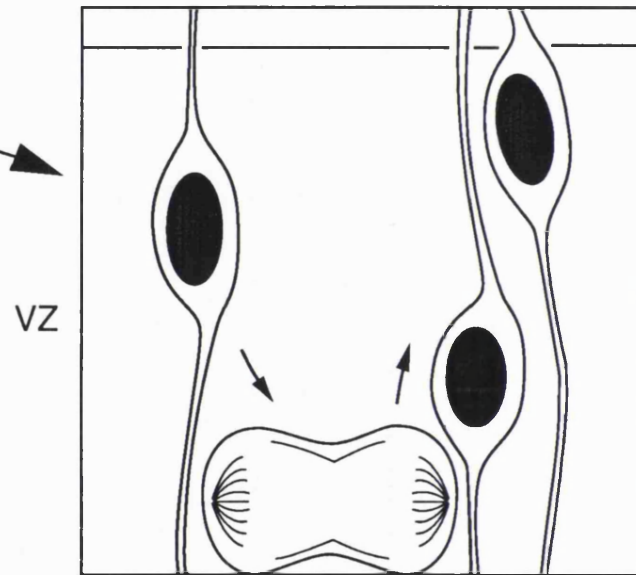
Ventricle

Developing Cerebral Wall

MZ

CP

IZ

RG

RG

VZ

Nc
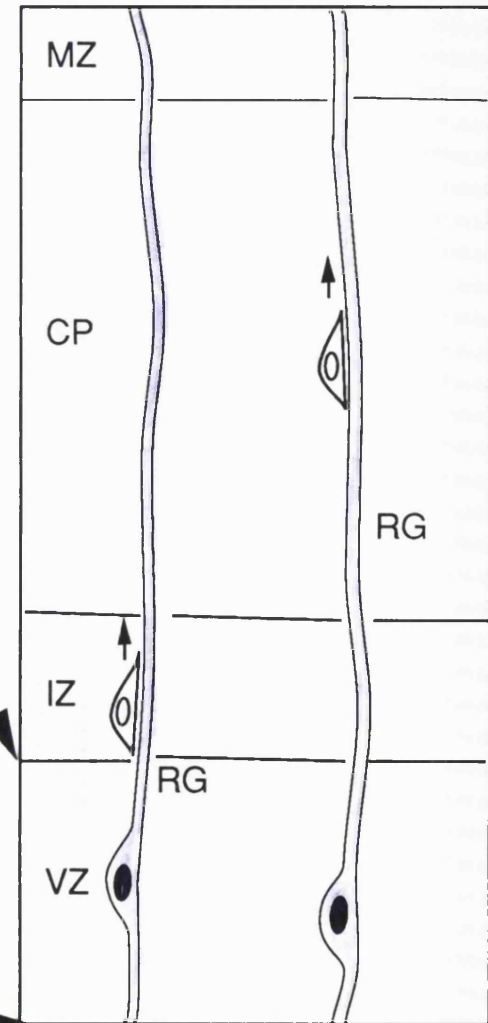
time. In the rat, for example, cortical VZ cells cycle every 13 hours on E12, 13-17 hours on E16, and 19 hours on E19 (Waechter and Jaensch, 1972), an increase also seen in the developing mouse cortex (Takahashi et al., 1993). Waechter and Jaensch (1972) and Kauffman (1968) have shown that this increase is mainly due to the lengthening of G1 phase of the cell cycle. Further to these findings, two populations of ventricular cells with different cell cycle times have been discovered in the VZ of developing rat cerebral cortex (Waechter and Jaensch, 1972; Altman and Bayer, 1990; Acklin and van der Kooy, 1993). The significance of this observation, however, remains to be determined.

Several workers have observed that the plane of cleavage of dividing ventricular cells has one of two orientations; in some cells, the plane is parallel to the ventricular surface, while in others it is perpendicular to this axis (Smart, 1973). Accordingly, some authors have suggested that the different plane of cleavages may have a bearing on the fate of daughters of a mitotic VZ cell; following a parallel cleavage, both daughters may migrate toward pial surface and re-enter S phase, while after a perpendicular cleavage, one daughter may become post-mitotic (a neurone for example) and leave the VZ (Martin, 1967; Smart, 1973; Tuckett and Morriss-Kay, 1985). Attractive as it may seem, however, this model has not been proven. There is evidence, for example, that some VZ cells do not divide at the ventricular surface at all. Smart (1973) reported that because of what he termed 'ependymal choke', in which the ventricular surface is packed with mitotic cells, some cells may be forced to divide at other sites within the VZ. Moreover, using timelapse videomicroscopy, Fishell et al. (1993) have shown that while both daughters of a mitotic VZ cell may remain within the VZ, they disperse randomly over considerable distances, each following an independent trajectory. In this study, however, the plane of mitotic cleavage of VZ cells was not analysed.

Also intriguing is that some VZ cells are linked together by desmosomal junctions (Zonlua adherens, Hirst et al., 1991; Shoukimas and Hinds, 1978), while others communicate through gap junctions (Dermietzel et al., 1989; Hinds and Ruffett, 1971; Keane et al., 1988). Lo Turco and Kriegstein, (1991), and Mienville et al. (1994) have discovered that with increasing developmental age, there is a gradual but prominent decrease in gap junctional communication between neighbouring rat telencephalic VZ cells. In other systems, such as the developing Xenopus embryo, such structures play an important part in the adhesion and synchronous development of neighbouring cells (Slack, 1991a). The role of such structures in the development of VZ cells, however, is not known.

1.3 Early hypotheses about generation of different cell types by Ventricular cells

The earliest hypothesis about how VZ cells generate different cell types was put forward by His (1889). His proposed that the ventricular zone, which he called the germinal matrix zone, contains two different cell populations. He based this idea on the observation that

1.2.1.2 Rat cortical histogenesis during embryonic days 14 and 16

The rat cerebral cortex develops as a laminated structure from the most rostral part of the neural tube. At E14, the cortex is composed mainly of two cellular layers; a layer lining the ventricular space and hence called the ventricular zone, and a layer, some 50 µm thick, situated at the presumptive pial surface, called the primordial plexiform layer (PPL) or the preplate (Bayer and Altman, 1991). These two layers differ markedly, however, in their cellular composition; while the VZ is packed with proliferating cells, the PPL contains only a few, differentiated, neurone-like cells. Cobas et al. (1994) report that a number of such PPL cells express the neurotransmitter GABA.

At E16, the embryonic cortex develops several more layers. Post-mitotic neurones migrate out of the VZ and form a layer known as the cortical plate (CP). CP bisects the PPL into two distinct layers: a superficial layer formed at the presumptive pial surface, called the marginal zone, and a layer juxtaposing the VZ, called the subplate. The marginal zone eventually develops into cortical layer I and contains a few neurones known as Cajal-Retzius cells. The subplate, by contrast, contains neurones that are thought to play an important role in the development of efferent and afferent cortical pathways (McConnell et al., 1994).

With further development, the cortical plate develops five more layers, layers VI-II. These layers develop in an inside-out manner such that layer VI is formed first with neurones belonging to layer V migrating past layer VI neurones to form layer V. Inturn, neurones of layer IV migrate past layer VI and V neurones to form layer IV and so on. Noteworthy, however, is that although E16 is thought to represent the peak of cortical neurogenesis, only cortical layer VI has begun to form at this embryonic age. Moreover, there appears to be latero-medial and caudo-rostral gradient in the development of the cerebral cortex, such that the lateral and dorsal tiers of E14 cerebral wall, for example, resemble E16 cortex.

nuclei of ventricular zone cells have one of two appearances; mitotic and non-mitotic nuclei. His called the mitotic cells, germinal cells, and the non-mitotic ones, spongioblasts, and assumed that these are separate populations giving rise to neurones and glial cells, respectively.

Reviewing this hypothesis, Schaper (1897) suggested that His's different cell populations were infact the same population but at different stages of the cell cycle. Schaper extended this suggestion to conclude that VZ cells are otherwise a uniform population of undifferentiated cells that remain undecided whether to generate neurones or glial cells, until they have left the ventricular zone. Schaper's view was held until the advent of cell-birth dating techniques, such as tritiated-thymidine labelling, in 1960's. Thymidine labelling studies revealed some important aspects of early cyto- and histogenic events within the developing CNS, thereby generating some new theories about the generation of different cell types within CNS. I shall begin by considering the ^{3}H-autoradiographic experiments.

1.3.1.Thymidine labelling as means of analysing the period of cytogenesis
1.3.1.1.Technique and interpretation of findings

Thymidine-labelling is a method of ascertaining whether cells were proliferating during a defined period of development. Cells are exposed for a brief period, typically one or two hours, to radioactively-labelled DNA precursors such as tritiated (^{3}H)-thymidine, or its non-radioactive analogue, bromo-dexoy uridine (Brdu). ^{3}H-thymidine labelled cells can be detected by the appearance of silver grains over their nuclei in histological sections processed for autoradiography. Brdu label, on the other hand, can be detected in the nuclei of labelled cells using antibodies to this compound. Any cell in S phase during the labelling period would incorporate the labelled DNA precursor, and, with subsequent divisions would pass this label to its progeny. Finding that cells are heavily labelled suggests that they must have stopped dividing soon after becoming labelled, because if they had continued to divide, the label would have become diluted amongst their progeny. Thus ^{3}H-thymidine labelling can show when cells become post-mitotic. Unfortunately, this type of birthdating can give no information on cell types that continue to divide. It has, therefore, been of more importance in birthdating neurones than in determining when neuroglia first appear.

1.3.1.2. Thymidine labelling: earliest conclusions about generation of cell types by the ventricular zone cells

Some of the early thymidine-labelling results were sometimes over-interpreted. For example, Fujita (1963), who managed to label almost all cells of six-day old chick VZ, during a ten hour pulse with thymidine, assumed that all such cells are functionally homogeneous. Also, because glial cells were found to appear later than neurones, early

autoradiographic data were interpreted as neurogenesis preceding gliogenesis. In 1966, Fujita went as far as suggesting that proliferating VZ cells first produce non-mitotic neuroblasts and only when neurogenesis ceases, do they switch to producing glial cells. Although wrong in his interpretation, Fujita's hypothesis underlined the fact that CNS neurones and glial cells have distinct periods of generation and differentiation during the development of the CNS. These are discussed below.

1.3.1.2.1. Period of neurogenesis in the mammalian CNS

A few authors have provided estimates of when neurogenesis in the mammalian CNS begins.

Schultze et al. (1974), for example, have used a series of calculations based on the number of neurones found in the mature rat ruber nucleus, as well as the cell cycle time of precursors generating those cells, to estimate that the proliferation of the first neuronal precursors in this nucleus begins on embryonic day 9 (E9). This is surprising, because, E9 represents the day on which the neural plate has just formed in these embryos (Hagemann and Schmidt, 1960).

At the other end of the spectrum, neurogenesis in most CNS regions of most mammals is thought to end around birth. For example, all neurones of the rat cerebral cortex are generated between E14 and birth (E21-22, Raedler and Raedler, 1978; Berry and Rogers, 1965). Exceptions to this observation have, however, been found. These include; post-natal generation of supragranular neurones in the cerebellar cortex of rodents; slow but continuous generation of neurones in adult olfactory bulbs (Altman 1966 and 1969; Hinds and McNelly, 1977); the dentate gyrus of the hippocampal formation (Altman and Das, 1965; Kaplan and Hinds, 1977) and the cerebellum. These exceptions aside, however, some authors have claimed that a limited number of neurones are generated throughout the adult CNS of rats and mice. Altman (1962), for example, could detect thymidine-positive neurones when he injected adult rat brains, although his identification of the thymidine-positive cells as neurones has been strongly challenged by Korr (1980).

Recently, Reynolds and Weiss (1992) showed that neurones can be produced de novo from dissociated striatal cells of 18 month-old mice, in vitro. This de novo production, however, depended on the presence of epidermal growth factor (EGF); it is not known what role EGF played in this production. Did it act as a survival factor for cells that were capable of producing neurones, in vitro, or did it induce the de novo production itself? Reynolds and Weiss (1992) interpreted their results as evidence for the presence of quiescent precursors capable of generating neurones, although, factors that may maintain this state of quiescence were not identified.

1.3.1.2.2. Period of gliogenesis in the mammalian CNS

In theory, gliogenesis in the mammalian CNS never ends; although the bulk of CNS glia are generated soon after birth, a small number of glial cells are produced throughout adulthood in the mammalian CNS. It has been shown that some 15,000 to 24,000 glial cells are produced every day in the adult mouse brain (Smart and Leblond, 1961), possibly from cells that have a 50 day cell cycle time (Notezel, 1962). As Korr (1980) points out, however, this generation may be part of a slow glial cell turnover, i.e. to compensate the brain for the loss each day of an equal number of glial cells.

The source of adult CNS glia is not clear, however. One possible source could be the differentiated glial cells themselves, which in contrast to neurones, have the ability to divide. This ability is not shared by all adult glia. There is no conclusive evidence, for example, that myelinating oligodendrocytes can divide (Sturrock, 1982). Astrocytes, by contrast, undergo rapid proliferation at the site of injury to the CNS and most gliomas of mammalian CNS, including man, are astrocytic in origin. Another source for adult glia, on the other hand, could be a transient population of undifferentiated cells (Dubois-Dalcq, 1987), similar to a precursor isolated from adult brains that is capable of generating oligodendrocytes and type-2 astrocytes in vitro (O-2A[adult] progenitor, ffrench-Constant and Raff, 1986a; Wolswijik and Noble, 1989; Wren et al., 1992).

In contrast to neurogenesis, it is not known when glial cells are first generated. Thymidine-labelling studies could not provide a clear answer because embryonically-labelled cells could not be conclusively identified as glial cells until early post-natal life (Mares and Buckner, 1978), by which time rapid proliferation of glial cells or their precursors had resulted in the dilution of thymidine label to a degree that could not be detected at light microscope level. Therefore, several workers decided to identify such cells using electronmicroscopy. Sturrock (1978), for example, could find a small number of thymidine-positive glial cells in the CNS tissue of 22-day-old rats that had been injected as early as E11. Similarly, Berry and Rogers (1966) and Schultze (1974), found thymidine-positive glial cells in cerebral cortex and corpus callosum of rats that had been labelled on E18.

More direct evidence for presence of glia during early stages of CNS development, however, was provided by Levitt et al. (1981and 1983), who found GFAP-positive mitotic cells (Eng et al., 1971; Bignami, et al., 1972) in the VZ of the developing monkey cerebrum. These authors have discovered that the frequency of such GFAP-positive cells increases with increasing embryonic age in the developing monkey occipital cortex; 47% of all mitotic VZ cells examined at E61 were GFAP-positive, a proportion that rose to 80% on E80. Levitt, et al. (1981 and 1983) regarded these GFAP-positive mitotic figures as precursors of astrocytes, and concluded that glial precursors co-exist alongside neuronal precursors at the peak of cortical neurogenesis (E80) in primates. This conclusion may have been supported by Waechter and Jaenisch 's (1972) discovery of two population of

VZ cells of different cell cycle times. However, the validity of regarding mitotic GFAP-positive cells of no clear morphology as astrocyte precursors is questionable, because, GFAP is also known to be expressed by some immature oligodendrocytes (Choi and Kim, 1984; Ogawa, et al., 1985). It is also interesting to note, however, that GFAP positive cells have not been found in the VZ of rat cortex during the peak of neurogenesis (E16); the first GFAP-positive cells detected in cell suspensions of rat cortex have been found on E18 (D. Maric and J. Barker, unpublished observations), a few days before cortical neurogenesis is over.

Nonetheless, detection of glial cells or their precursors during the period of neurogenesis was significant in one respect; it refuted the hypothesis that neurogenesis precedes gliogenesis. Two important question, however, remained unresolved. First, what may be the role of glial cells that are present during neurogenesis? This question was partially answered by Rakic (1972). Using Golgi impregnation and electron microscopy, Rakic (1972) found that during their departure from the ventricular zone, cortical neurones become closely apposed to a distinct population of bipolar glial cells, the processes of which spanned the entire radial width of the developing monkey occipital cortex. Rakic proposed that post-mitotic neurones use the processes of such cells, whose nuclei were normally located within the VZ or subventricular zone (SVZ), to migrate to their relevant cortical lamina (figure. 1.1). Accordingly, he called these cells, radial glia, cells that are known to eventually differentiate into GFAP-positive astrocytes (Schmechel and Rakic, 1979; Voight, 1989; Gray and Sanes, 1992). Rakic's observation, therefore, was the first demonstration of a role for glia in the development of neurones.

The second question, however, was more fundamental: what was the lineage relationship between glial and neuronal precursors? This question was addressed by cell lineage studies.

However, before presenting the results of such studies, it is worth considering some general concepts in the study of cell lineage, terminology used when interpreting its results, as well as techniques with which such studies have been carried out.

1.4 Study of cell lineage
1.4.1. concepts and terminologies

In a literal sense, a cell's lineage is its genealogical relationship to its ancestors traced back to the fertilised egg. The lineage of a cell, however, cannot be studied in a retrograde manner, rather, by labelling its ancestors at different stages of development, a cell lineage study can ask: what other cell types can a particular ancestor of this cell generate, and, at what point in development is a particular ancestor of this cell fated to generate cells of one phenotype only; i.e. when does the lineage of one cell type diverge from that of others? Two extreme models can be considered: divergence could occur at the last mitosis of a precursor, in which case the precursors remains multipotential up to its final division, or it

could occur several cell cycles prior to this final mitosis, which would suggest an early restriction in the developmental potential of the precursor in question. It is worth noting, however, that cell lineage studies follow the fate of cells in their normal undisturbed environment; they only, therefore, analyse the presumptive fate of a cell, not its developmental potential (see below).

Returning to the question of divergence of neuronal and glial lineages within the VZ, one could address this question by following the fate of a VZ cell labelled at a particular stage of neural development and ask whether it generated both neurones and glia, or just a single cell type. If the labelled cell is found to give rise to both neurones and glia, then its fate was not specified at the time of labelling i.e. the neuronal and glial lineages had not diverged at that particular phase of development. It may, however, be possible that the fate of this cell was specified, but that subsequently the phenotype of some of its progeny were altered by environmental factors.

If the progeny of the labelled cell are all found to be neurones, however, this is an indication, but not proof, that at the time it was labelled, the VZ cell was specified to producing only neurones.

For two reasons, the VZ cell that generated neurones only cannot be regarded as 'committed' (Stent, 1985) to production of neurones. First, this cell may have had the potential of generating glia as well as neurones, but that in its natural environment it was restricted to generating neurones only. Moreover, it may have generated glial progeny that died in this environment. To some extent this can be resolved by performing a similar lineage analysis with the tissue transplanted to an ectopic site, or by growing the cells in culture, or by ablating the cell's normal neighbours in vivo. If, in these new environments, the VZ cell in question continues to generate only neurones, then this a strong indication, but still not proof, that it was committed to generating neurones. In theory, it may never be possible to prove that a cell is committed; unidentified factors or conditions may exist that could alter the developmental potential of this cell.

The second reason for not calling the VZ cell of the last paragraph committed in its fate, is that its progeny, though uniform in cell type, may be heterogeneous in other respects; the VZ cell in question may generate neurones of different neurotransmitter phenotypes. As far as the latter phenotye is concerned, therefore, the VZ cell was not even specified, let alone committed to this fate. When analysing the results of a cell lineage study, therefore, the fate in question must be clearly defined.

1.4.2. Using cell lineage studies to address other developmental questions

Apart from analysing the phenotypic potential of a precursor cell, cell lineage studies can be used to investigate other developmental questions, two in particular. In the first of these, it may be possible to resolve whether a precursor cell divides arithmetically, also known as asymmetrically or geometrically (symmetrically). If, for example, a labelled VZ

cell generates 10 cells in a matter of 48 hours, then this suggests that it or some of its progeny have undergone geometrical division, because the shortest known cell cycle time of a VZ cell is approximately 10-11 hours. It is, however, important to draw a distinction between the terms arithmetic/ geometric and asymmetric/ symmetric. While the former pair have a mathematical meaning, the latter are usually used in a biological sense; an asymmetrically dividing precursor cell would generate a dividing stem cell, with a developmental potential identical to its parent, and another daughter cell that would be restricted in its developmental potential, which may or may not continue to divide. A symmetrically dividing precursor, by contrast, would always generate two daughters of equal developmental potential, regardless of whether one or both of them continue to divide.

In the second of these, cell lineage studies could be used to investigate the migration pathway of clonally-related cells (Halliday and Cepko, 1992; Gray and Sanes, 1991; Austin and Cepko, 1990 Levison and Goldman, 1993; Luskin, 1993), or their possible restriction to a particular anatomical/ physiological compartment (Fraser et al., 1990; Grove et al., 1992). Luskin (1993), for example, has demonstrated that a population of neuronal progenitors located in the anterior post-natal rat subventricular zone, migrate along a discrete pathway to reach the olfactory bulbs where they differentiate into granule and peri-glomerular interneurones. Fraser et al. (1990), on the other hand have demonstrated that prior to the formation of the rhombomeres in the chick hindbrain, the progeny of single labelled cells in the hindbrain would contribute to several rhombomeres; if labelled after the formation of such structures, however, this progeny would be restricted to the rhombomere in which the single cell was labelled. This observation has raised the possibility that some form of barrier may prevent cells of neighbouring rhombomeres from mixing. Also, Grove et al. (1992) have shown that clonally-related neurones contribute to more than one functional region of the rat hippocampal formation.

1.4.3. Possible ways of studying cell lineage/ cell fate

Although numerous strategies have evolved for following the fate of cells, in vivo or in vitro (Reviewed by Rossant, 1987, and Price, 1989), only a few have been found to be feasible for studying cell lineage in the developing vertebrate nervous system. These strategies are discussed below.

1.4.3.1. Direct strategies
1.4.3.1.1. Visualisation of cell development under normaski optics

Perhaps the most direct method of cell fate analysis is the continuous observation of embryonic development under normaski optics. This method has been used to describe the entire cleavage pattern of the fertilised egg of the nematode, *Caenorhabditis elegans*. It has been shown that all *C. elegans* embryos undergo an identical pattern of cleavage and

cytogenesis to generate 810 somatic cells, 302 of which are neurones (see review of Sternberg et al., 1992). Although direct, the nomarski optic method of cell fate analysis is limited to transparent embryos, such as that of *C. elegans* or the Zebrafish that develop in a non-uterine environment.

1.4.3.1.2 Analysis of the fate of isolated cells

Another direct approach would be to follow the fate of single cells in vitro, either by growing individual cells in separate wells of a multiwell culture dish, or by seeding cells at clonal densities on large tissue culture plates. Temple (1989), for example, has used the first approach to study the fate of single embryonic (E13-14) rat septal cells. She identified three kinds of progenitors: those that generated either neurones or astrocytes, and a third that generated both cell types. Wren et al. (1990), on the other hand, have used timelapse cinematography to follow the fate of oligodendrocyte-type 2 astrocyte (O-2A) progenitors plated at clonal densities. They have shown that perinatal O-2A cells give rise to a second population of O-2A progenitors of different morphology and cell cycle kinetics; these were called adult O-2A progenitors (^{adult}O-2A).

As noted in section 1.4.1 , however, these forms of fate analysis in vitro are not a lineage analysis *per se*, but a test of developmental potential of cells under a particular condition. In Temple's study, for example, survival of isolated single cells was enhanced by the presence of conditioned-medium from embryonic striatal cells that lined the walls of each microwell. Also, analysis of cell fate at clonal densities is limited by the degree of cellular migration in vitro. Wren et al. (1990), for example, were unable to analyse some of the progenies of the perinatal O-2A cells simply because they had migrated out of the cinematographic recording field. Nonetheless, as also discussed in section 1.4.1, fate analysis in vitro can be crucial in interpreting the results of lineage studies in vivo.

1.4.3.2. Indirect strategies
1.4.3.2.1. Use of chimeric animals

Inter-species chimeras have been used in classic embryological studies in order to investigate the contribution of a cell or groups of cells to a particular embryonic tissue/ structure (for a review see LeDouarin, 1988). As an example, Goldowitz (1989) generated chimeric mice by implanting fused 8-cell-stage embryos of two different species of mice, Mus Caroli and Mus Musculus, into the uterus of pseudopregnant females and allowed the chimeric animals to develop to term. Using species-specific DNA sequences as probes, he was subsequently able to calculate the percentage contribution of cells of either genotype to any particular organ, or a particular region of CNS, in the chimeric offspring. An analysis of hippocampal cells, for example, showed that while the cells of the superficial hippocampal layers were M.Caroli derived those of the deeper granule layers were mostly M.Mus. in origin. Accordingly, Goldowitz (1989) suggested that in the hippocampus "

lineage is coincidental with cytoarchitechtonic areas". Grove et al. (1993), however, have demonstrated that clonally-related cells can contribute to more than one hippocampal cytoarchitectonic area.

While not denying or ignoring its contributions to our knowledge of embryology, chimeric technology is, for several reasons, unsuitable for cell lineage studies. First, embryo fusions are limited to very early (pre-implantation) stage of development. Fusion at these early stages, when even the three germ layers have not formed, would result in widespread distribution of cells of a particular species or genotype throughout the chimeric animal, making cell lineage analysis at single cell level almost impossible. Second, finding that a particular organ is made up predominantly of cells of a particular genotype could be the result of differential adhesion, survival, or asynchronous development of cells of that genotype at the fusion stage. Third and finally, though practical, the detection of genetic markers such as species-specific DNA probes (Goldowitz, 1989) or the differential appearance of heterochromatin in chick-quail chimeras, is time consuming.

1.4.3.2.2. Use of lineage labels

It is possible to follow the fate of a cell or groups of cells after they have been labelled with distinct lineage labels. This technique has several advantages; fate can be analysed at single cell level, both within the cell's natural environment or when grown in bulk cultures, in vitro. The last of these would not only remove the stringent needs of single cell culturing, but also would allow one to study the interactions of the labelled cell with its environment (neighbouring cells, or response to growth factors). Such an approach has been used in the work presented in this thesis inorder to analyse the fate of embryonic cortical cells in vitro.

However, according to Rossant (1987), every lineage label should have at least three characteristics: it should be heritable; ubiquitous in distribution i.e. every cell type should be capable of carrying or expressing it; and finally, it should be easily detectable. A fourth characteristic can be added to this list; administration or presence of a lineage label should not affect the normal development, or the developmental potential of the labelled cell, even though this would be difficult to ascertain. In this respect, two main labels have been used in cell lineage studies of vertebrate nervous system: vital dyes and genetic labels.

1.4.3.2.3. Use of vital dyes

In this technique, single cells are injected under pressure or by electrophoresis with vital dyes such as the enzyme Horse radish peroxidase (HRP, Weisblat, 1978), or conjugated fluorescene dextrans (e.g. lysinated rhodamine dextran (LRD), Gimlich and Braun, 1985). Following intracellular injection, these dyes become distributed throughout the cytoplasm, and due to their large molecular size, remain confined to the labelled cell. Moreover, with each division, they are inherited by the progeny of the labelled cell.

One advantage of this method of fate analysis is that because the position of the labelled cell is known, the stereotactic as well as the genelogical relationship of the labelled cell and its progeny can be determined. The prime disadvantage, however, is that as the embryo grows, the dye would become diluted to undetectable levels. This seems not to be a problem in amphibian embryos which do not grow appreciably in size. There are also other disadvantages; injection of vital dyes under electrophoresis is expensive and requires experties. Moreover, dyes can only be injected into large cells or cells of large embryos. It has thus been possible to inject single retinal cells in the mouse and Xenopus (Holt et al. 1988; Turner et al. 1990), as well as cells of chick, quail and Xenopus neural tube (Fraser, et al., 1990; Bronner-Fraser and Fraser, 1988 and 1989; Hartenstein 1989; Soula et al., 1993).

1.4.3.2.4. Use of genetic labels
1.4.3.2.4.1. DNA microinjection

The prime advantage of a genetic label is that it would be inherited in an undiluted form by the progeny of the labelled cell. This, in theory, allows one to analyse the fate of labelled cells over an infinite number of divisions.

In early attempts at genetic labelling, short fragments of DNA of known sequence or those encoding a hormonal-gene, were microinjected (Lo 1983) into preimplantation embryonic cells (Gordon and Ruddle, 1981; Lo 1983). At some point after injection, such DNA fragments would have integrated into the chromosomal DNA of the labelled cell and would thus have been inherited by its progeny. The labelled progeny could then be detected by their expression of the transgene whose product could be detected either by antibodies or by in situ hybridisation. Generating a transgenic in this way, however, poses two major problems. First, the physical injection poses the same problems as dye injection (see above), making such a technique limited to cells that are large and readily accessible. Second, the number of DNA copies injected can not be controlled; high levels could be toxic to the injected cell, or integration of a high copy number may perturb normal host gene function.

1.4.3.2.4.2. Retrovirus mediated gene transfer

Although similar in principle, gene transfer using retroviruses has circumvented many of the problems posed by DNA microinjection. In this technique, cells are infected with non-pathogenic replication-defective retroviruses that carry a specific marker gene (see section 1.8.6 for strategies for making such viruses). The technique centres around the fact that retroviruses are natural carriers of genetic material. Upon binding and entry into a cell by receptor-mediated endocytosis, they reverse transcribe their RNA genome into linear DNA which becomes integrated into the host cell chromosomal DNA (described in more detail in section 1.8.5). If part of the viral RNA were to encode a transgene, then the infected cell

and its progeny would become permanent carriers of such a transgene. Furthermore, since each retroviral particle generates only a single DNA copy of the transgene, an infected cell always carries a single copy of the provirus, so long as the multiplicity of infection is low.

Several retroviruses carrying foreign marker genes have been constructed. The BAG virus (Price et al., 1987), for example, carries *lac-Z*, a bacterial gene coding for enzyme β-galactosidase. Cells infected with this virus incorporate this gene and produce the enzyme β-galactosidase, the presence of which has no obvious deleterious effect on mammalian cells.

1.4.3.2.4.2.1. Methods of detecting retroviral transgenes or their product

Cells labelled with a retrovirus can be detected in at least two ways. The first and more complicated approach is in situ detection of the viral DNA. Just as in DNA microinjection technique, the entire sequence of the viral genome and of the transgene would be known, hence, viral DNA fragments can be detected and amplified by polymerase chain reaction (PCR), once cellular DNA has been digested with the relevant restriction enzymes. Furthermore, since viral DNA integrates into random sites within the host cellular DNA, two separate infections can be resolved by comparing the length of the DNA fragments generated when cellular DNA is subjected to enzymatic digestion.

The second approach is by detecting the product of a particular transgene. The enzyme β-galactosidase, expressed by BAG-labelled cells, for example, can be detected either by antibodies raised against this protein or by incubation of labelled cells with X-gal, a chromogenic substrate for this enzyme. In either case, the presence of the enzyme is a true reflection of the presence of the transgene as shown by work presented in chapter III of this thesis.

1.4.3.2.4.2.2. Disadvantages of the retrovirus method

Like other methods of lineage tracing, retroviral-mediated gene transfer technique has some disadvantages. These are:

(i) Since virus is injected into the vicinity of cells to be labelled, the exact location and identity of cells that become infected is not known;

(ii) The viral genes may not be expressed in some infected cells. So far this phenomenon has been noted only for infected early pre-implantation embryonic cells (Savatier et al., 1990);

(iii) Because clonally-related cells are identified by their clustering, lineage analysis by retroviral labelling is limited to cell populations that do not migrate or disperse over large distances within the embryo; and finally,

(iv) Only dividing cells can be labelled with retroviruses, since viral integration takes place during or after a cell's mitosis (Roe et al., 1993; Hajihosseini et al., 1993).

These drawbacks aside, this technique has proved invaluable in studying cell lineage in structures inaccessible to conventional dye injection methods. The results of such lineage studies in the nervous system, using both retroviral and dye labelling techniques, are reviewed below.

1.4.4. Studies of cell lineage in the vertebrate nervous system
1.4.4.1. Neural crest cell lineage studies

During the formation of the neural tube, the lateral folds of the neural plate give rise to a specialised population of migratory neuroepithelial cells, called neural crest cells (figure 1.1), such that with the closure of the neural tube, neural crest cells come to lie on its dorsal aspect. In some vertebrates, such as mouse, the entire population of neural crest cells are pinched off the lateral walls of the folding neural plate, forming a separate colony of cells outside the neural tube. In avian embryos, such as quail, however, cells exhibiting identical developmental potentials to those of neural crest cells can also been found in the dorsal roof of the closed neural tube; these cells, like those outside the neural tube, migrate along distinct pathways to populate several peripheral sites within the embryo (Bronner-Fraser and Fraser, 1988 and 1989). Despite their morphological similarity to NE cell of the neural tube, neural crest cells exhibit a much broader developmental potential, giving rise not only to most neurones and glial cells of the peripheral nervous system (PNS), but also to a variety of peripheral connective tissue and glandular cells, such as the skin melanocytes and chromaffin cells of the adrenal medulla (LeDouarin, 1982).

Because neural crest cell and their derivatives show a wide degree of dispersion within the developing vertebrate embryos, it has proved difficult to study their lineage with retroviral vectors. Neural crest cell lineage has, therefore, been investigated mainly by chimeric transplantations (LeDouarin, 1975 and 1978) or more recently by in situ injection of vital dyes into single neural crest progenitor cells (Bronner-Fraser and Fraser, 1988 and 1989; Fraser and Bronner-Fraser, 1991). Moreover, due to the small size and inaccessible nature of mammalian neural tube, studies of neural crest cell lineage in vivo, have been restricted to relatively larger avian embryos. Although these studies have produced substantial evidence that neural crest derivatives arise from multipotential precursors, almost all studies of neural crest cell lineage have also found subpopulations of developmentally-restricted precursors that could generate only one or distinct combinations of neural crest derivatives (e.g. Artinger and Bronner-Fraser, 1992; Anderson and Axel, 1986). For example, Fraser and Bronner-Fraser (1991) report that on some occasions the progeny of single neural crest cells, labelled as they migrated out of the neural tube, could give rise to sympathetic and sensory neurones as well as Schwann cells, while on other occasions could generate only one of such cell types, i.e in these cases, clonally-related cells were found to be restricted to either sympathetic ganglia or DRG.

Clonal studies of neural crest cells in vitro have also provided evidence for existence of both multipotential and developmentally-restricted neural crest precursor cells (Cohen and Konigsberg, 1975; Baroffio, et al. 1988 and 1991; Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989). For example, Sieber-Blum (1989) has shown that some neural crest precursors can generate clones comprising adrenergic and DRG-like neurones together with pigmented cells. Cohen and Konigsberg (1975), by contrast, have discovered clones containing only pigmented cells. These observations have led several authors (e.g. Anderson, 1989; Bronner-Fraser, 1992) to draw an analogy between generation of different cell types by neural crest cells to that of the haematopoetic system, in which multipotential precursors gradually give rise to restricted intermediate precursors, some of which behave like stem cells (see a later section). In this respect, Stemple and Anderson (1992) have been able to isolate and expand a neural crest stem cell-like precursor from E10.5 rat embryos. Stemple and Anderson (1992) report that these self-renewing cells can generate neurones as well as Schwann cells in vitro. However, unlike the haematopoetic system, in which stem cells persist into adulthood, no such counterparts has been found in neural crest derived structures.

1.4.4.2. Lineage analysis in the rodent and amphibian retina

The neural retina is a laminated structure that contains a variety of specialised neurones and glial cells, not found in other regions of the CNS. At least seven different retinal cell types are known. These are: retinal photoreceptors (rods and cones) of the outer nuclear layer; amacrine, bipolar and horizontal cells, as well as Muller's glia, found in the inner nuclear layer; and retinal ganglion cells of the ganglion cell layer. In rodents, the retinal ganglion cells, horizontal cells and cone photoreceptors are generated before birth, while bipolar cells, amacrine cells, Muller cells and rod photoreceptors are generated at or after birth (Hinds and Hinds, 1978 and 1979; Sidman, 1961).

Turner and Cepko (1987) and Turner et al. (1990) have studied the lineage relationships of these different retinal cell types in mouse. These authors report that at all stages of retinal development, the majority of retrovirally-labelled retinal precursors give rise to clones that contain several different cell types. For example, neonatally-labelled precursors (Turner, et al., 1990) were found to generate small clones of rod photoreceptor cells in almost any combination with Muller, amacrine and bipolar cells. Clones generated by labelled embryonic (E13-14) cells, on the other hand, were found to contain every possible combination of all retinal cell types, although no clones, whether embryonic or neonatal, contained retinal astrocytes, consistent with reports that they are derived from a different lineage (Watanabe and Raff, 1988). These observations suggested that throughout the development of the retina, retinal cell types are generated from multipotential precursors. Furthermore, these precursors are thought to remain multipotential up to their final round

of division; some small (e.g. two-cell) neonatal clones, interpreted as the last round of division by such precursors, still had a mixed phenotype.

Similar conclusions were derived from cell lineage studies of the amphibian (Xenopus) retina. Holt et al. (1988) and Wetts and Fraser (1988) have shown that single retinal cells injected with HRP or LRD at stage 23-27, produce a variety of different clone types, each of which is mixed in its phenotypic composition.

The idea that there is no degree of developmental restriction on behalf of retinal precursors throughout development has, however, been challenged by Williams and Goldowitz (1992). By analysing a series of computer simulated data about type and composition of retinal clones, these authors report that the combinations of cell types in retinal clones reported by lineage studies are less diverse than would be predicted by chance. Williams and Goldowitz (1992), therefore, use this comparison to suggest that with progressive development, retinal precursors become increasingly restricted in the diversity of cell types they can generate. There is also some direct evidence for early specification of some retinal precursors. Guillemot and Cepko (1992), for example, report that between 10-20% of mitotic cultured embryonic chick retinal precursors express neuronal markers several cell cycles before their terminal round of division.

1.4.4.3. Chick optic tectum and spinal cord

Several workers have studied the fate of retrovirally-labelled VZ cells in the chick optic tectum (Gray et al., 1988; Galileo, et al., 1990; Gray and Sanes, 1991, and, Gray and Sanes, 1992), a structure that receives retinal inputs and which is laminated in a way remarkably similar to that of mammalian cerebral cortex. The results of these studies have highlighted two important aspect of tectal development: (a) the pattern of migration by clonally-related cells, and (b) an indication of types of precursors present in the developing tectal VZ. In the first of these, it has been shown that the progeny of single VZ cells labelled on the third embryonic day (stage 17 of chick development, Hamburger and Hamilton, 1951) show four distinct patterns of migration (figure. 1.2). Initially (at stage 25, ~40 hours after labelling) the progeny of such labelled cells arrange themselves in a tight radial cluster within the VZ. With the development of the tectal intermediate zone some 2 days later (at stage 30), however, some of the progeny migrate tangentially and differentiate into multipolar efferent neurones. This is surprising, because during this stage of tangential migration, radial migration by some of the progeny is ongoing. With the continuation of radial migration, a proportion of the progeny come to populate the developing tectal plate where they differentiate into either neurones or astrocytes. The fourth and final migratory pattern takes place at stage 35, when some progeny of the labelled cell migrate again in a tangential plane within the most superficial layer of the tectum, the optic stratum (figure. 1.2) ; Gray and Sanes (1991) show that some of these cells differentiate into astrocytes.

Figure 1.2 Development and migration patterns of clonally-related cells in the chick optic tectum (modified from Gray and Sanes, 1991). Gray and Sanes (1991) have shown that the progeny of a single tectal cell, labelled with a retrovirus on E3, initially become arranged in a radial column within the VZ, some 40 hours after labelling (day 5). With the formation of tecto-bulbar axons in the IZ of E7 tectum, however, some of the progeny migrate tangentially along these axons; these cells differentiate into multipolar tectal efferents. Concurrent with the development of the tectal plate at stage 35, some of the progeny of the labelled cell continue their radial migration along the tectal radial glial cells into the TP where they give rise to either neurones or astrocytes. A second wave of tangential migration occurs on E9 a stage at which the first retinal axons innervate the chick optic tectum (shown as striped lines alone the most superficial layer of the tectum, the stratum opticum, SO); cells that migrate tangentially during this period differentiate mostly into tectal astrocytes. These observations suggest that migratory pathway of a cell may play an important part in the determination of its phenotype. Noteworthy also is that in a separate study, Gray and Sanes (1992) provide evidence that most tectal radial glial cells themselves differentiate into astrocytes. Abbreviations: IZ, Intermediate zone; MZ, marginal zone; TP, Tectal plate; SO, stratum opticum; VZ, ventricular zone.

Second Wave of
Tangential
Migration

Radial
Glia

First Wave of
Tangential
Migration

Radial
Displacement

(SO)

SO

TP

MZ

IZ

TP

IZ

VZ

MZ
IZ

VZ

VZ

VZ
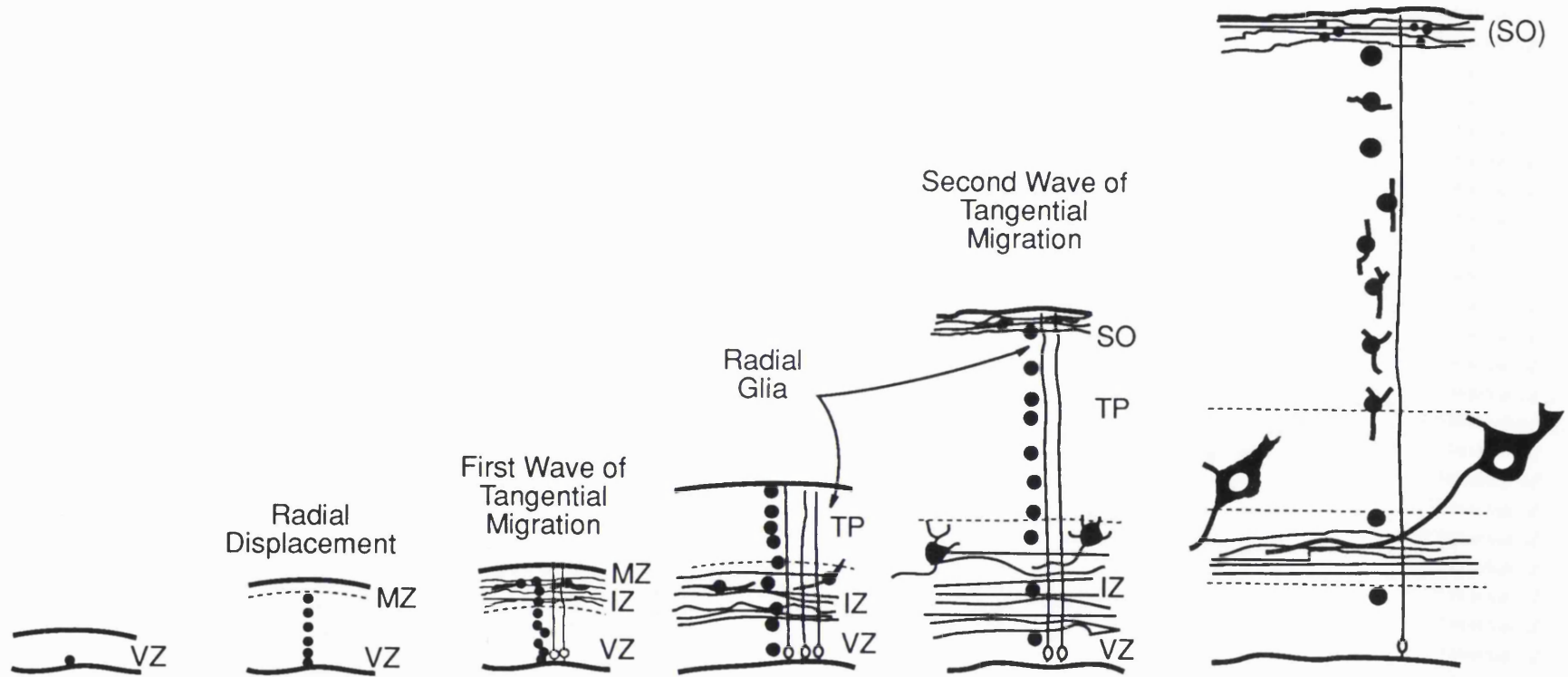
Embryonic Day	3	5	7	8	9	18
Stage	17	25	30	32	35	44

The second important result concerns the phenotypic composition of clones derived from E3-labelled tectal cells. In this respect, Galileo et al. (1990) report that four types of clones are generated by such labelled cells: clones containing only neurones (23% of all clones) or glial cells (3%), clones composed of neurones and glia (39%) and finally clones which contain neurones, glia and cells that could not be identified (23%). One important observation was that frequently radial glial cells and neurones could be found within the same clone, typically only one radial glial cell per such clone. It is concluded, therefore, that in contrast to retina, the developing tectum contains both bipotential precursors and precursors that appear restricted in their developmental potential.

A similar conclusion has been reached by cell lineage studies of chick spinal cord. Leber et al. (1990), for example, who labelled chick neural tube cells between stages 11 to 18, report that while some clones were mixed in their phenotype, containing neurones together with a variety of glial cells, others were composed purely of neurones. In this study, Leber et al. (1990) also show that the developmental death of spinal motoneurones is independent of their lineage.

1.4.4.3. Rat striatum

Halliday and Cepko (1992), have studied cell lineage in the developing rat striatum by analysing the progeny of striatal precursor cells some 2 to 5 days after retroviral labelling at E15. At all stages of analysis, the authors find that some clones contain radial glial cells together with cells that resemble neurones, suggesting that at E15, the presumptive rat striatum may also contain bipotential precursor cells. This is not certain, however, because in Halliday and Cepko's study, which has focused mainly on the degree of migration by clonally-related cells, different cell types were identified solely by their early morphological appearance.

1.4.4.5. Rodent cerebral cortex

Because of its inaccessibility to insitu single cell dye injections, study of cell lineage in cerebral cortex has only been possible with retroviral labelling; in every such study, a low number of embryonic cortical cells (VZ cells) lining the cerebral walls have been labelled by injecting retroviruses into the ventricular space. The progeny of such cells have then been analysed at times ranging from days to months after injection. Initial studies of cortical cell lineage determined that virally-labelled cells falling within a 500 µm radius of each other in transverse sections of cortex, were derived from a single infected precursor cell, and thus were part of the same clone. Using light-microscopic morphological criteria, these studies also showed that the majority of such clones contain only a single cell type (mouse cortex: Luskin et al., 1988; rat cortex: Price and Thurlow, 1988; Walsh and Cepko, 1988). For example, Price and Thurlow (1988), who labelled cortical precursors on E16, could find clones containing only neurones or grey matter astrocytes, although a

few (10% of all clones) clones of neurones and non-neuronal cells were also observed; in these latter clones, neurones were found to be in radial alignment with non-neuronal cells situated in the white matter.

Subsequent to these studies, however, several workers questioned the accuracy of identifying different cell types using light-microscopic morphology only (Grove et al., 1993; Luskin et al., 1993, and Parnavelas et al., 1991). These authors used electronmicroscopic criteria or immunolabelling with defined cell type-specific antibodies inorder to identify cells within each clone, only to confirm the earlier findings. Parnavelas et al. (1991), for example, found that clones derived from virally-labelled E15/16 cortical VZ cells, are composed of single cell type i.e. clones contained only neurones, oligodendrocytes or astrocytes. Previous findings were, however, extended by Parnavelas et al. (1991), who showed that most cortical neuronal clones were either of pyramidal or non-pyramidal neurones type, an observation confirmed by an ultrastructural lineage analysis of the mouse cortex by Luskin et al. (1993) and Mione et al. (1994). These observations suggested that as early as E15 in both mouse and rat, cortical VZ contains separate developmentally-restricted precursors for neuronal and glial cell. Using a combination of morphology and immunolabelling with anti-GFAP antibody, Grove et al. (1993) were able to provide evidence that even the macroglial subtypes (astrocytes of the grey and white matter, and oligodendrocytes) arise from separate precursors.

These findings clearly contrasted with lineage studies of most other CNS regions in which most precursors were shown to be either multipotential (retina) or at least bi-potential (chick tectum, spinal cord, rat striatum). Walsh and Cepko (1992) have suggested that this marked discrepancy may be due to methodology. These authors suggest that because of widespread dispersion of neurones throughout the developing cerebral cortex and the fact that cortical clones are defined by spatial parameters, some heterogeneous clones, e.g. of neuronal and glial cells, or clones of different neuronal subtypes (Parnavelas, et al., 1992) may actually appear homogeneous, i.e. as clones containing only a single cell type. Although there is substantial evidence for migration of neural precursors within the VZ (Fishell et al., 1993) and of *some* neurones in the cerebral cortex (Walsh and Cepko, 1988; Austin and Cepko, 1990; Walsh and Cepko, 1992), cortical cell lineage studies in vitro (Luskin et al.., 1988; Williams et al.., 1991, and results presented in chapters II and IV of this work) show that cortical precursors of comparable age, continue to generate phenotypically homogeneous clones in vitro. These in vitro observations, therefore, discount the methodological explanation and support the notion that most cortical precursors are indeed restricted in their developmental potential.

1.4.4.6. Conclusions from cell lineage studies of vertebrate central nervous system

Collectively, the above cell lineage studies suggest that there are regional differences in temporal divergence of lineages; neuronal and glial lineages diverge early in development

for such regions as the cerebral cortex, or as late as final mitosis, in the case of retina, with some structures such as the spinal cord showing an intermediate status between these two extremes. It should be noted, however, that these observations are generalisations; a small number of cortical precursors are found to be bipotential, giving rise to neurones and oligodendrocytes (Price et al., 1987; Williams et al., 1991), or neurones and astrocytes (this study), and, a small but considerable number of retinal clones are found to contain only one cell type (Turner et al., 1987). Also, both bipotential and developmentally-restricted precursors have been found as early as the neural plate stage in some amphibian embryos (Soula et al., 1993).

1.4.4.7. Possible explanations for regional differences in temporal divergence of lineages

The regional differences in temporal divergence of lineages may somehow be related to the evolution of different regions, or the rostrocaudal position of a particular structure within the CNS. It is of interest, for example, that cerebral cortex is the most evolutionary advanced region of the brain, found only in higher vertebrates. Also, for example, cranial neural crest cells are more restricted in their developmental potential than those of the trunk (Bronner-Fraser, 1992). There could, however, be another explanation; it may be that the VZ itself is regionalised early in development, with cells in one region behaving differently from those of their neighbouring regions. In this respect, Fishell et al. (1993) have found that neural precursors of the cerebral cortical anlage do not migrate into adjacent presumptive striatum. Similarly, after a particular stage in chick development, clonally related cells fail to transgress from one rhombomere to another rhombomere within the chick hind brain (Fraser et al., 1990). There is also increasing evidence for early regionalisation of VZ within the neo-cortex itself. Keller et al. (1989) and Barbe and Levitt, (1991) report that cortical and subcortical cells fated to become part of the adult Limbic system express a cell-surface protein, Limbic system associated protein (LAMP), that is not found on any non-limbic precursors. Arimatsu et al. (1992) also find that neurones of the lateral but not dorso-medial embryonic rat neocortex, express a 29 kDa protein in vivo and in vitro. Finally, Dehay et al. (1993) have discovered that neuronal precursors of the VZ fated to become the striate (part of the visual) cortex, have a higher proliferative rate than other non-striate areas of cortex, thus explaining why area 17 of the visual cortex has twice as many neurones than the adjacent area 18 (Rockel et al., 1980).

In any event, regardless of how late or early lineages diverge, the central question posed by all cell lineage studies is: what factors govern the divergence of lineages themselves? This is a question of factors that govern cell fate determination.

1.5 Factors governing cell fate determination

A polarised model of factors that determine the fate of a cell would divide these into cell-intrinsic or genetic factors, and environmental or epigenetic factors. In the former, fate

may be determined by products of specific gene/s in a precursor cell, while in the latter, interaction of a cell with its environment may determine its fate. However, it is often difficult to draw such a distinction; gene expression, under the control of transcription factors, can be influenced by extracellular factors (see an excellent review by Lemke, 1992) and extracellular factors may affect a cell's fate by inducing distinct patterns of gene expression. Nonetheless, for the sake of identifying cell fate determining factors, they may, at a given stage of development, be divided into genetic and epigenetic factors. If, for example, two daughters of a precursor cell adopt different phenotypes because of an asymmetrical inheritance of a particular transcription factor, then in this case fate has been determined by a cell-intrinsic or genetic factor. Two daughters of a precursor could, however, adopt different phenotypes through their exposure to different extracellular matrix molecules or different concentration of a particular growth factor, in which case, environmental or epigenetic factors have been critical in determining cell fate. Noteworthy is that in this example, stereotactic position of the two daughters has played an important role in their fate and therefore positioning of a cell itself can be considered as an epigenetic factor.

With the above points in mind, some example of genetic and epigenetic factors are reviewed below.

1.5.1 Genetic factors

Most of our knowledge of genes that play critical roles in cell-fate determination, comes from mutational studies; in these studies absence or alteration of a particular phenotype is correlated with induced mutation of a particular gene. This type of analysis has, until recently, been limited to invertebrate species whose nervous systems are much simpler than that of vertebrates. Two invertebrate species in particular have been studied extensively; these are the fruit fly Drosophila melanogaster, and the nematode worm, C. elegans. For example, it has been shown that generation of neurones and neuronal diversity in the Drosophila CNS is controlled by a distinct and sequential pattern of gene expression (see review of Doe, 1992). Several 'proneural genes' have been shown to commit groups of cells within the ventral epidermis, which are fated to become epidermal cells, to the neuronal lineage and thus production of neuroblasts. Example of these genes include, achaete, scute, lethal of scute and daughterless, all of which encode transcription factors, basic helix-loop helix proteins (b-HLH). Also daughters of a Drosophila neuronal precursor acquire separate identities as a consequence of inheriting a distinct subset of gene products from by their parent. Moreover, dorso-ventral patterning of the Drosophila nervous system is also under genetic control; homeobox (Hox) genes such as prospero give Drosophila neurones a segmental identity.

Fortunately, many of these cell-fate determining genes have been conserved throughout the evolution and only recently has the function of their homologues in the development of

the vertebrate nervous system been discovered. The role of homeobox genes (called homeotic in vertebrates) in the formation of rhombomeres in the chick hind brain, and segmentation of mouse forebrain are only two such examples (see Puelles and Rubenstein, 1993; and a recent review by Keynes and Krumlauf, 1994).

As for involvement of distinct genes in lineage determination in the mammalian nervous system, Duncan et al. (1992) have found that *Id*, a homologue of Drosophila gene *extramacrochaetae* , is expressed specifically in proliferating neural precursors. Bernard et al. (1992), on other hand, have suggested that members of proto-oncogene family *myc*, which also have an HLH domain, may channel multipotential neural precursors into either neuronal or glial lineages. Bernard et al. (1992) show that while at E10-12, VZ cells of the mouse telencephalon and mesencephalon express c-*myc*, L-*myc* and N-*myc*, at E13-14, L-*myc* and N-*myc* expression become restricted to subpopulations of VZ cells. In this respect, Bernard et al. (1992) have been able to induce differentiation of immortalised neural cells into either neurones or glial (GFAP-positive) cells by inducing expression of L-*myc* or N-*myc* in these cells, respectively. Wakamatsu et al. (1993), on the other hand, show that transition of N-Myc protein from the nucleus to the cytoplasm of neural cells in vivo is closely associated with differentiation along the *neuronal* pathway. On the surface, the findings of Bernard et al. (1992) and Wakamatsu et al. (1993) seem to be contradictory. However, these two studies could be reconciled if rather than cytoplasmic N-Myc protein having a direct role in neuronal differentiation *per se*, its absence from the nucleus could signal differentiation of neural cells along the glial lineage. Although this hypothesis is speculative, it would be consistent with the role of *myc*s as nuclear DNA-binding proteins.

1.5.2. Epigenetic factors

There are a variety of ways by which the extracellular environment could influence the fate of precursor cells. Most of these, however, comprise direct or indirect cell-cell interactions. Examples of direct cellular interactions include, gap junctional communication and adhesion of neighbouring cells by cell adhesion molecules (CAMs), such as Neural-CAM and N-Cadherin (For a review of their structure and function see Anderson, 1990; Doherty and Walsh, 1992). Fate determining molecules may be passed from one cell to another through gap junctions. Alternatively, physiological coupling through gap junctions may ensure that a group of cells undergo synchronous development. Lo Turco and Kriegestein (1991), for example, have shown that during early phases of cortical development, cortical neuroblasts are physiologically coupled in groups of 15 to 19 cells, and that a decrease in uncoupling is coincidental with the onset of neuroblast migration out of the VZ.

Beside their mere anchoring of different cells, CAMs could also play an important part in fate determination, since (like integrins, see below) their cytoplasmic tail is associated with

cytoskeletal proteins, some of which are coupled to and can activate second messengers signalling molecules (see review of Gumbiner, 1993).

In contrast to direct cell-cell interaction, indirect interactions would be mediated by extracellular matrix (ECM) molecules and growth factors produced either by neighbours of the precursor cell in question or pre-existing cells. The response of the such factors would be mediated by the relevant functional receptors expressed on the cell surface of the precursor cell. Such receptors would be coupled to second messenger or other 'effector' molecules that would ultimately affect gene expression within the precursor cell. Termination of this response at some later time point may occur by extinction of the extracellular factor, or, by down-regulation, conformational change or uncoupling of the receptor from the internal effectors.

1.5.2.1. Extracellular matrix molecules (ECMs)

ECM molecules, such as glycoproteins; thrombospondin, entactin, laminin, fibronectin and tenascin/ J1, and, chondritin- or heparan sulphate proteoglycans, have been detected within developing neural tissues (Hunter et al., 1992; Sheppard et al. 1991), and are known to influence various aspects of CNS and PNS development (reviewed by Sanes, 1989, and Reichardt and Tomasselli, 1989). Examples of such roles include: promotion of neurite outgrowth, axonal guidance and synapse formation, cell migration, survival, proliferation and differentiation. In this respect it is known that the effects of glycoproteins: laminin, fibronectin and tenascin, are mediated by integrin receptors (reviewed by Hynes, 1992). The diversity of function of such molecules may be explained by the fact that integrin receptors can exist as different non-covalent dimers of α and β subunits, linked via their intracellular carboxy tail to a variety of cellular signalling or cytoskeletal molecules (see reviews by Burridge, et al., 1988; Hynes 1990, and, Juliano and Haskill, 1993).

Beside binding to cell-surface receptors, some ECM molecules may have other crucial roles. They could bind and concentrate growth factors whose action may depend on a particular concentration or prolonged presence, or, they could protect growth factors from proteolytic degradation. For example, such a relationship exists between bFGF and heparin/ heparan sulphate proteoglycans (Gospodarowicz and Cheng, 1986; Saksela et al., 1988, see also section below).

ECM and substrate molecules could also be involved directly in phenotypic choice of a precursor cell. Stemple and Anderson (1992) have isolated a neural crest-derived multipotential precursor cell capable of generating Schwann cells, neurones and unidentified undifferentiated cells, in vitro. These authors have discovered that when grown in the presence of fibronectin alone, this precursor fails to generate neurones, while additional presence of poly-D-lysine can circumvent this restriction.

1.5.2.2. Growth factors

Before discussing examples of involvement of growth factors in the phenotypic choice of neural precursors, it is worth considering some general concepts about growth factors.

By definition, a 'growth' factor should only affect two aspects of cellular function: increase in cell size, and increase in cell number. In this respect, growth factors are known to have a role in normal and abnormal cellular growth. For example, constant attrition of cells from intestinal walls and skin, requires a constant but controlled rate of cellular growth; epidermal growth factor (EGF) and fibroblast growth factor (FGF), that are known to be mitogens for such cells in vitro, may play a role in this controlled growth. As for abnormal growth of cells, several tumour cells are known to express high levels of growth factors. For example, glial cell growth factor, which is now thought to be a from of FGF, is produced by astrocytomas in vitro (Powell and Klagsburn, 1993). Moreover, some growth factors are products of oncogenes themselves; int-2, a close relative of bFGF, is one such example (Dickson et al., 1984). What is not clear, however, is whether abnormal levels of growth factor expression is the cause or the consequence of deranged cellular growth.

In any event, it is now known that action of molecules we now call growth factors is not limited to cellular growth. Outside the nervous system, generation and normal function of diverse cell types in the haematopoetic system provides an excellent example of this concept. In this system, specialised cell types arise from pluripotential stem cells via subpopulations of precursor that are more restricted in potential than their parent cells (Metcalf, 1993). Factors must, therefore, exist that (a) control the divergence of lineages, e.g. the lymphoid from the myeloid lineage (b) maintain precursor cells in a stem cell mode of division so as to ensure a constant supply of all cell types and (c) control the size of any particular population within blood, and, (d) regulate the function of distinct cell types. As far as the last two points are concerned, it has been shown, for example, that granulocyte-macrophage colony stimulating factor (GM-CSF) is a mitogen specific for bipotential granulocyte-macrophages progenitors, and granulocytes-colony stimulating factor (G-CSF), a close relative of leukaemia inhibitory factor (LIF), interleukine-6 (IL-6) and Oncostatin M (Rose and Bruce, 1991), is a mitogen for progenitors 'committed' to the granulocyte lineage only. Similarly, erythropoeitin produced by adult liver and kidney, controls the rate of red blood cell production from erythroid progenitors. Also, during wound repair, clot formation induces platelets to release platelet derived growth factor (PDGF), which is then thought to induce the proliferation and migration of fibroblasts to the site of the wound.

Within the nervous system, the classic studies of Hamburger and Levi-Montalcini (1949) that led to the discovery of NGF, raised the possibility that soluble growth factors may be involved in the development of neural cells. These studies showed that NGF acts as a target derived neurotrophic factor that can promote the survival of chick sensory and

sympathetic neurones during a period of naturally occuring cell death in vivo (see a review by Hamburger, 1993). Since these observations, several more NGF-like factors have been identified: these include brain derived neurotrophic factor (BDNF) and neurotrophic factors 3, 4 and 5 (NT-3, NT-4/5) all of which have been shown to promote the survival of distinct populations of sensory and motor neurones (Barde, 1989). Moreover, the molecular nature of these survival effects are being carefully dissected. It is now known, for example, that the biological effects of NGF and NGF-related factors are mediated mostly by high affinity receptors, belonging to the receptor tyrosine kinase family, which have tyrosine kinases (or trks) domains in their cytoplasmic tail ; NGF acts via trkA, BDNF via trkB, and NT-4/5 and NT-3 via trkC respectively (reviewed by Glass and Yancopoulos, 1993, and, Schlessinger and Ullrich, 1992).

Because of their effects on neuronal survival and process outgrowth, NGF and the NGF-like factors are referred to neurotrophs, suggesting that this is their only function. This is not justified, however, since pleiotropic effects of such factors are constantly being discovered. For example, NGF can induce neuronal differentiation of immortalised pheochromocytes   (PC-12)(Tischler and Green, 1975). Another example of a neurotroph with multiple functions is ciliary neurotrophic factor (CNTF). Although its was first identified for its survival effect on chick parasympathetic ciliary neurones (Hefland et al., 1976), CNTF is now known to be capable of maintaining the pluripotency of embryonic stem cells (Conover et al., 1993). Moreover, it can induce a bipotenital optic nerve progenitor, called the O-2A progenitors, to differentiate into astrocytes (Hughes et al., 1988; Lillien et al., 1988). Indeed the O-2A progenitor has been an excellent model for identifying factors that may regulate the phenotypic choice of   multipotential neural precursors. This system is considered in detail below.

1.6. The optic nerve Oligodendrocyte-type 2 astrocyte (O-2A) progenitor and cell fate determining factors

1.6.1. Study of macroglial cell lineage in the optic nerve and discovery of the O-2A progenitor cells

The study of macroglial cell lineage in the optic nerve was prompted by the discovery that cultures of postnatal rat optic nerve contain two types of morphologically and serologically distinct astrocytes (Raff et al., 1983a). The first type, called type-1 astrocytes, were found to have a fibrous appearance, bind anti-Rat neural antigen (Ran-2) antibody (Bartlett et al., 1981) but not bind ligands for ganglioside, Tetanus toxin (Mirsky et al., 1978), or the antibody A2B5 (Eisenbarth et al., 1979). The second type of astrocytes (type-2 astrocytes), on the other hand, resembled cultured neurones and oligodendrocytes, bound Tetanus-toxin and A2B5 antibodies, but not Ran-2. Furthermore, while type-1 astrocytes were found to proliferate in response to EGF or extracts of bovine pituitary, type-2 astrocytes showed no such response (Raff et al., 1983a). Subsequent studies provided

evidence that the lineage of type 1 and type 2 astrocytes diverge early in development, probably around embryonic day 17 in the rat (Raff et al. 1984; Abney et al., 1981; Miller and Raff, 1984; Miller et al., 1985; Williams et al., 1985). In this respect, Raff et al. (1983a) and Temple and Raff (1985) showed that precursors that generated type-2 astrocytes were also capable of producing oligodendrocytes, cells that had a chandelier like appearance and bound A2B5 as well as the oligodendrocytic marker Gal-C (Raff et al., 1978; Ranscht et al., 1982). Accordingly, this bipotential precursor, which bound A2B5 but not anti-GFAP or Gal-C antibodies, was called the oligodendrocyte-type 2 astrocyte (O-2A) progenitor.

Over the following decade, studies of optic nerve cell lineage focused on characterising factors that control the proliferation and phenotypic choice of this bipotential precursor. As with the earlier studies, however, these efforts were aided by two important factors. First, absence of neuronal cell bodies from the optic nerve provided the investigators with a purified population of glial cells, and second, expression of distinct morphological as well as serological traits by the different glial cells meant that such cells could be identified readily in culture. The major findings of such efforts are discussed below.

1.6.2. Initial studies of O-2A progenitor differentiation in vitro

In 1983, Raff et al. (1983b) showed that in the absence of 10% Foetal Calf Serum (FCS), very few type-2 (A2B5$^+$, GFAP$^+$) astrocytes develop in bulk cultures of perinatal optic nerve cells, cultures that were known to contain O-2A progenitors. Instead, more oligodendrocytes (A2B5$^+$, Gal-C$^+$) could be found in the absence compared to the presence of FCS. This suggested that FCS somehow influences the phenotypic choice of the O-2A progenitor, such that in its presence, O-2A progenitors give rise to type-2 astrocytes and in its absence, they differentiate into oligodendrocytes, as if oligodendrocyte differentiation was the default path of O-2A development. Temple and Raff (1985), who studied the fate of single O-2A progenitor cells, in the presence and absence of FCS, showed that indeed FCS can induce differentiation of type-2 astrocytes from O-2A progenitors. In their study, however, differentiation of O-2A progenitors into type-2 astrocytes required 20% FCS, or conditioned medium collected from bulk cultures of optic nerve cells grown in 10% FCS. In the presence of 10% FCS alone, or absence of serum altogether, O-2A progenitors differentiated into oligodendrocytes (Temple and Raff, 1985). These observations suggested that in addition to FCS, cell-cell interactions may also play a role in type-2 astrocyte differentiation. However, it is known that O-2A progenitors do not become exposed to high serum concentrations in vivo. The question was, therefore, what factors present in serum induce type-2 astrocyte differentiation? Moreover, if cell-cell interactions are involved, interaction of O-2A progenitors with which cell types induces their differentiation into type-2 astrocytes?

Several studies had shown that it is only in the second post-natal week of development that cells with type-2 astrocyte phenotype (A2B5$^+$/ GFAP$^+$)) appear in the optic nerve in vivo (Abney et al., 1981; Miller et al., 1985, Williams et al., 1985), suggesting that factors that induce type-2 astrocyte differentiation become abundant only around this period. In this respect, Hughes and Raff (1987) discovered that a 25 kD protein present in extracts of optic nerves obtained on or after, but not before, the second post-natal week of development can indeed induce type-2 astrocyte differentiation in vitro. Subsequently, it was shown that the type-2 astrocyte differentiation inducing factor present in extracts of post-natal optic nerve is ciliary neurotrophic factor (CNTF, Hughes et al., 1988). Moreover, this factor, which could also be found in whole postnatal rat brain extracts, was shown to be produced by type-1 astrocytes (Lillien et al., 1988).

However, despite the widespread presence of CNTF in post-natal optic nerves (Stockli et al., 1991), it is was not clear why only a subpopulation of O-2A progenitors differentiate into cells with type-2 astrocyte characteristics in vivo, since, O-2A progenitors are found to persist in the optic nerve for several weeks if not months after birth.

In this respect, several authors found that the differentiation of O-2A progenitors into type-2 astrocytes in response to CNTF or brain/ optic nerve extracts, differed in two respects from that induced by FCS. First, O-2A progenitors differentiated far more rapidly into type-2 astrocytes in response to CNTF or brain or optic nerve extracts (e.g within 6 hours) than FCS (approximately 3 days). Second, unlike FCS, CNTF or brain/optic nerve extracts had a transitory effect; the majority of cells that expressed GFAP in response to CNTF or brain/optic nerve extracts went on to differentiate eventually into oligodendrocytes (Hughes and Raff, 1987). This observation, together with the finding that CNTF fails to induce differentiation of type-2 astrocytes in purified cultures of O-2A progenitors (Lillien et al., 1990), suggested that non-O-2A cells or factors produced by them, may be involved in the stable conversion of O-2A progenitors into type-2 astrocytes, in vitro (Lillien and Raff, 1990). Lillien et al. (1990) were able to isolate such factors. They showed that stable differentiation of O-2A progenitors into type-2 astrocytes in response to CNTF, in vitro, depends on the presence of extracellular matrix prepared from optic nerve cells that had been cultured for 9-12 days. Furthermore, only matrix produced by optic nerve meningeal cells was found to be effective. It was postulated, therefore, that matrix may play a 'geometrical' role in the differentiation of type-2 astrocyte from O-2A progenitors in vivo, i.e. presence of matrix may ensure that only a subpopulation of O-2A progenitors differentiate into type-2 astrocytes, or, that type-2 astrocyte production is restricted to distinct regions of the optic nerve in vivo. ffrench-Constant and Raff (1986b), for example, have identified the nodes of Ranvier as one site where type-2 astrocytes may be found, although the existence of a type-2 astrocyte in vivo remains controversial.

1.6.3. Factors that govern the division and proliferation O-2A progenitors

In addition to demonstrating the role of FCS in the phenotypic choice of O-2A progenitors, both Raff et al. (1983) and Temple and Raff (1985) also found that many peri- or neonatal O-2A progenitors stopped dividing soon after being cultured. This was intriguing, because, as already mentioned, O-2A progenitors can divide for weeks in the optic nerve in vivo. In search of reasons for this discrepancy, Noble and Murray (1984) noted that the premature differentiation of cultured perinatal O-2A progenitors can be delayed by growing such progenitors on a purified monolayer of cortical type-1 astrocytes, or in the presence of conditioned medium collected from cultures of type-1 astrocytes. This suggested that a soluble factor produced by type-1 astrocytes may have a mitogenic effect on O-2A progenitors. Subsequently, it was shown that premature differentiation of O-2A progenitors can be prevented by PDGF (Noble et al., 1988; Richardson, et al., 1988; Pringle et al., 1989) and that glial cells, possibly type-1 astrocytes, are capable of producing this factor (Richardson et al., 1988; Pringle et al., 1989). Consistent with these findings was that PDGF mRNA is detected in the developing optic nerve (Pringle et al., 1989) and that O-2A progenitors possess PDGF receptors of the α type (Hart et al., 1989; Mckinnon et al., 1990; Pringle et al., 1992). Agresti et al. (1991) however, have shown that unidentified components of type-1 astrocyte matrix, which lack PDGF, can also act as a mitogen for O-2A progenitors.

In any event, Noble and Murray (1984) and others noted that while O-2A progenitors continue to divide in the presence of cortical astrocytes, after a while a considerable number leave the cell cycle and differentiate into oligodendrocytes. This suggested that after a period in culture, some O-2A progenitors become unresponsive to PDGF. To date the exact nature of this sudden unresponsiveness has not been determined, although Hart et al. (1989 and 1989a) have shown that this is not due to either loss, or uncoupling of PDGF receptors from the intracellular second messengers they normally activate in O-2A progenitors. It may be that this unresponsiveness in some O-2A progenitors is induced by those progenitors that continue to divide in the presence of PDGF. In this respect, McKinnon et al. (1993) have demonstrated that a TGF-β like substance produced by cultured O-2A progenitors inhibits the proliferation of other O-2A progenitors cultured in the presence of PDGF. In any event, O-2A progenitors that continue to divide in the presence of PDGF in vitro, may be reproducing what is required of some progenitors in vivo, i.e. while some O-2A progenitors differentiate into oligodendrocytes during the first postnatal week, others need to remain as progenitors inorder to produce type-2 astrocytes in the second postnatal week (figure. 1.3).

Raff et al. (1985) have suggested that through PDGF, type-1 astrocytes may drive an intrinsic schedule of division and differentiation in O-2A progenitors, i.e. in the presence of PDGF, O-2A progenitors of a particular age may count a certain number of divisions before differentiating into oligodendrocytes. In support of this hypothesis, Raff et al.

Figure 1.3 Proposed cellular interactions that determine the phenotypic choice of Oligodendrocyte-type 2 Astrocyte (O-2A) progenitors in the rat optic nerve. Although the exact origin of O-2A progenitors is not known, such progenitors are known to be present in the developing optic nerve as early as E17 in the rat. Results of in vitro studies suggest that proliferation of pre-natal O-2A cells is influenced by PDGF, produced by type-1 astrocytes. It is also thought that around the time of birth, some O-2A progenitors become unresponsive to PDGF and differentiate in to oligodendrocytes. However, a proportion continue to proliferate as undifferentiated progenitors and in the presence of CNTF, thought to be produced by type-1 astrocytes during the second post-natal week, give rise to type-2 astrocytes. In addition to CNTF, stable conversion to O-2A progenitors into cells with type-2 astrocyte phenotype is thought to require contact with extracellular matrix molecules produced by optic nerve mesothelial cells.

There is in vitro evidence that perinatal O-2A progenitors may give rise to a slowly-dividing progenitors that have characteristics of adult O-2A cells (Wren et al., 1990).
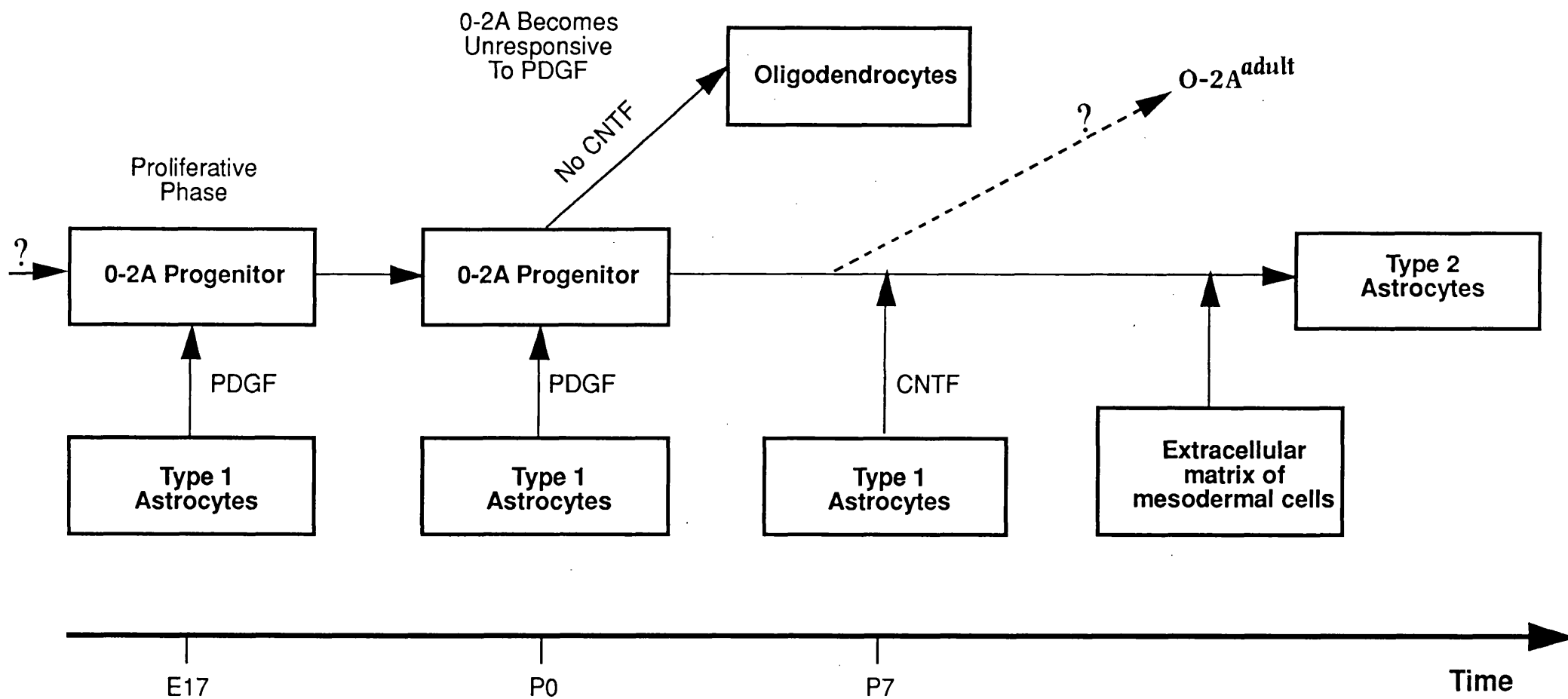
(1985) demonstrated that just like perinatal O-2A progenitors (Noble and Murray, 1984) E17-derived O-2A progenitors differentiate prematurely into oligodendrocytes if cultured in the absence of type-1 astrocytes. In the presence of type-1 astrocytes, however, these embryonic progenitors undergo a number of divisions before producing the first oligodendrocytes after 4 days in culture, a time corresponding to the day of oligodendrocyte development in vivo (E21). Similarly, E19 progenitors were found to divide for 2 days in culture before generating oligodendrocytes (Raff et al., 1985). However, as both these and other studies have shown, differentiation of O-2A progenitors need not depend on a certain number of divisions, or any division at all; Temple and Raff (1985), for example, show that differentiation of single O-2A progenitors into oligodendrocytes or type-2 astrocytes, or, transdifferentiation of GFAP$^+$ type-2 astrocytes into oligodendrocytes can occur in the absence of any cell division (Temple and Raff, 1985).

Bogler et al. (1990) have recently added a second dimension to our knowledge of factors that may influence the division and proliferation of O-2A progenitors. These authors have discovered that the intrinsic differentiation of O-2A progenitors, described above, can be overridden by culturing such progenitors in the presence of PDGF together with bFGF. Bogler et al (1990) showed that as long as both these factors are present, O-2A progenitors would behave like immortalised cells; cells that divide symmetrically and undergo self-renewal. Bogler et al. (1990), however, reported that bFGF alone does not prevent the premature differentiation of perinatal O-2A progenitors. This contrasts with the findings of Lillien et al. (1990) findings, who showed that ECM of cultured perinatal optic nerve cells or bFGF alone can directly inhibit the differentiation of O-2A progenitors into oligodendrocytes, Also, differentiation of O-2A^{adult} progenitors (ffrench-Constant and Raff, 1986a; Wolswijk and Noble, 1989) into oligodendrocytes is known to be inhibited by bFGF (Wolswijk and Noble, 1992; Mackinnon, et al., 1990). Although these differences may be due to the different concentrations of bFGF used (100ng/ml in Lillien et al.'s versus 5-10 ng/ml in Bogler et al.'s study) or the age and method of O-2A progenitor isolation, they may also highlight a pleiotropic effect by bFGF. In this regard, work presented in chapter IV of this thesis also shows that bFGF may have more than one effect on CNS neural progenitors, specifically neuronal precursors of embryonic rat cerebral cortex. As a prelude to presentation of this work, however, it is necessary to consider the general biology of bFGF and its related growth factors in some detail.

1.7. The family of Fibroblast growth factors (FGFs): general characteristics
1.7.1. FGF subtypes

The first description of fibroblast growth factor (FGF) comes from the work of Gospodarowicz (1974), who discovered that an extract of bovine pituitary gland is mitogenic for resting 3T3 fibroblasts. Subsequently, it was shown that bovine pituitary

and brain contain two different forms of FGF; one that has a basic isoelectric point (pI, 9.6) and the other an acidic pI (5.6) (Gospodarowicz et al., 1978, 1982 and 1984, and Thomas et al., 1984). Accordingly, these two FGFs, were named basic and acidic fibroblast growth factors. Since then, 5 more FGFs have been characterised. These are: int-2 (FGF-3); hst/ K-fgf (FGF-4); FGF-5; FGF-6, and KGF (short for keratinocyte growth factor, FGF-7) (Table 1.1). Although each of FGFs 1 to 7 is encoded by a distinct gene, there is a remarkable degree of amino acid sequence homology not only between different FGF subtypes (Esch et al., 1985), but also between the same FGF type in different species. Bovine acidic and basic FGF, for example, share 55% sequence homology (Thomas and Gimenez-Galleggo, 1987). Moreover, there is a high degree of homology between the amino acid sequence of bFGF from diverse species as Xenopus, human, bovine and chick (Kimelman and Kirschner, 1987; Mitrani et al., 1990).

1.7.1.1. Existence of FGF subspecies and their cellular compartmentation

Frequently different forms of one particular FGF can be found; bFGF, for example, can be found as either 18, 22.5, 22 or 24 kDa proteins. Initially, it was thought that the higher molecular weight forms of bFGF are artifacts of methods of isolation. It is now known, however, that the different bFGFs are distinct translation products of the same bFGF mRNA transcript; translation of the 18 kDa form begins from an AUG codon, while those of higher molecular weight species begins from more 5', CUG codon (Florkiewicz and Sommer, 1989; Prats et al., 1989). Moreover, it has been shown that the 18 kDa form of bFGF is found predominantly in the cell cytoplasm, while the higher MW forms are mostly nuclear. This compartmentation, which is due to the possession of a nuclear localisation signal by the high MW but not the 18 kDa bFGF species (Quarto et al. 1991), has led some authors to suggest that the nuclear and cytoplasmic forms of bFGF may have different biological functions (Logan, 1990; Renko et al., 1990).

1.7.1.2. Secretion and release of FGFs

Although most FGFs are secreted to the cell exterior, the two most prominent types of FGF, namely, acidic and bFGF, lack a hydrophobic secretory sequence (Abraham et al., 1986a and 1986b; Jaye et al., 1986) and remain confined to the cytoplasm of cells that express them; when this sequence is supplied artificially, however, these factors can become secreted (Blam et al., 1988). Recent evidence, however, suggests that proteins lacking a secretory signal sequence may be secreted by novel mechanism (Kandel et al., 1991). One such mechanism may be co-secretion with other proteins; for example, secretory granules of chromaffin cells of the adrenal medulla that normally store noradrenaline, also contain bFGF (Westermann et al., 1990). In any case, it has been postulated that lack of secretion of acidic and basic FGF may be significant in itself; these factors could have an autocrine role in cells that express them (Halaban et al., 1988;

Table 1.1 Types of FGF; their isolation and characteristics

FGF subtype	Isolation and characterisation	Chromosomal location of the gene	Physical and biological properties
FGF-1 (acidic FGF)	- First isolated from extracts of bovine pituitary and brain using heparin sepharose columns (Armelin, 1973; Gospodarowicz, 1974). Such extracts were found to be mitogenic for 3T3 fibroblasts.	5 (human)	≈ 146 amino acids long (bovine pit.) - Protein's MW ≈ 16.7 kDa - This factor and bFGF (FGF-2) are the only members of the FGF family not to possess a peptide secretory signal sequence
FGF-2 (basic FGF)		4 (human) 3 (mouse)	≈ 157 amino acids long (bovine pit.) - Protein's MW ≈ 18 kDa, although several different MW species ranging from 18 to 24 kDa have been found - because of its high binding affinity for heparin, was also initially called Heparin binding growth factor (HBGF). Also, called Astroglial Growth factor because of its mitogenic effect on cultured astrocytes.
FGF-3 (Int-2)	- First isolated in a proviral integration assay for Mouse Mammary Tumour virus (Dickson et al., 1984) - Xenopus homologue isolated by Kiefer et al. (1993)	7 (mouse)	- Protein's MW ≈ 28 kDa
FGF-4 (hst or Kfgf)	- Isolated first from human stomach cancer cells (Taira et al., 1987) and from Kaposi sarcoma cells (Delli Bovi et al., 1987) - - NIH 3T3 cells transfected with cDNA for this factor, produced FGF-4	11 (mouse)	- 206-216 amino acids long; Protein's MW ≈ 22 kDa
FGF-5	- First isolated by Zhan et al. (1988) by selection of NIH-3T3 cells transfected with DNA from human cell lines when grown in the absence of FGF and PDGF	4 (human)	- 267 amino acids long
FGF-6 (hst-2)	- Isolated first by Marics et al. (1989) in a screen of a mouse cosmid library using an hst-specific probe	12 (human) 6 (mouse)	- Gene found to encode a 198 amino acid protein
FGF-7 (KGF)	- Discovered first by Rubin et al. (1989) as a factor present in conditioned medium of a line of foetal human lung cells that was mitogenic for epithelial but not mesodermal cells. - First cloned by Finch et al. (1989)	Not known	- Protein's MW ≈ 28 kDa although Mason et al. (1994) have found a 23 kDa protein

Neufeld et al., 1988; Sherman et al., 1993) or may play an important part in tissue repair when they become released from ruptured cells.

1.7.1.3. High binding affinity for components of the extracellular matrix

Some FGFs, particularly bFGF, are known to have a high binding affinity for extracellular matrix molecules such as heparin. Indeed, acidic and basic FGF were first isolated from bovine pituitary and brain by using heparin-sepharose columns. Accordingly, these FGFs were also known as heparin-binding growth factors (HBGFs). Although not all FGFs bind heparin, it has been demonstrated that some, such as bFGF, have a high binding affinity not only for pure heparin, but also for sulphated forms of glycosaminoglycans (GAGs) and heparans associated with the proteoglycans of the extracellular environment. This association is thought to be important in at least three respects. First, it has been shown that binding of FGF (particularly bFGF) to extracellular matrix molecules, protects it from thermal denaturation, exposure to extremes of pH and proteolytic degradation and enzymatic cleavage by enzymes such as trypsin and plasmin (Gospodarowicz and Cheng, 1986; Rosengart et al., 1988; Saksela et al., 1988; Sommer and Rifkin, 1989). Matrix bound FGF can, however, be liberated by enzyme heparinase. These properties may serve to prolong the presence of FGF in the extracellular environment or allow FGF to travel freely as a stable complex within tissue fluids, thereby enhancing its biological functions. Second, as FGFs are found to have numerous functions, binding to ECM may play a critical role in localising the effects of FGF to a particular cell layer or structure. (c.f. Stable in vitro conversion of O-2A progenitors into a type-2 astrocyte phenotype only when CNTF is presented to such progenitors in the presence of an bFGF-like matrix component , Lillien et al, 1990). Third and finally, binding heparin may be a prerequisite for binding of FGFs to their high affinity receptors and that heparin-like molecules, bound to cell-surface membranes, may themselves act as FGF receptors (Klagsburn and Baird, 1991). In this regard, it is worth describing the nature and type of FGF receptors in more detail.

1.7.2. FGF receptors

FGF receptors can be classed into low affinity and high affinity receptors. The low affinity receptors, as already noted, consist of heparan sulphate proteoglycans (HSPG) attached to the cell surface membrane. Although the mechanism of signal transduction via these low affinity receptors is unknown, several studies show that FGF bound to HSPG can be directly internalised (Gannoun-Zaki, et al., 1991; Carmen et al., 1990; Ferguson et al., 1990) and transported to the cell nucleus (Walicke and Baird, 1991)

1.7.2.1. High affinity FGF receptors (FGFRs)

1.7.2.1.1. Receptor genes and receptor subtypes

To date, four different but highly-related high affinity FGF receptor genes, termed *FGFR-1* to *4*, have been characterised (Houssaint et al., 1990; Avivi et al., 1991; see review by Robinson, 1991). The protein product of all such genes are capable of binding two members of the FGF family, namely aFGF (FGF-1) and FGF-4. However, of these genes, the product of *FGFR-1* gene (homologous to *flg* in mouse, and *cek-1* in chick) has the highest binding affinity for acidic and basic FGF (FGF-2) and is thus thought to act as a high affinity receptors for these FGFs. The protein product of *FGFR-2* gene (homologous to *bek* in mouse, and *cek-3* in chick), by contrast, acts mainly as a high affinity receptor for KGF (FGF-7). Less is known about the binding affinities of FGFR-3 and FGFR-4 (Keegan et al., 1991; Partanen et al., 1991). Partanen et al. (1991), however, have shown that FGFR-4 binds FGF-6 and acidic but not basic FGF.

Structural analysis of FGFR types, shows that at least 6 (potentially 12) structurally different FGFRs exist, suggesting that a single *FGFR* gene may produce two or more distinct FGFRs. Miki et al. (1992) have shown that *FGFR-2* gene (also termed *bek*)can yield two different FGFRs; one of these, KGFR, demonstrated a high binding affinity for KGF and aFGF, while the other, FGFR-2, could not bind KGF but showed a high affinity for both bFGF and aFGF. These and other authors have suggested that these receptor isoforms arise through alternative splicing of the same transcript and that the alternative splicing may be a mechanism for restricting the action of one particular FGF type to particular cell type (Orr-Urtreger et al., 1991 and 1993). In this respect, Miki et al. (1992) also show that although their FGFR-2 transcript could be detected in a variety of cell types, the KGFR transcript was restricted to epithelial cells. Similarly, Orr-Urtreger et al. (1993), have demonstrated that in the mouse embryo, one transcript of *bek* is localised to surface ectoderm and epithelia of inner spaces, while the other is found primarily in superficial mesenchymal cells. These observations are consistent with reports that KGF (FGF-7) acts as a mitogen specifically for epithelial- but not mesodermally-derived cell types (Finch et al., 1989 and Rubin et al., 1989). However, not all cells show this type of specific receptor localisation; Pertovaara et al. (1993), for example, have discovered that teratocarcinoma cells express all four FGFR types; only after treatment with retinoic acid do these cells down regulate expression of some of the FGFR types.

1.7.2.1.2. FGFR Structure and signal transduction

High affinity FGF receptors are of the receptor tyrosine kinase type (see reviews of Schlessinger and Ullrich, 1992, and, Ullrich and Schlessinger, 1990). These receptors normally contain three distinct domains; an extracellular domain, to which the growth factor binds, a single transmembrane domain, and, an intracellular (carboxy) domain,

Figure 1.4 Structure and signal transduction by FGF receptors. FGF receptors contain three distinct domains; an extracellular domain, normally composed of two or three highly glycosylated Immunoglobulin-like domains with which FGF protein interacts; a transmembrane and juxtamembrane domain, through which bound FGF's signal is transmitted; and an intracellular domain composed of two kinase subdomains that are separated by inter-kinase domains. Isoforms of FGF receptors usually differ in the number of their IG domains. For example, mouse KGFR contains two, while human KGFR has three such IG domains (Miki et al., 1992). As a member of the receptor tyrosine kinase family, signalling by FGF receptors is thought to depend on the phosphorylation of tyrosine residues present in the interkinase regions of the cytoplasmic domain. Phosphorylation of such tyrosine residues results in their interaction with cell signalling proteins such PLC-γ via their SH-2 domains (Schlessinger and Ullrich, 1992). This interaction leads to activation and/or phosphorylation of a cascade of second messenger molecules, resulting in release of Ca^{2+} ions from intracellular stores and/or ultimately to altered patterns of gene expression via regulation of transcription factor activity.

Extracellular
Domain

Transmembrane
Domain

Intracellular
Domain

Domain I

Domain II

Domain III

Kinase I

Kinase II

Ⓟ tyr

C terminus

PLC

SH-2

SH-2

SH-3

PLC

Phospholipase C-γ molecule

Cascade of
intracellular
second messages

Release of calcium from
internal store
or
Altered gene expression ?
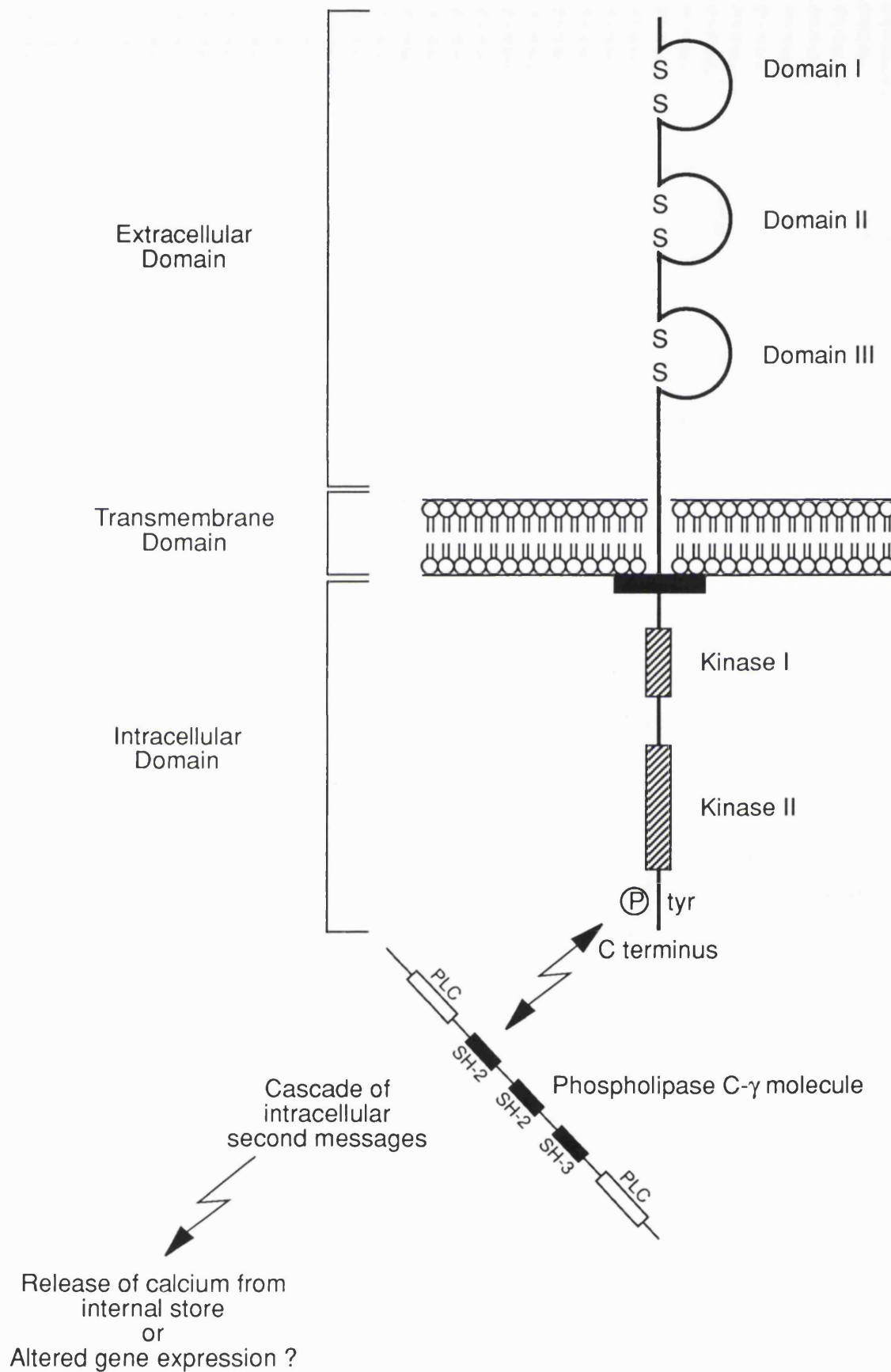
which contains one or more tyrosine kinase domains separated by interkinase domains (figure. 1.4); typically, FGFRs have two such tyrosine kinase residues.

Binding of FGF to the extracellular domain of its receptors is thought to have at least two immediate consequences. First, it causes a dimerisation of two FGFR receptor molecules, and second, it results in the transmission of a signal via the transmembrane domain to the intracellular tail of the receptor. The combined consequence of these events is that tyrosine residues, present in the interkinase domains of the intracellular tail, become phosphorylated. This phosphorylation then exposes sites on the intracellular tail that have a high binding affinity for specific cell signalling molecules, such as src or phospholipase C (PLC-γ) (Mohammadi et al., 1992). Upon interaction of the receptor tail with these signalling molecules, which occurs via their SH-2 (src homology -2) domains, the signalling molecules become activated and inturn activate a cascade of second messengers in the cell cytoplasm, a cascade that ultimately leads to alteration of gene expression within the cell nucleus (figure. 1.4).

1.7.3. Biological roles of FGFs

Almost all organs, tissues and cultured cells examined have been found to contain FGF in one form or another (Rifkin and Moscatelli, 1989). It would not be surprising, therefore, to find that FGFs can influence almost every aspect of cellular development and functioning, ranging from cell proliferation and differentiation to cell migration, adhesion and metabolism (see review of Rifkin and Moscatelli, 1989). In only a very few systems, particularly during development, however, has a concise and direct effect of FGF or FGF subtypes been described. Before reviewing possible roles of FGF, particularly bFGF, in the development of the vertebrate nervous system, it is worth describing two well-characterised effects of FGF outside the nervous system; its role in mesoderm induction and the development of the limb bud.

1.7.3.1. Role of FGF in the induction of mesoderm in Xenopus embryo

Upon fertilisation, the Xenopus egg becomes polarised into two halves; the animal half and the vegetal half. Cells of the animal half have an ectodermal, while those of the vegetal half have a endodermal fate. At the 64 cell stage of development, however, vegetal cells induce their equitorial animal pole-derived neighbours to acquire a mesodermal fate, an induction that can be demonstrated in vitro; blastula stage animal pole (animal cap) cells produce mesodermal cells when co-cultured with cells of the vegetal half (Smith, 1993). Moreover, animal caps give rise to distinct mesodermal derivatives depending on the anatomical source of vegetal cells, i.e. co-culture with more dorsal vegetal cells yields more ventral mesodermal derivatives, such as blood cells, while co-culture with more ventral vegetal cells induces derivatives such as muscle and notochord.

In search of factors mesoderm inducing factors, several studies showed that factors such as bFGF and members of TGF-β family (such as TGF-β2 and activin) can mimic the effects of vegetal cells in the animal cap assays (Kimelman and Kirschner, 1987; Slack et al., 1987; Rosa et al., 1988; Asashima et al., 1990, and Smith et al, 1990). In the presence of bFGF as opposed to TGF-β, however, animal cap cells tended to produce the ventral mesoderm derivatives. The idea the bFGF may be involved in mesoderm induction was supported by the detection of bFGF mRNA and protein in the developing Xenopus embryo (Kimelman and Kirschner, 1987; Slack and Isaacs, 1989). Interestingly, Shiruba et al. (1991) demonstrated that coinciding with the time of mesoderm induction, there was a cytoplasmic to nuclear translocation of bFGF in cells of the mesoderm forming regions of the embryo. The strongest evidence for involvement of FGF, however, came from the study of Amaya et al. (1991) who showed that blocking FGF function by over-expression of a mutant FGFR that lacks the cytoplasmic tail and hence can not dimerise (as described in section 1.7.2.1.2), prevents formation of ventral mesoderm derivatives (in this case tail structures) in vivo. However, since several workers had shown that mesoderm is induced by a diffusable factor (Grunz and Tuckle, 1986; Gurdon, 1989; Slack, 1991), it was difficult to envisage bFGF as such a candidate, since, it is not secreted from cells. Moreover, Slack (1991) found that neutralising antibodies against bFGF, fail to inhibit the normal mesoderm inducing activity of vegetal cells. It thus seemed as though a secreted type of FGF is involved in mesoderm induction. In this regard, initially FGF-3 (int-2) and FGF-4 (hst/ K-fgf) were tested for their mesoderm inducing properties. Although FGF-3 is the closest relative of bFGF amongst the secreted FGFs, Paterno et al. (1989) discovered that FGF-4 was a better mesoderm inducer in animal cap assays. The search for a secreted FGF capable of inducing mesoderm in Xenopus, however, ended with the isolation of a novel type of FGF from Xenopus embryos (Isaacs et al., 1992). This type, which was initially called Xenopus FGF, is now known to be related to mammalian FGFs 4 and 6.

Several groups have shown that beside their role in mesoderm induction, FGFs are also important in differentiation of mesodermal derivatives, or even the anteroposterior specification of the Xenopus embryo. For example, bFGF can induce frog animal cap cells to express *Xhox-3* (Ruiz i Altaba and Melton, 1989), a homeobox gene that is involved in the axial patterning of the Drosophila embryos. These findings have been the subject of a recent reviewed Smith (1993).

1.7.3.2. Role of FGF in the development of the vertebrate limb bud

The vertebrate limb development begins with the formation of limb buds at specific sites along the embryo body. At these sites, interaction of three cell populations contribute to the development of the limb bud (figure. 1.5). The first of these is a population of ectodermal cells which are induced by the underlying mesodermal cells to become what is

Figure 1.5 Schematic structure of the vertebrate limb bud and cellular interactions that are important to its development. Interactions between three different cell populations, namely, cells of the progressive zone, ZPA and AER cells are thought to contribute to the development of the vertebrate limb. Progressive zone cells act as precursors for the bone and muscle of the developing limb. AER cells are thought to have two roles: first, they maintain a population of progressive zone cells in an undifferentiated state, so that the more distal structures can develop from these precursor cells. Second, AER cells are thought to maintain ZPA cells and induced them to produce a factor that can pattern the developing limb along its antero-posterior axis. ZPA cells inturn are thought to maintain AER cells.

AER's effects are thought to be mediated by a diffusable growth factor; evidence suggests that this may be FGF-4 (see text)

ZPA ⟶ Sonic

Proliferation

Proliferation

AER ⟶ Growth factors

Anterior

Proximal

Apical Ectodermal Ridge

Progress Zone

Distal

Zone of Polarizing Activity

Posterior
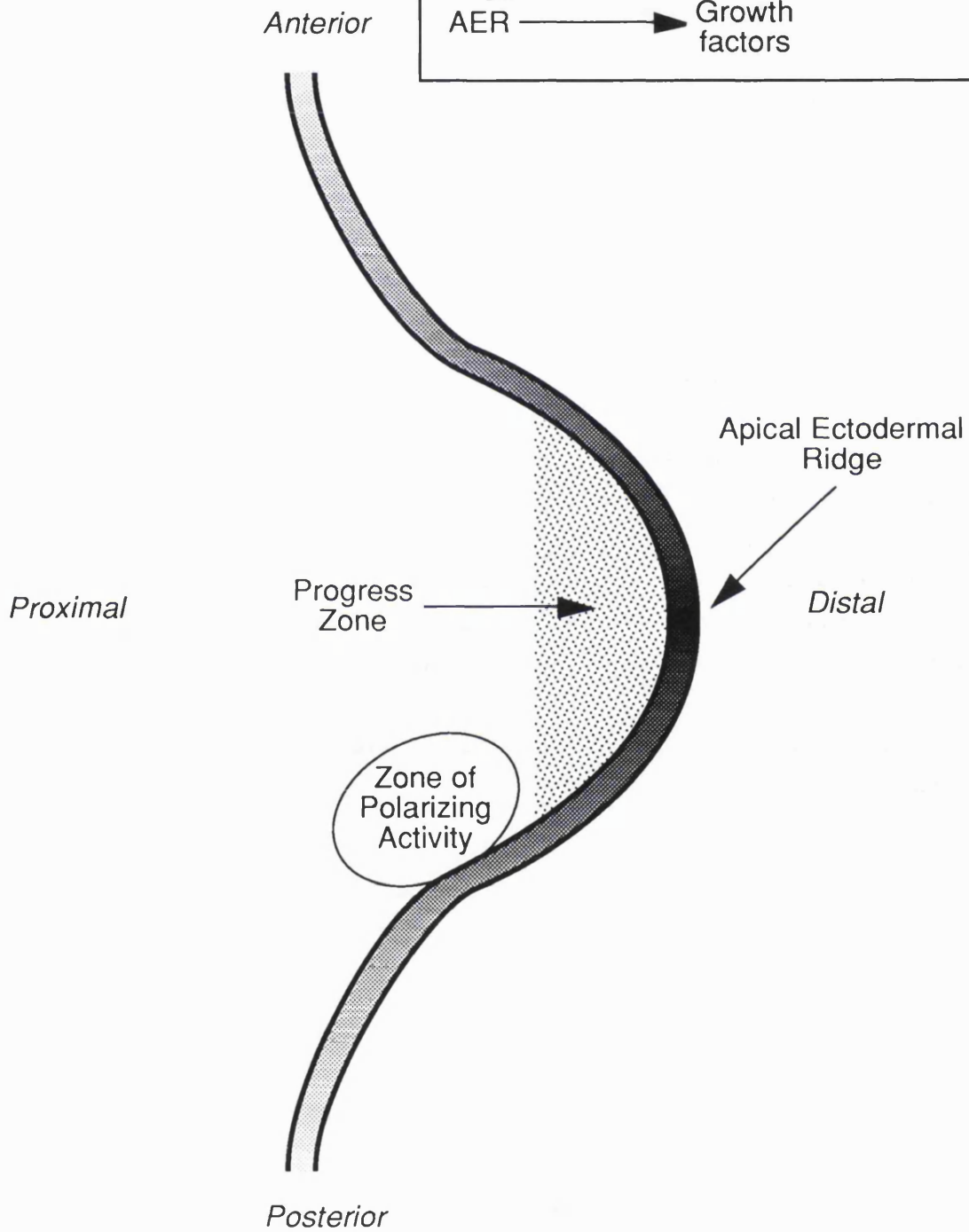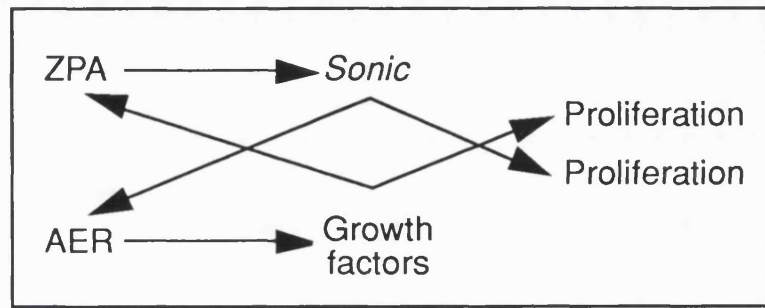
known as the apical ectodermal ridge cells (AER); these cells are located at the most lateral rims of the developing limb buds. The second, are a highly proliferative population of mesodermal cells that act as precursors of the bone and muscle of the developing limb; these cells are located medial to the AER cells. The third population, which is also mesodermal in origin, is located at the most posterio-lateral margins of the developing limb buds in what is known as the zone of polarizing activity (ZPA, figure. 1.5); ZPA cells are known to be important in antero-posterior (thumb to little finger) patterning of the limb buds.

AER cells are thought to have two important roles in the development of the limb bud. First, they maintain cells of the progressive zone in an undifferentiated state. This in turn, has two consequences: a) by remaining in an undifferentiated state, progressive zone cells can act as progenitors of more distal structures within the developing limb bud, and b) by remaining in this undifferentiated state for a particular period, they can adopt a distinct morphogenetic fate, as postulated by the progressive zone model of Summerbell et al. (1973).

The second role of AER cells is to induce ZPA cells (figure. 1.5), to inturn induce patterning of the limb bud cells along the antero-posterior axis.

Both of these effects of AER cells are thought to be mediated by diffusable factors and FGF has been recognised as one such factor (Niswander and Martin, 1993). For example, it has been shown that bFGF can act as mitogen for progressive zone cells and maintain their in vivo pattern of gene and antigenic expression, in vitro (Watanabe and Ide, 1993). Moreover, if AER cells are removed from the developing limb buds, FGF-4 can prevent the loss of chick ZPA cells (Niswander et al., 1993). Also, bFGF can support the survival of mouse ZPA cells, in vitro (Anderson et al., 1993). The involvement of FGF is further supported by the presence and pattern of FGF/ FGFR expression in the developing limb bud in vivo. bFGF, for example, has been found in extracts of developing limb buds (Munaim et al., 1988; Seed et al., 1988) and FGF-4 mRNA is expressed specifically in AER cells of the posterior half of the limb bud (Niswander and Martin, 1992). Moreover, FGFR-1 and 2 have also been found in the developing limb bud (Peters et al., 1992).

Recent studies of the antero-posterior patterning of the developing limb, however, have been able to provide further support for the involvement of FGF. Previously it was thought that retinoic acid is the morphogen responsible for mediating ZPA's patterning effect on the progressive zone cells. Evidence for this came from transplantation studies in which the presence of ectopic ZPA cells or retinoic acid at the anterior half of limb bud, could cause duplication of digits (Summerbell, 1983). Two recent studies (Krauss et al., 1993; Riddle et al., 1993), however, have shown that ZPA's induction is meditated by a secreted protein product of gene, *sonic hedgehog,* a homologue of Drosophila segment polarity genes, whose expression can be turned on by retinoic acid.

Because expression of *sonic hedgehog* is co-incidental with detection of FGF-4 transcripts in AER cells, and that as already noted FGF-4 can substitute for AER cells effect on ZPA in vivo, it has been hypothesised that FGF-4 may be required for expression of *sonic hedgehog*.

1.7.3.4. Possible roles of bFGF in the development of vertebrate PNS and CNS

Detection of FGF immunoreactivity and differential expression of transcripts for FGFs and their receptors in the nervous system, has lead many groups to investigate possible roles of FGF in the development of PNS and CNS. These efforts, which have focused mainly on acidic and basic FGF, show that these factors affect the proliferation, survival and differentiation of precursors cells. Three specific areas are worthy of mention: bFGF effects on neural-crest derived precursors, its possible role in generation/ regeneration of the neural retina, and, its effects on cultured neural precursors of the developing CNS, in particular those of the rodent telencephalon. The last of these is particularly relevant because it would serve as prelude to work presented in chapter four of this thesis, namely, an investigation of the effects of bFGF on embryonic rat cortical precursors. This section will, therefore, also include a description of a) the pattern of bFGF/ bFGFR immunoreactivity and mRNA expression in the developing rodent telencephalon, and b) previous investigation of bFGF's effects on neural cells derived from this area of CNS.

1.7.3.4.1. Effects of bFGF on neural-crest derived precursors
1.7.3.4.1.1. Survival and differentiation of Schwann cell precursors

Although it is known that Schwann cells, the myelin forming cells of the PNS, are derived from neural crest cells, it is not known when the Schwann cell lineage *pre se* diverges from that of other neural crest-derived cells (see review of Jessen and Mirsky, 1992). A recent study by Jessen et al. (1994), however, suggest that in the rat this may occur on embryonic day 14. Jessen et al. (1994) have isolated Schwann cell precursors from E14 rat sciatic nerves that are distinct from their neural crest parents in that they express the antigen GAP-43, and also differ from mature Schwann cells in that they have a characteristic flat appearance in culture and do not express S-100. Thus the newly identified precursors may represent the transitory phase between multipotential neural crest cells and committed Schwann cells. A peculiar feature of this precursor, however, is that it dies within 5-7 hours of its isolation from embryonic peripheral nerves, a death that can prevented by conditioned-medium of neuronal cultures. Jessen et al .(1994) have discovered that amongst a range of growth factors tested, only bFGF is capable of preventing the death of such precursor cells, in vitro. Moreover, these authors show that this death is apoptotic and that once precursors pass a critical stage of their development in vitro, they do not rely on bFGF *per se* for their survival; thereafter, the survival of such

cells can be supported by co-culturing with neurones. In the absence of neurones, however, bFGF is again required for their transition to S-100 bearing Schwann cells.

The findings of Jessen et al.(1994), therefore, suggest that bFGF can act not only as a critical survival factor for Schwann cell precursors but also as a factor that induces Schwann cell differentiation, at least under the conditions of their culture. In this respect, the axons of embryonic DRG neurones, are known to produce bFGF (Gonzalez et al., 1990) and may play this vital role in vivo. The hypothesis about Schwann cell development put forward by Jessen et al. (1994), therefore, is that contact with embryonic axons and sequestration of bFGF by Schwann cell precursors may, in the first instance, commit multipotential neural crest cells to the Schwann cell lineage, and, in the second instance, regulate the number of Schwann cells that ultimately myelinate an axon.

1.7.3.4.1.2. Transdifferentiation of Schwann cell precursors into melanocytes

It has been reported that in the presence of bFGF, embryonic quail peripheral nerve or DRG-derived Schwann cell precursors that can be recognised by mono-clonal antibody 1E8 (Bhattacharyya et al., 1991 thought to bind myelin glycoprotein P_0), can transdifferentiate into melanocytes, in vitro (Stocker et al., 1991; Sherman et al., 1993). This effect can be mimicked by phorbol esters such as TPA, and inhibited by TGF-β1 (Stocker et al., 1991). However, the study of Sherman et al. (1993) shows that not all Schwann cell precursors in cultures of embryonic quail peripheral nerve or DRG express the Schwann cell marker, 1E8, i.e. melanocytes could have been generated not by transdifferentiation but by a bipotential precursor that could generate Schwann cells and melanocytes. A clonal analysis would be able to resolve between these possibilities. If such an analysis were to demonstrate that bFGF or TPA induce transdifferentiation, however, then this would reinforce the argument that no precursor can be called 'committed' in its fate (discussed in section 1.4.1), i.e. DRG or PN-derived Schwann cell precursors that appeared 'committed' to Schwann cell lineage were induced to generate melanocytes, a cell type not normally found in these structures.

1.7.3.4.1.3. Phenotypic choice of the sympathoadrenal precursor cells

By using neural crest specific antibody, HNK-1(Abo and Balch, 1981) or monoclonal antibody SA-1(Carnahan and Patterson, 1991a), several workers have been able to isolate a bipotential precursor from embryonic rat adrenal glands and sympathetic ganglia that can generate adrenal chromaffin cells or sympathetic neurones in vitro (Axel and Andreson, 1986; Carnahan and Patterson, 1991b). In vivo, the phenotypic choice of this precursor, called the sympathoadrenal precursor, depends on its environment; in adrenal glands, it generates chromaffin cells and in sympathetic ganglia it produces sympathetic neurones.

In search of factors that may induce this phenotypic choice in vivo, several workers showed that

in the presence of high glucocorticoid concentrations, cultured sympathoadrenal precursors generate chromaffin cells, recognised by their expression of epinephrine-synthesising enzyme, PNMT. In the absence of glucocorticoids, or presence of NGF, however, the precursors generate neurofilament positive, catecholamingeric neurones (Anderson and Axel, 1986). This observation, together with the finding that NGF can cause a transdifferentiation of chromaffin cells into sympathetic neurones, suggested that NGF may be the in vivo factor that promotes neuronal differentiation. Korsching and Thoenen (1988), however, were unable to find any NGF in embryonic sympathetic ganglia, suggesting that factors other than NGF may be regulating this phenotypic choice. In this respect, Togari et al. (1985)'s discovery that both aFGF and bFGF can induce the neuronal differentiation of a pheochromocytes cell line (PC12 cells) suggested that FGFs may be the regulatory factors. Further evidence, however, was provided by Stemple et al. (1988) and Claude et al. (1988) who showed that FGF was not only a mitogen for chromaffin cells but could also cause their transdifferentiation into neurones. Moreover, Kalcheim and Neufeld (1990) showed that both acidic and basic FGF can be found in the developing avian sympathetic ganglia.

Subsequent to these studies, Birren and Anderson (1990) were able to elucidate the exact role of FGF and NGF in the phenotypic choice of sympathoadrenal precursors. These authors showed that generation of sympathetic neurones from *v-myc*-immortalised sympathoadrenal precursors, depends on the sequential action of FGF and NGF, such that, FGF specifies the bipotential precursor along the neuronal lineage and by upregulating NGF receptors, makes the specified precursors dependant on NGF. Subsequently, NGF acts not only as a survival factor, but also promotes the full differentiation of the specified precursors into sympathetic neurones (Birren and Anderson, 1990). Moreover, it is now known that FGF and NGF antagonise the effects of glucocorticoids, such that development of sympathoadrenal precursor along the chromaffin lineage may depend on a critical concentration of glucocorticoids. The molecular details of this antagonism have been described in a recent review by Anderson (1993).

1.7.3.4.2. Role of bFGF in generation of neural retina and differentiation of rod photoreceptors

The vertebrate retina develops from an outgrowth of the neural tube, called the optic vesicle. During its formation, however, the optic vesicle invaginates in the shape of a punched football, resulting in the formation of two closely apposed sheets of cells. The outer, more posterior sheet develops into a pigmented layer of cells, called retinal pigmented epithelium (RPE), while the inner (more anterior) sheet gives rise to sensory (neural) retina (NR, described in section 1.4.4.2). Although these two sheets are thus derived from the same neuroepithelium and are continuous at their peripheral borders, it is

not known what factors cause one to adopt a markedly different fate from the other. There is evidence, however, that FGFs may play a role in this developmental process.

The first line of evidence is that following removal of embryonic chick NR, presence of FGF can induce RPE cells to transdifferentiate and generate a 'normal', albeit inverted NR, in vivo (Park and Hollenberg, 1989). In order to characterise the precise role of FGF, however, several groups have studied this phenomenon in vitro. Guillemont and Cepko (1992), for example, have investigated the effects of acidic and basic FGF on explants of stage 17-18 and 24-25 chick RPE, i.e. at stages (Hamilton and Hamburger, 1951) before and after the appearance of pigmented cells in vivo. These authors report two major findings. First, in the presence, but not absence of FGFs, cultured RPE cells (from stage 24-25) lost their pigment and gradually developed into a highly folded structure. Furthermore, in the presence of serum in addition to bFGF, these folded structures gave rise to differentiated ganglion cells. The second and more dramatic finding, however, was that stage 17-18 explants never developed pigments in the presence of FGF, giving rise directly to cells that had a neuronal morphology. These observations suggested that absence of FGFs or unresponsiveness to FGFs may be important in the development of RPE anlage into a pigmented epithelium, in vivo. To reverse the argument, presence of FGF may be important in the development of NR, although this has not been demonstrated directly.

Similar observations have been reported by Pittack et al. (1991) although these authors emphasise that in addition to bFGF's presence, physical configuration of the RPE explants determines the degree of transdifferentiation into neuronal phenotypes; flattened RPE explants attached to the culture substratum gave fewer differentiated neurones than explants that were prevented from flattening. The study of Opas and Dziak (1994) suggests that bFGF and matrix may play separate roles in the transdifferentiation of RPE into NR. These authors suggest that while FGF provides the transdifferentiation signal *per se*, full differentiation of RPE cells into retinal neuronal phenotypes may depend on cell-matrix or cell-cell interactions. A role of matrix in this process has also been shown by Reh et al. (1987) who found that laminin alone can promote the transdifferentiation of RPE cells into retinal ganglion cells, in vitro. Even this effect of laminin, however, may have been induced by FGFs since binding to laminin can upregulate FGF-receptor expression in neural cells (Drago et al., 1991a).

Lillien and Cepko (1992) have described a separate effect of bFGF; they show that both acidic and basic FGFs, are potent mitogens for embryonic (E15-E18) rat NR precursors and that with increasing developmental age, these precursors become less responsive to FGF and more responsive to another peptide growth factor, TGF-α. In addition to FGF and TGF-α, EGF is also found to be a mitogen for NR precursors (Anchan et al., 1991).

Consistent with possible roles of bFGF in the development of NR, is the detection of bFGF (Mascarelli et al., 1987), FGFR mRNA (Heuer et al., 1991) and FGF binding sites

(Jeanny et al., 1987; Cirillo et al., 1990) in distinct regions of the developing chick and mouse retinas. Cirillo et al. (1990), for example, describe two types of FGF-binding sites; one that is sensitive to heparinase treatment and is associated with matrix of retinal basement membranes, and a second found exclusively in cellular layers of NR that is insensitive to heparinase; the latter possibly corresponds to high affinity FGF receptors Noteworthy also is the pattern of int-2 (FGF-3) expression in embryonic mouse retina. Wilkinson et al. (1989) report that int-2 transcripts are found exclusively in NR between embryonic days E14.5 to E17 (and possibly until birth, but not beyond) in a pattern that suggests a role in differentiation rather than proliferation of NR cells. These observations suggest that FGF and FGFR expression may be exclusive to NR. Wanaka (1991), however, report that at E12, FGFR mRNA is expressed both in the pigment epithelium as well as the neuroblast layer of rat retina.

1.7.3.4.2.1. Possible role of FGF in rod photoreceptor differentiation

Cell lineage studies have shown that throughout retinal development, different retinal cell types (described in section 1.4.4.2) are generated by precursors that remain multipotential up to their terminal round of division. However, it is also known that retinal cell types are not generated at random; distinct cell types are produced during specific periods of retinal development. The question is, therefore, what factors induce multipotential NR precursor cells to differentiate into a particular cell type in preference to another? There is strong evidence that temporal and spatial cellular interactions play an important role in production of particular retinal cell types (Harris and Messersmith, 1992; see also review of Reh, 1992a). In this respect, several groups have focused their attention on factors that promote the generation of rod photoreceptors, a cell type produced predominantly in early postnatal life. The results of such studies show that a factor present in neonatal NR, promotes differentiation of retinal precursors into rod photoreceptors. Reh et al. (1989), for example, have shown that within 3 days of their transplantation into postnatal rat eyes, E14 mouse NR precursors give rise to rod photoreceptors, several days before their normal schedule of rod photoreceptor generation in vivo (Reh, 1989). Moreover, Watanabe and Raff (1990 and 1992) and Altshuler and Cepko (1992), have shown that a diffusable, but highly localised factor produced by neonatal (but not postnatal day 3 NR cells) can promote an increasing number of E15 NR precursors to develop into rods, in vitro. Finally, Reh (1992b) has shown that close proximity of E10-12 NR precursors exclusively to rod photoreceptors in vitro, can induce such cells to become rods. Although the exact nature of this rod-promoting factor is still not known, the study of Hicks and Courtois (1992) suggests that it may be bFGF. These authors have shown that in the absence of any significant effect on cell division or survival, bFGF can induce a six-fold increase in the number of dissociated new born (but not post-natal day 3) rat NR cells that develop into

rods (Hicks and Courtois, 1992). However, it remains to be shown whether bFGF has a similar role in rod photoreceptor determination in vivo.

1.7.3.4.3. Possible role of FGF in the development of rodent telencephalon

The strongest evidence that FGFs may be involved in the development of distinct parts of CNS, such as the telencephalon, comes from studies that have analysed the spatial and temporal expression of FGFs in the developing CNS. These studies, which have focused mainly on acidic and basic FGF, are considered below.

1.7.3.4.3.1. Developmental expression of acidic and basic FGF and their receptor in the rat telencephalon

Several workers have detected immunoreactivity for both acidic and basic FGF and for their receptor-proteins in the developing rat cortex (Fu et al., 1991; Gonzalez et al., 1990; Weise et al., 1993). This immunoreactivity is first detectable between E11 and E13, becomes strongest around E16-E17 and falls dramatically before birth (Fu et al., 1991; Weise et al., 1993). Fu et al. (1993), report that during this developmental period, aFGF immunoreactivity is absent from the neuroepithelial cells of VZ and other undifferentiated cells. It is, however, detectable in the cytoplasm (and occasionally nuclei) of migrating neuroblasts and cells that resemble glia. Gonzalez et al. (1990) also reported positive immunoreactivity for aFGF at E18 in cells that resemble glia. Weise et al. (1993), however, emphasised that bFGF immunoreactivity is limited to differentiating cortical neurones. Furthermore, these authors used anti-FGFR antibodies to show that most cortical neurones that express FGF also express FGFR in vivo and in culture (Weise et al., 1993).

However, FGF (acidic and basic) is also detectable in the postnatal and adult rat brain and spinal cord, in subpopulations of neurones (Caday et al., 1990; Elde et al., 1991; Grothe et al., 1991; Stock et al., 1992). Elde et al. (1991) and Grothe et al. (1991), for example, have detected acidic and basic FGF immunoreactivity in sensory and motor nulcei of brainstem and spinal cord. Similarly, Stock et al. (1992) have observed aFGF immunoreactivity in distinct populations of subcortical and to a lesser degree in some cortical neurones. Like Weise et al. (1993), however, these authors emphasise that immunoreactivity is absent from glial cells and is found only in neurones.

Presence of FGF has also been investigated at mRNA level. Powell et al. (1991) report that whole embryonic and adult rat brains express different species of bFGF mRNA; embryonic brains contain a predominant 1.8 kb species, while adult brains contain smaller fragments, ranging in size from 1.0 to 6.0 kb . Moreover, in the embryonic brains, the 1.8 kb species is most abundant between E17 to E19, becoming undetectable at E20. These and other authors have also investigated the distribution of FGFR and FGFR mRNA. In the study of Powell et al. (1991), for example, a 4.3 kb FGFR-1 transcript was shown to

be present between E13 and E21. However, this transcript was also detectable at postnatal day 45. Wanaka et al. (1991) have studied FGFR mRNA expression in more detail; they show that FGFR transcripts first become detectable at E12 and that between this age and E17, the transcripts remain confined to the ventricular zone (they call ependymal layer). At E17, however, the transcripts can be detected both in the VZ and the cortical plate. Although these authors do not say so, their results seem to show a rostro-caudal gradient of FGFR expression, with highest expression in the more rostral (developmentally younger) cortex. Finally, Reid et al. (1990) have been able to PCR amplify two distinct forms of bFGF receptor mRNA from E10 mouse neuroepithelial cells.

The temporal expression of some other FGFs in the developing murine CNS has also been investigated, in particular that of int-2 (FGF-3) and KGF (FGF-7)(Wilkinson et al., 1988, and 1989; Mason et al., 1994). Mason et al. (1994), for example, report that at E14.5, FGF-7 expression in the mouse CNS is restricted to the VZ and cortical plate of the rostral cortex. Moreover, int-2 is also expressed in the developing mouse cortex (I. Mason, personal communication).

Although further qualitative and quantitative studies of FGF / FGFR gene and protein expressions are needed to correlate their presence with a particular aspect of telencephalic development, the present findings provide an important clue about the role of FGF; it is striking that the reported patterns of FGF immunoreactivity in the developing cortex coincide with the period of cortical neurogenesis, in vivo.

In any case, presence of FGF and its receptors within the developing CNS has encouraged several groups to investigate its effects on neural precursors, not just from the developing telencephalon but also other regions of the developing brain. These investigations, which have been carried out exclusively in culture, are discussed below.

1.7.3.4.3.2. Studies of acidic and basic FGF's effects on neural precursor cells

To date, almost all investigations of FGFs' effects on neural precursors have been in carried out in vitro; typically, dissociated embryonic cells have been cultured at a particular cell density in the presence and absence of FGF. The clearest observation reported by all such studies is that FGF (mainly bFGF) is a survival factor for neural precursors, as cultures grown in the absence of FGF either do not survive or contain significantly fewer cells. This survival effect has been scored either by gross visual comparison of cultures or more accurately by comparing cell counts from randomly chosen fields of view in controls versus FGF-treated cultures. In this way, FGF (mainly bFGF) has been shown to promote the survival of neural precursors from: E10 mouse telen- and mesencephalon (Murphy et al. 1990; Drago et al. 1991); E14 rat mesencephalon (Ferrara et al., 1989; Engele and Churchill Bohn, 1991, 1992), cerebral cortex (Gensburger et al., 1987; Deloulme et al., 1991), striatum (Cattaneo and McKay, 1990); E17 rat hypothalamus (Petroski et al., 1991); and E18 rat hippocampus (Walicke and Baird, 1988). On the

surface, these observations would suggest that FGF acts as a uniform survival factor for all neural precursors. Walicke (1988) has shown, however, that some neural precursors, such as those of the presumptive septum and subiculum, show no such response. Moreover, neural precursors derived from some CNS regions show a greater response than others; in Walicke's study, bFGF caused a 60% increase in the survival of cultured E18 hippocampal cells as compared to 39% for entorhinal, and 25% for parietal cortical cells.

Some authors have asked whether FGF is also a mitogen for neural precursors. Typically, this has been tested by comparing the number of cultured cells that incorporate ^{3}H-Thymidine within a defined period, in presence and absence of FGF (Gensburger et al., 1987; Murphy et al., 1990; Deloulme et al., 1991; Drago et al., 1991). The results of this approach, as admitted by most authors themselves, however, is difficult to interpret; detection of more thymidine-positive cells in the presence of FGF may reflect survival of a population of greater proliferative potential rather than a mitogenic effect *per se*. Moreover, thymidine incorporation itself, is not always an indication of division; bFGF could merely induce increased cellular DNA synthesis without such cells proceeding through mitosis. Thus, an increased rate of thymidine incorporation by a bulk population of cells *alone* is not an accurate indicator of a mitogenic effect by FGF.

Because FGF's effects have been tested mostly in bulk cultures of neural precursors, in which both neuronal and non-neuronal cells develop, a few groups have asked: what particular cell types respond to FGF's survival and/or mitogenic effects? To address this question, several workers have used cell type specific antibodies in order to identify cells that survive or incorporate labelled thymidine in the presence of bFGF. Gensburger et al. (1987), for example, stained their 5 day cultures of E13 cortical cells grown in the presence or absence of bFGF with anti-neurofilament antibodies. These authors report that in control cultures, only 8.5% of all neurofilament positive cells counted were labelled with radioactive thymidine, where as in the presence of 5 ng/ml bFGF, this proportion was 31%, thus concluding that bFGF had acted as a mitogen for neuronal precursors. Engele and Churchill Bohn (1991), on the other hand, have investigated the effects of acidic and basic FGFs on E14.5 mouse mesencephalic dopaminergic neurones by staining their cultures with antibodies against the enzyme tyrosine hydroxylase (TH). These authors could find between 190 to 210% more TH-positive cells in the presence of FGFs, concluding that both acidic and basic FGF had acted as a survival factor for dopamingeric neurones. Furthermore, these authors also find that both FGFs act as a mitogen for astrocytes since 20-40% more GFAP positive-thymidine labelled cells could found in the presence of basic and acidic FGFs, respectively.

Although these types of analyses are useful in determining whether FGF has any effect on neural precursors, they do not show directly how FGF may influence the behaviour of particular type/s of precursor cells. For example, discovery of more neurones in cultures

grown in the presence of bFGF can be interpreted in two different ways: bFGF could have acted as a survival factor for neurones or neuronal precursors, or, bFGF could have simultaneously enhanced the survival and neuronal differentiation of non-neuronal precursors. Murphy et al. (1990) indeed find that bFGF can induces neuronal differentiation of some undifferentiated E10 mouse neural cells. Thus, only a clonal analysis in which the fate of single cells is analysed can demonstrate whether or how bFGF can influence distinct cell types or their precursors. To date, only two groups have attempted such a clonal approach; these are the studies of Kilpatrick and Bartlett (1993) and Vescovi et al. (1993).

Kilpatrick and Bartlett (1993) have grown single E10 mouse telencephalic and mesencephalic neural cells in separate wells of a multiwell plate to discover that the survival of such isolated precursors depends on the presence of bFGF and serum. Moreover, three different clone types were found to survive under these conditions. Cells of the first type, designated clone type-A, were found to be amorphous and were not followed further. Cells in the second type, called 'non-proliferating type-B' were found to stop dividing after 10 days in culture and gave rise mostly to neurones. Most cells of the third clone type, called 'proliferating type-B' clones, however, maintained a proliferative potential as long as bFGF and serum were present. Although some proliferating type-B cells underwent spontaneous differentiation, most were found to differentiate only when bFGF and serum were withdrawn, or if cultures were supplemented by condition medium of an astrocyte cell-line. However, when cells did differentiate, two types of clones were generated: clones composed only of neurones, or clones composed of neurones and astrocytes. Despite prolonged periods of cultures, however, no oligodendrocytes were generated.

Although Kilpatrick and Bartlett (1993) have drawn a variety of conclusions about survival, proliferation and differentiation requirements of distinct precursors cells, presence of serum throughout their experiments makes is it difficult to attribute any one of these specific requirements to bFGF.

The second clonal study is that of Vescovi et al. (1993). These workers have tested the effects of bFGF on E14 rat striatal cells whose proliferation (and perhaps survival) depends on the presence of EGF. Vescovi et al. (1993) have shown that after 8 days of treatment with, and subsequent withdrawl of EGF, bFGF can act both as a mitogen and a survival factor for a subpopulation of precursor cells, which, in the continuous presence of bFGF give rise to two types of clones; clones composed of only neurones, and clones composed of neurones and astrocytes. Although results of Vescovi et al. (1993) show that bFGF has acted both as a survival factor and a mitogen for two distinct precursors, a unipotent neuronal precursor and a bi-potential neurone/ astrocyte precursor, it is difficult to know how much of this effect is due to pre-treatment with EGF. The precedence for this critisicm comes from the Cattaneo and McKay's work on the effects of NGF and bFGF on E14 striatal precursor cells in vitro. These authors show that while NGF alone

has no effect on such precursors, and bFGF alone acts as a survival factor, the combined presence of bFGF and NGF induces their proliferation.

Against this background, work presented in this thesis has employed a novel method of clonal analysis in order to investigate the effects of bFGF on rat embryonic cortical precursor cells. This involves following the fate of single retrovirally-labelled precursors, grown amongst a population of unlabelled cells, in the presence or absence of bFGF. This method has several advantages over the clonal analyses described above. First, it removes the stringent needs of single-cell culturing. In Kilpatrick and Bartlett's study, for example, serum was an absolute requirement for survival of isolated single cells. As another example, in Temple (1989)'s study, survival of single isolated E14 rat septal cells, placed in the bottom of a microwell, depended on the presence of embryonic striatal cells lining the walls of each microwell. Second, by identifying the progeny of single labelled cells (defined as a clone) with cell type-specific markers, it would be possible to determine the developmental potential of individual precursors in vitro and whether FGF has any influence on this potential. Third, it would be possible to determine which precursor type is affected by FGF. Fourth and finally, by analysing the size of particular clones types, it may be possible to quantify any mitogenic effect by bFGF.

In this respect, chapter three of this thesis examines the developmental potential of rat embryonic cortical precursors, while chapter four analyses the effects of bFGF on these precursors in vitro. However, both these studies rely on genetic labelling of single cells with a replication-defective retrovirus carrying a detectable histochemical marker gene. In this regard, it is essential to understand how a such a gene transfer is achieved. This understanding in turn depends on knowing the retroviral life cycle, its anatomy and how replication-defective retroviruses have been generated.

1.8 Retroviruses and overview of the retroviral life cycle

In 1911, Rous found that a filterable agent could cause cancer in chickens. It was not until early 1970's, however, that these filterable particles were identified as retroviruses. Since then, at least three different classes of retroviruses have been recognised: Retroviruses that cause tumors (Oncoviridae); those that cause non-neoplastic disease (Lentiviridae); and those that induce foamy vacuolization of cells (Spumiviridae)(Weiss et al., 1985).

Retroviruses are mobile genetic elements that carry two plus (sense) strand RNA molecules as their genome, a feature that distinguishes them from DNA viruses or minus-strand RNA viruses (Varmus and Brown, 1989). The life cycle of retroviruses, like that of most other viruses, is in two phases, an extracellular phase, in which they propagate horizontally from one cell to another, and a cellular phase on which their replication depends (figure 1.6). The cellular phase begins when retroviruses bind and enter host cells and reverse transcribe their RNA genome into viral DNA, the stage from which

Figure 1.6 The retroviral life cycle. Retroviral life cycle is in two phases, a cellular and an extracellular phase. The cellular phase, on which the viral replication depends, begins with binding and entry of viral particles into host cell cytoplasm. While in the cell cytoplasm, viral particles are uncoated and retroviral RNA genome is reverse transcribed into viral DNA; only one molecule of viral DNA is generated by each viral particle. The viral DNA gains access to the nucleus as part of a nucleoprotein complex, where it integrates into host cell DNA. As a consequence of this integration viral genes are transcribed into viral RNA along with host cellular genes. The viral RNA is translated into viral proteins, from which a new viral particle is assembled. Included in this particle also is a copy of viral RNA. Newly-generated viral particles leave their host by budding from its membrane thus becoming enveloped in a layer of host cell membrane.

The Retroviral Life Cycle

Binding and Entry

Host Cell

Cytoplasm

Viral DNA
(part of
nucleoprotein
complex)

Nucleus

Uncoating

Reverse
Transcription

Viral
RNA

Integration into
Host DNA

Transcription

Viral RNA

Assembly

Translation

Association with
Host Cell
Membrane

Viral Proteins
and RNA

Release
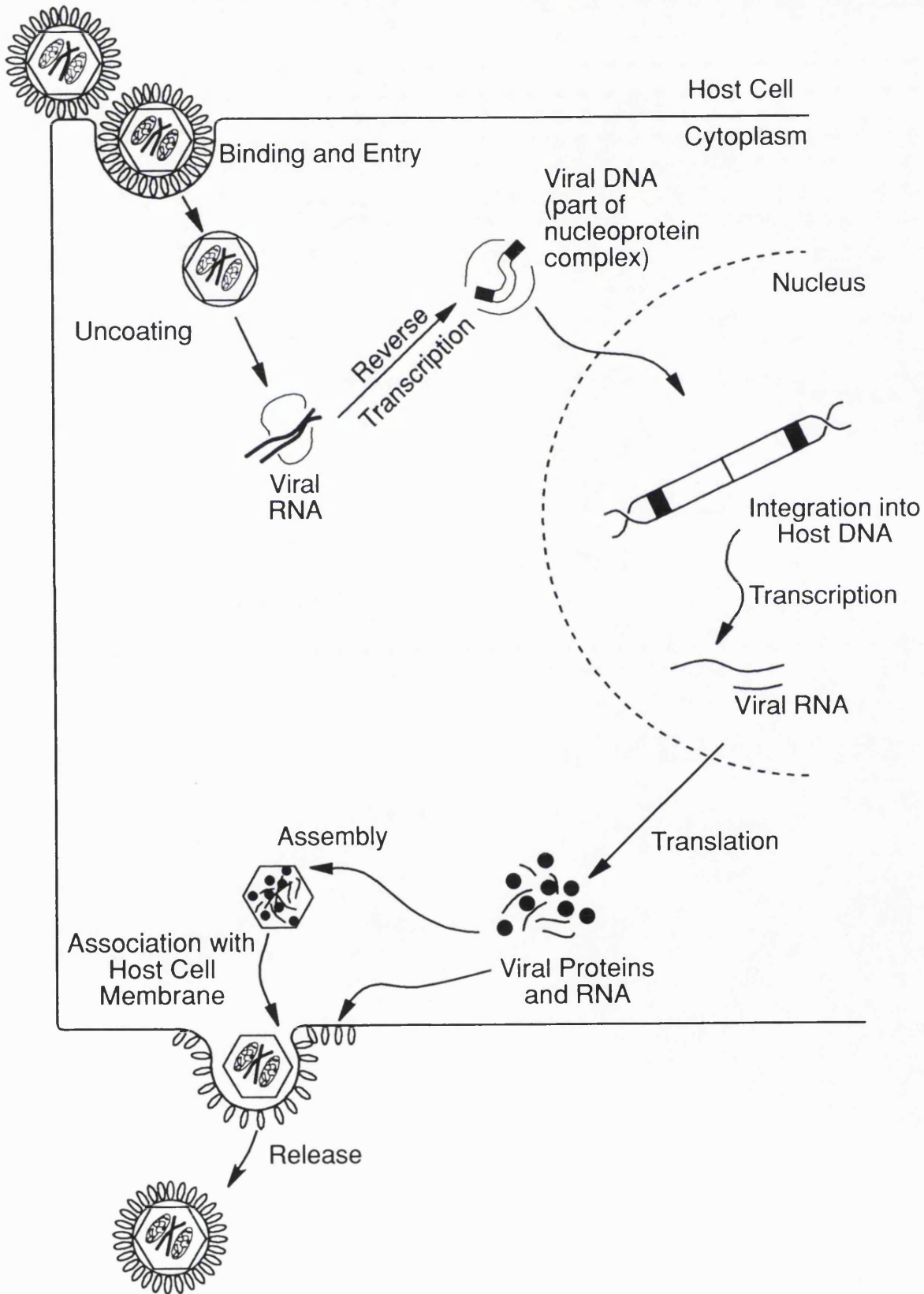
retroviruses derive their name. The viral DNA then integrates into host chromosomal DNA, where it is efficiently transcribed and translated into viral proteins. New viral particles are assembled from these proteins and leave the cell by budding off its membrane, thus completing the cellular phase of the retroviral life cycle (figure 1.6). The work presented in this thesis involves replication-defective viruses derived from a particular mouse virus, the Molonley-Murine Lukeamia Virus (Mo-MLV). The following sections, therefore, consider Mo-MLV in more detail.

1.8.1 The Retroviral genome

The genomic map of a wild type Mo-MLV and its provirus are shown in figure 1.7. Each viral RNA strand ends in a short sequence of terminal repeats (R) that plays an important part in the transfer of newly synthesised viral DNA strands during the reverse transcription process (see below). Each R sequence is inturn juxtaposed medially by two unique sequences termed U5 at the 5', and U3 at the 3' end of the viral RNA. During reverse transcription, these unique sequences are duplicated at either ends of the viral DNA strand, where, together with the R sequences, they form what are called Long Term Repeats (LTRs) (figure 1.7) The sequences that intervene between the unique sequences can be divided functionally into two main categories; those that are required in *cis* and act as regulatory or recognition sequences, and those that encode viral enzymes and structural proteins and are required in *trans* during various stages of the viral life cycle. Example of the first category include the transfer RNA (tRNA)-primer binding site (Pbs) just 3' of U5 that acts as the origin of reverse transcription, and the psi (Ψ) sequence that signals packaging of genomic viral RNA into virions. *gag*, *pol* and *env* genes, by contrast, fall into the second category.

1.8.2 Transcription and translation of viral genes into functional proteins

Expression of viral genes depends on the integration of viral DNA into host chromosomal DNA; when integrated, the viral DNA is called the provirus.

The proviral genes are transcribed under the influence of powerful transcription initiation signals present in the U3 domain of the 5' LTR. Two main mRNA species are generated from the provirus (figure 1.7). The transcription of the first, full length genomic mRNA, begins at the R region of the 5' LTR and ends at the polyadenylation site close to the 3' LTR, R sequence. This transcript has two functions; its serves as a template for translation of *gag-pol* , and is also be packaged into virions as genomic RNA. Translation of *gag-pol* from this template is initiated from an AUG translation signal just 5' of *gag* genes. The second viral mRNA species is transcribed in a spliced form and encodes *env* (figure 1.7)

Translation of both *gag-pol* and *env* genes from the above mRNAs produces several polyproteins that are subsequently cleaved to form functionally distinct proteins. Proteolytic cleavage of gag products yields three proteins; Matrix (MA), Capsid (CA) and Nucleocapsid (NC), all of which have important structural roles in the formation of a viral

Figure 1.7 Structure of wild type Mo-MLV genome and virion. At the top of this figure, the RNA genome of a wild type Mo-MLV is shown; retroviral particles contain two such RNA strands. Pbs is the primer biding sites for the enzyme reverse transcriptase, while Ψ sequence is important in the packaging of viral RNA into viral particles.

Reverse transcription of the viral RNA genome yields one molecule of double stranded viral DNA at the ends of which the U3 and U5 sequences have been duplicated to form what are known as long term repeat sequences, or LTRs. Sequences at the ends of the LTR molecules are important in the subsequent association of viral DNA with host nuclear DNA. Furthermore, LTRs contain powerful promoter sequences that can drive the transcription of genes downstream of themselves.

Transcription of viral DNA within the host cell nucleus yields a genomic and a sub-genomic viral RNA molecule.

Structure of a viral particle is shown at the bottom of this figure; colour coding is used to denote the contribution made by the products of *trans* acting viral genes, *gag, pol* and *env* to the structure of the viral particle, described in detail in the text. Abbreviations: U, unique sequence; R, repeat sequence; SU, viral surface protein; TU, transmembrane protein; MA, matrix protein; CA; capsid protein: NC, nucleocapsid protein; RT, viral enzyme reverse transcriptase; IN, viral enzyme integrase: and PR, viral enzyme protease.

Viral RNA

3'   3'

| R | U5 | | gag | Pol | env | | U3 | R |

Pbs

Ψ Sequence

Poly A

Reverse Transcription

Viral DNA

5'   3'

| U3 | R | U5 | | gag | Pol | env | | U3 | R | U5 |

LTR
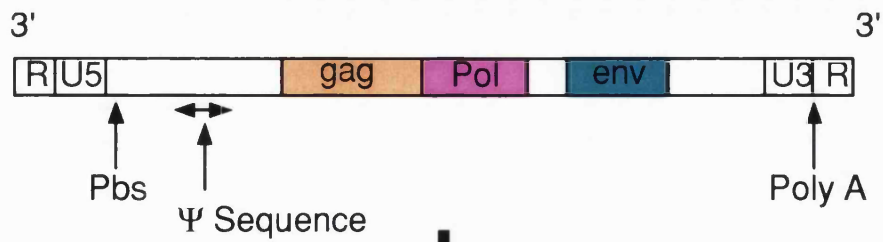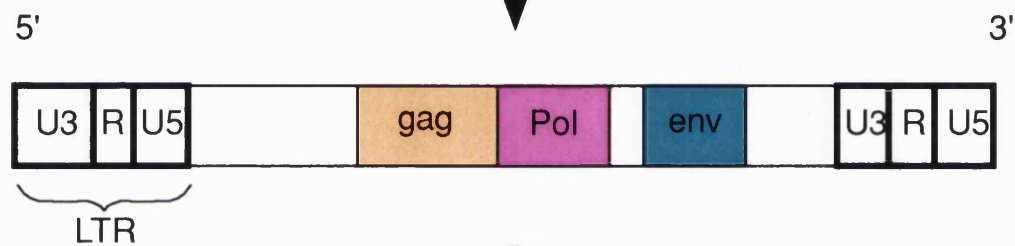
Integration and Transcription

Genomic RNA

| R | U5 | | gag | Pol | env | | U3 | R |

Subgenomic RNA

| env | | U3 | R |
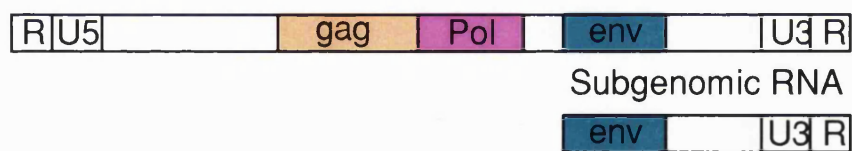
Translation, Proteolytic cleavage, Packaging and Assembly

Virion

SU
TU
MA
tRNA
CA
RT   IN
NC
PR
RNA Genome
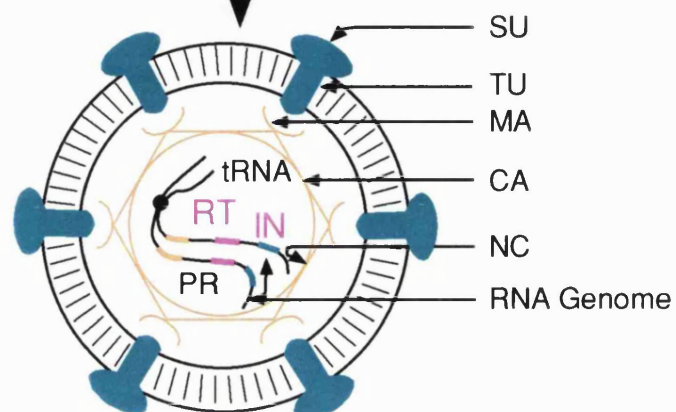
particle (figure 1.7). Cleavage of pol-derived polypeptides produces viral enzyme protease (PR) and a polypeptide of two other viral enzymes; reverse transcriptase (RT) and Integrase (IN). RT has a vital role in production of viral DNA from an RNA genome, and IN, which has endonuclease activity, is vital to the integration of viral DNA into host cell DNA.

Protein products of *env* are; transmembrane protein (TU), and surface viral protein (SU). Like gag and pol derived proteins, TU and SU are also generated initially as a polyprotein precursor that is cleaved by cellular proteases. SU becomes further glycosylated within the host cytoplasm before its insertion into the cell membrane, where it subsequently plays a critical role in specific binding of viruses to new host cells.

1.8.3 Structure, assembly and production of a virion

Free Mo-MLV retroviral particles in the extracellular environment are approximately 100 nm in diameter and are referred to as virions. Mo-MLV virions are enveloped spherical particles consisting of a core of viral RNA and nucleoproteins, wrapped in a capsid protein shell and surrounded in turn by a lipid bilayer. The lipid envelope, studded with viral envelope proteins, is acquired from host cell membranes as viral particles leave the cell.

The production of viral particles begins with the packaging of two copies of viral genomic RNA in association with other viral proteins and cellular tRNAs, into a nucleoprotein core. The signal for packaging of viral RNA is provided by the psi (Ψ)sequence, although the packaged sequences downstream of this signal do not necessarily have to be viral genes, a feature that has been exploited in producing replication-defective recombinant retroviral vectors. During the packaging process, viral RNAs are pulled into and wrapped by the nucleocapsid protein, NC, which has a high affinity for nucleic acids. Packaged viral RNA is a dimer of two RNA molecules linked at their 5' ends. Two possible mechanisms are thought to be responsible for this 5' linkage, (i) interaction of gag proteins, or (ii) base pairing of tRNA associated with one RNA strand, with some complementary sequences on the second RNA strand.

Three important viral enzymes, RT, IN and PR, and some host transfer RNAs are also packaged with viral RNAs. As a consequence of this packaging, the viral enzyme reverse transcriptase (RT) and host cell-derived transfer RNAs become physically associated with the viral RNA.

The packaged nucleoprotein core, which forms a 'C' or crescent shaped structure (in C-type retroviruses) is then enclosed within an icosahedral-shaped (in MLV) capsid protein, CA. In contrast to B and D type retroviruses, in which the assembly of virions occurs at random sites within host cells, in C type retroviruses assembly takes place close to host cell membrane. At these sites, CA is thought to become associated with an area of host cell membrane enriched with viral envelope glycoproteins. Although the precise nature of this association is unknown, it is thought that CA forms a linking bridge with host cell

membrane via the viral matrix protein, MA. Following this association, the viral core gradually evaginates from the host cell surface becoming surrounded by a layer of host cell membrane. Presence of viral glycoproteins, however, is not always necessary for this process, since viral cores can become associated with, and bud away from, areas of host cell membrane devoid of these glycoproteins (Varmus and Brown, 1989).

After leaving a cell, virions undergoes a maturation phase in which the viral nucleoprotein core is remodelled and becomes more electron dense. It is at this stage, for example, that viral protease, PR, cleaves the RT-IN polyprotein into separate molecules of RT and IN.

1.8.4 Infection of host cells and generation of viral DNA by reverse transcription
1.8.4.1 Binding and entry of viral particles

The term infection is an ambiguous one; it could define solely the process of viral entry into the host cell cytoplasm, or it could mean 'productive' infection where a viral particle becomes successfully established inside a host cell and completes its life cycle. Infection is used in the former context throughout this and the following chapters.

Infection of host cells begins by an electrostatic interaction of viral particles with the host cell membrane. This could be a non-specific or a specific interaction. In the former, viral particles may become loosely attached to lipids, proteins or carbohydrates on the cell surface and subsequently become endocytosed into pinocytic vesicles. The more specific interaction, however, involves binding of viral surface glycoproteins to distinct receptors expressed on host cell membrane (see reviews of Kabat, 1989, and Sommerfelt and Marsh, 1989).

1.8.4.2 Viral receptors

Although many viral receptors remain uncharacterised, several proteins have been shown to act as viral receptors. The Mo-MLV receptor, for example, is shown to be a 110kDa hydrophobic protein with 14 potential membrane spanning domains that is encoded by the *Rec-1* gene found on mouse chromosome 5 (Albritton et al., 1989). Although this receptor interacts with the gp70 subunit of MLV surface glycoprotein, SU, it is normally a transporter of cationic amino acids into cells (Wang et al., 1991). The binding-specificity of this protein for ecotropic MLV has been demonstrated by transfecting cells not normally prone to infection by MLV, with cDNAs coding for gp70-receptor; when exposed to MLV particles, these cells become infected with MLV. Furthermore, human gp70 receptor introduced into these cells can not bind MLV, although it shares 88% homology with mouse gp70 receptor (Yoshida et al., 1993).

Another well-characterised viral receptor is the CD4 molecule found on T lymphocytes; this 60 kDa protein has been shown to interact specifically with the HIV. As in the case of gp70 receptor for MLV, CD4 is not a protein expressed exclusively to bind HIV particles.

Rather, it is a serological marker of helper T lymphocytes distinguishing them from other T cell subtypes such as the CD8-bearing cytotoxic T cells of the immune system. Unlike gp70 receptor, however, CD4 is not a transmembrane protein and therefore is only thought to mediate binding of HIV. In this respect, some non CD4-bearing cells can become infected with HIV and most recently a second protein has been identified that may control subsequent entry of HIV particles into cells. Other examples of specific interactions between multifunctional receptors on host cell membranes and viral particles include: HSV-1's interaction with fibroblast growth factor receptors (Kaner et al., 1990), and binding of Influenza viruses to sialyated cell surface proteins via their HA surface molecule.

Binding of retroviruses to distinct cellular receptors is important in one other respect; it can restrict their host or even tissue range of infection. This is a particularly important factor in the design of retroviral vectors that deliver genes of interest to distinct hosts or cell types. On this basis, murine leukemia viruses can be divided into several subgroups; ecotropic, amphotropic and xenotropic. Ecotropic MLVs can only infect mouse and rat cells, while amphotropic MLVs show a broader host range that includes many mammals, including man, and even some avian species. By contrast, Xenotropic MLVs infect most non-murine species. In all cases, this restriction is conferred by the viral surface proteins encoded by the *env* gene and acquired during assembly and release of virions from their hosts.

1.8.4.3 Entry

Binding of viral particles to host cell membranes usually results in the entry and release of these particles into the cytoplasm. Naturally, this import of viral particles would be more efficient through specific rather than non-specific interaction of virions with the host cell membrane. Recent studies, however, suggest that mere binding by virions to cellular receptors is not sufficient for their entry into cells. For example, Wang et al. (1991) transfected cells non-permissive to MLV infection with cDNA for the Ecotropic MLV receptor, ecoR; they found that although many ecoR-expressing cells bound MLV, not all were able to import viral particles, suggesting that some additional cellular components linked to these receptors are required for viral entry. Interestingly, a strain of wild type mice have been isolated from lake Casitas in California that are resistant to infection by certain murine retroviruses (Gardner et al., 1991).; this resistance has been linked to the presence of the gene, Fv-4 (or AKvr-1) in these mice, that bears significant homology to the *env* retroviral genes. It is thought that Fv-4 products expressed in the cytoplasm, may interfere with viral binding and entry. In this regard, it is also noteworthy that packaging cell lines that normally express viral envelope proteins in their cytoplasm are also resistant to superinfection by viruses encoding the same *env* prototype (see below).

1.8.4.4. Release of viral particles into the cytoplasm

Viral core proteins become released into cell cytoplasm only when their host-derived membrane is removed. This may occur in several ways: direct fusion of this lipid bilayer with the host cell membrane at the cell surface, or inside pinocytic vesicles, or by degradation of this membrane inside the lysosomal vesicles. Uncoating of virions inside lysosomes requires low pH and therefore can be inhibited in the presence of weak bases such as chloroquine (Varmus and Brown, 1989).

1.8.4.5 Generation of viral DNA from viral RNA by reverse transcription

Reverse transcription is the process by which one molecule of double-stranded viral DNA is generated from two identical plus (sense) strands of viral RNA (Hu and Temin, 1990; Panganiban and Fiore, 1988). This process has several peculiar features. For example, almost all elements necessary for this process are contained within a virion. These include; two molecules of reverse transcriptase enzyme, transfer RNAs and genomic RNA templates, all existing in a complex encapsidated by CA.

The viral enzyme reverse transcriptase (RT), is similar in some respects to cellular DNA polymerases, in that it synthesize DNA from either an RNA and DNA template. Unlike DNA polymerases, however, RT is not inhibited by factors such as aphidicolin.

The signal that activates reverse transcription is thought to be viral-derived. gag-derived proteins, especially CA, are thought to play a role, although this process can be triggered by permeablising free virions with chemicals such as Nonident P-40 or melitin. It may be that a sudden influx of deoxynucleotides or even divalent cations into the CA complex trigger this process, since cations, such as manganese ions are known to be an absolute requirement for the activity of reverse transcriptase enzyme. The important point though is that reverse transcription can take place largely independent of the host cell derived factors. Despite earlier suggestions that S-phase of the cell cycle may be crucial to this process, it is now known that both infection and reverse transcription can take place even in G2 of the cell cycle (Roe et al., 1993). Reverse transcription can, however, be affected by external factors. For example, drug AZT is a powerful inhibitor of this reaction and has been widely used in anti-HIV therapy (Mitsuya et al., 1985).

Another feature of reverse transcription, is a series of DNA strand transfers (or 'jumps') between the two RNA templates during synthesis of two viral DNA strands, a minus and a plus DNA strands. These and other key regulatory features of this complex process are outlined in figure 1.8.

Reverse transcription has at least two important roles in the viral life cycle. First, it generates a double-stranded DNA molecule at the ends of which the unique sequences have been duplicated. Together with Rs, these duplicated sequences form the viral LTRs which are vital to the subsequent association and integration of viral DNA into host chromosomes. Second, the intermolecular DNA transfers provide an opportunity for

Figure 1.8 Steps involved in reverse transcription of viral RNA into viral DNA.

The opposite figure outlines steps involved in the reverse transcription of viral RNA into a molecule of double stranded viral DNA. This account is mostly true of Mo-MLVs,although with minor differences it would be true of all Retroviruses. Viral DNA is indicated by heavier lines and capital letters.

The first step (step 1) in reverse transcription is initiation of minus-strand viral DNA synthesis by the enzyme reverse transcriptase. This process begins from the primer binding site (Pbs) on one of the RNA templates and continues in a 3' to 5' direction up to a strong stop signal at the 5' end of viral RNA. The strong stop signal is exposed by degradation of the U5-R sequences of the viral RNA template by the ribonuclease activity of RNase H, a component of the reverse transcriptase enzyme (step 2). This cleavage has two consequences; it frees the ends of the nascent minus-DNA fragment (encoding the U5-R sequences of the RNA template), and also ensures that no further minus-DNA synthesis is initiated from the primer binding site, so that eventually, only one molecule of double-stranded DNA is produced by each virion.

The first transfer of DNA strands occurs when R of the nascent minus-DNA fragment becomes associated with the 3' R sequence of the second RNA template (step 3). Minus-DNA synthesis thereby continues towards the 5' end of this RNA template, until it reaches the Pbs of this RNA strand. It is noteworthy that this first DNA 'jumping' could have been intramolecular as well as intermolecular; i.e the 3' end of the same RNA template on which minus strand DNA synthesis began could be used for the homologous recognition.

While the minus DNA strand is being synthesized, the RNase H component of the reverse transcriptase enzyme frees the 3' end of this newly synthesized DNA from its association with the RNA template by cleaving the RNA molecule at the junction of 3' U3 and its upstream polypurine tract sequence (step 4). The exposed U3 sequence on the minus-strand DNA is then recognised and used by this reverse transcriptase enzyme to initiate plus strand DNA synthesis (step 5).

The plus-DNA synthesis continues along the minus-strand DNA until it reaches the Pbs sequence. It is thought that by another DNA transfer, this newly synthesized Pbs on the plus-strand DNA becomes associated with the Pbs sequence at the 3' end of the incomplete minus strand DNA (step 6). This association allows the minus- and plus-strand DNA fragments to use each other as templates for completing their DNA synthesis.

A comparison of minus and plus-strand viral DNA synthesis, therefore, shows that while synthesis of the minus-DNA is largely a continuous process, plus-DNA is produced from joining of discontinuously-generated DNA fragments.

Abbreviations: Pbs, primer binding site; ppt, polypurine tract.

Reverse transcription of viral RNA to Viral DNA

tRNA

r - u5 - Pbs   ppt - u3 - r

R- U5-

← tRNA

r - u5 - Pbs   ppt - u3 - r

1

▼

tRNA

r - u5 - Pbs   ppt - u3 - r

R- U5-

← tRNA

    Pbs   ppt - u3 - r

  RNAse H

2

▼

tRNA   R- U5-

                              ← tRNA

r - u5 - Pbs   ppt - u3 - r

tRNA

Pbs   ppt - u3 - r

3

▼

tRNA   PPt-U3-R-U5

                    ←   tRNA

r - u5 - Pbs   ppt - u3 - r

                        RNAse H

tRNA

Pbs   ppt - u3 - r

4

▼

                     U3- R-U5-Pbs

                    PPT-U3-R- U5-   →

←

r - u5 - Pbs   ppt   tRNA

5

▼

tRNA

Pbs   ppt - u3 - r

▼

U3- R-U5-Pbs   →

                      →   PPT-U3-R- U5-

                    ←

6

▼

U3- R-U5-Pbs   PPT-U3-R- U5-

U3- R-U5-Pbs   Double stranded viral DNA   PPT-U3-R- U5-

7
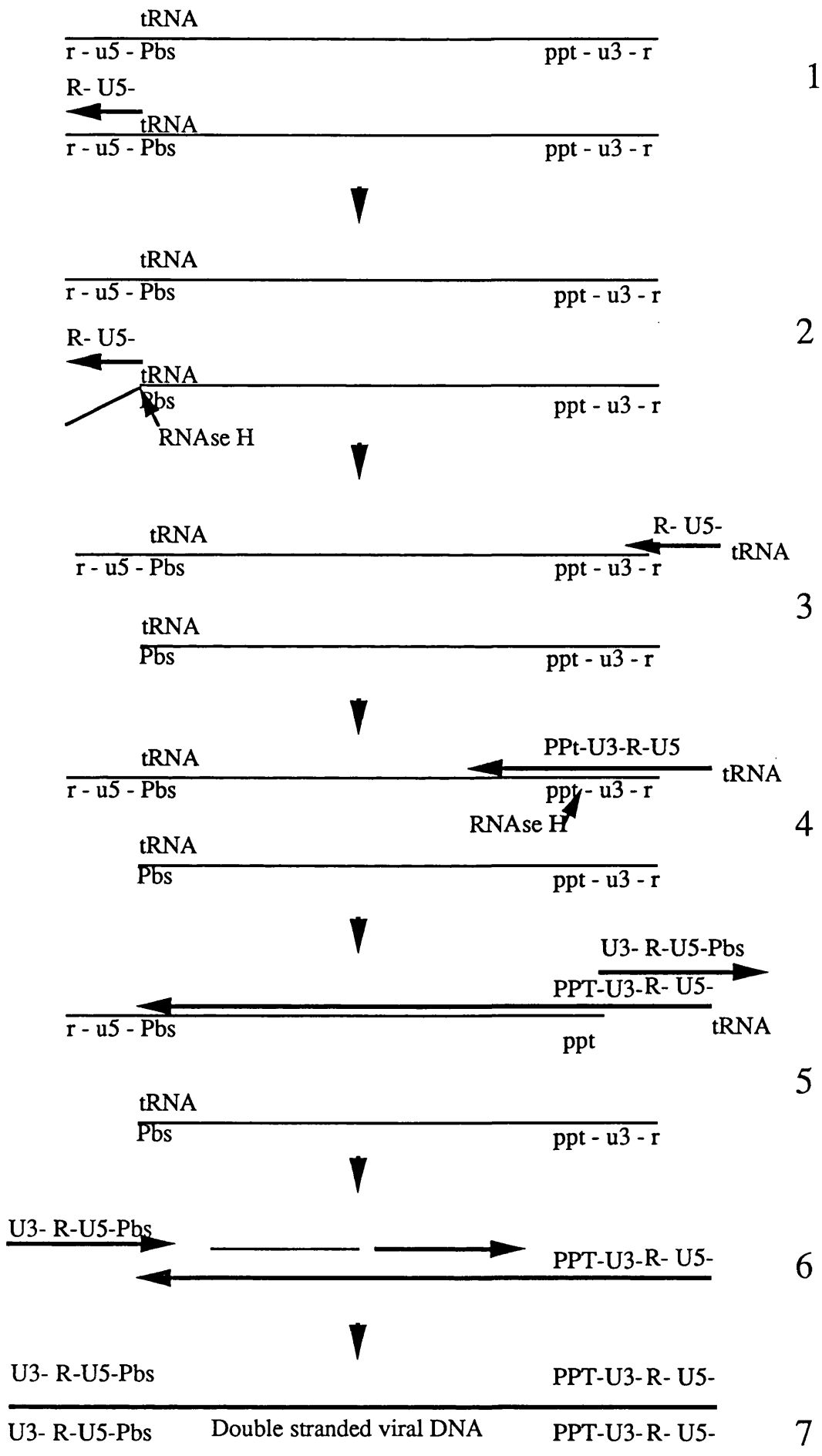
recombination between genes encoded on two separate viral RNA strands. This mechanism is implicated in the generation of helper viral particles (discussed below) and acquisition of cellular proto-oncogenes during evolution of oncogenic retroviruses.

1.8.5. Integration of viral DNA into host chromosomal DNA

Retroviral genes encoded by viral DNA are expressed efficiently only after viral DNA has integrated into the host chromosomal DNA (see reviews by Panganiban, 1985, and Goff, 1993). For this process, viral DNA produced by reverse transcription of viral RNA, migrates to the host nucleus as a 160S nucleoprotein complex (Bowerman et al., 1989). This complex contains important factors required for the integration process, amongst them, correct forms of viral DNA molecules and the viral integrase enzyme (IN; Roth et al., 1988). At least three forms of viral DNA molecules have been isolated from such nucleoprotein complexes; two of these are circularised and one is a blunt ended linear DNA. The circularised forms, which may contain one or two LTRs, are thought to arise by covalent joining of the ends of a linear molecule. It was only recently that the question as to which of these forms is the true precursor for integration was resolved; Fujiwara and Mizuuchi (1988), and Brown et al. (1989) showed that linear viral DNA is preferred to the circularised intermediates in in vitro integration reactions. Indeed, much of our knowledge about the dynamics and requirements of the integration process comes from in vitro integration studies, in which integration complexes are extracted from acutely infected cells and incubated with lambda phage DNA.

Both in vitro and in vivo studies show that the integration is a sequential multi-step process that depends on the activity of viral enzyme, Integrase (IN) (Brown et al., 1989). Importance of IN, which has both DNA-binding and endonuclease properties to the integration reaction can be demonstrated by mutating the most 3' sequences of *pol* , which encodes IN; although these mutant viruses can infect and produce reverse transcribed viral DNA inside infected cells, the viral DNA fails to integrate into the host cell DNA (Donehower and Varmus, 1984).

1.8.5.1 Steps and requirements of the integration reaction

Integration of viral DNA into host DNA occurs by a co-valent association of viral DNA LTRs with host DNA, a process in which the viral enzyme integrase (IN) plays a crucial role. The molecular details of the integration process, outlined in figure 1.9, suggest that viral integration takes place largely independent of host cells. However, there is evidence that host cell cycle is also crucial to viral integration. For example, S phase, the host DNA synthesis phase, has been long been recognised as a crucial phase for integration by many authors (Harel et al. 1981; Fritsch and Temin, 1977; Miller et al. 1990) although no particular factor, either abundant or scarce during this phase is known to influence viral integration. For example, viral integration is known not to require ATP or even de novo

Figure 1.9 Steps involved in integration of viral DNA into host chromosomal DNA. The viral DNA is thought to gain access to the nucleus as part of a nucleoprotein complex. Moreover, it is known that linear viral DNA molecules are the true precursors for viral integration and that the enzyme IN plays a crucial role in the integration process.

During the first step in the integration reaction, viral and host DNA are bound and brought together by IN (step 1). IN then cleaves two base pairs from the 3' ends of both viral DNA strands to expose a specific dinucleotide sequence, CA. This cleavage, which requires the presence of divalent cations (Magnesium ions are preferred by MLV integrase), is thought to involve activation of a phosphodiester bond that would be readily attacked by water molecules (Goff, 1993). Mutational studies show that varying the position of CA within the first 10-15 nucleotides at ends of the 3' DNA strands, or its substitution with TA, will sill result in cleavage of all sequences 3' of these nucleotides by IN. Furthermore, IN of different retroviruses normally remove different numbers of nucleotides before consistently exposing a CA dinucleotide at the 3' ends of viral DNA strands. It is thought, therefore, that CA dinucleotide is somehow recognised by IN, although recent studies suggest that nucleotide sequences downstream of CA play an important part in this recognition (Goff, 1993).

Having exposed the specific dinucleotide sequences, IN then cleaves cellular DNA in a staggered fashion (step 2). The first interaction of viral and host DNAs then takes place when the adenine nucleotide of the exposed 3' ends of viral DNA are joined to 5' ends of host DNA by hydroxyl bonding (step 3). Meanwhile, cellular endonucleases remove two base pairs from the 5' free ends of the viral DNA and duplicate four bases at the free 3' ends of the host DNA. Integration is the completed when the 3' host and 5' viral DNA ends are bonded together and the gaps in between filled by host enzymes (step 4).

Integration of Retroviral DNA into Host DNA
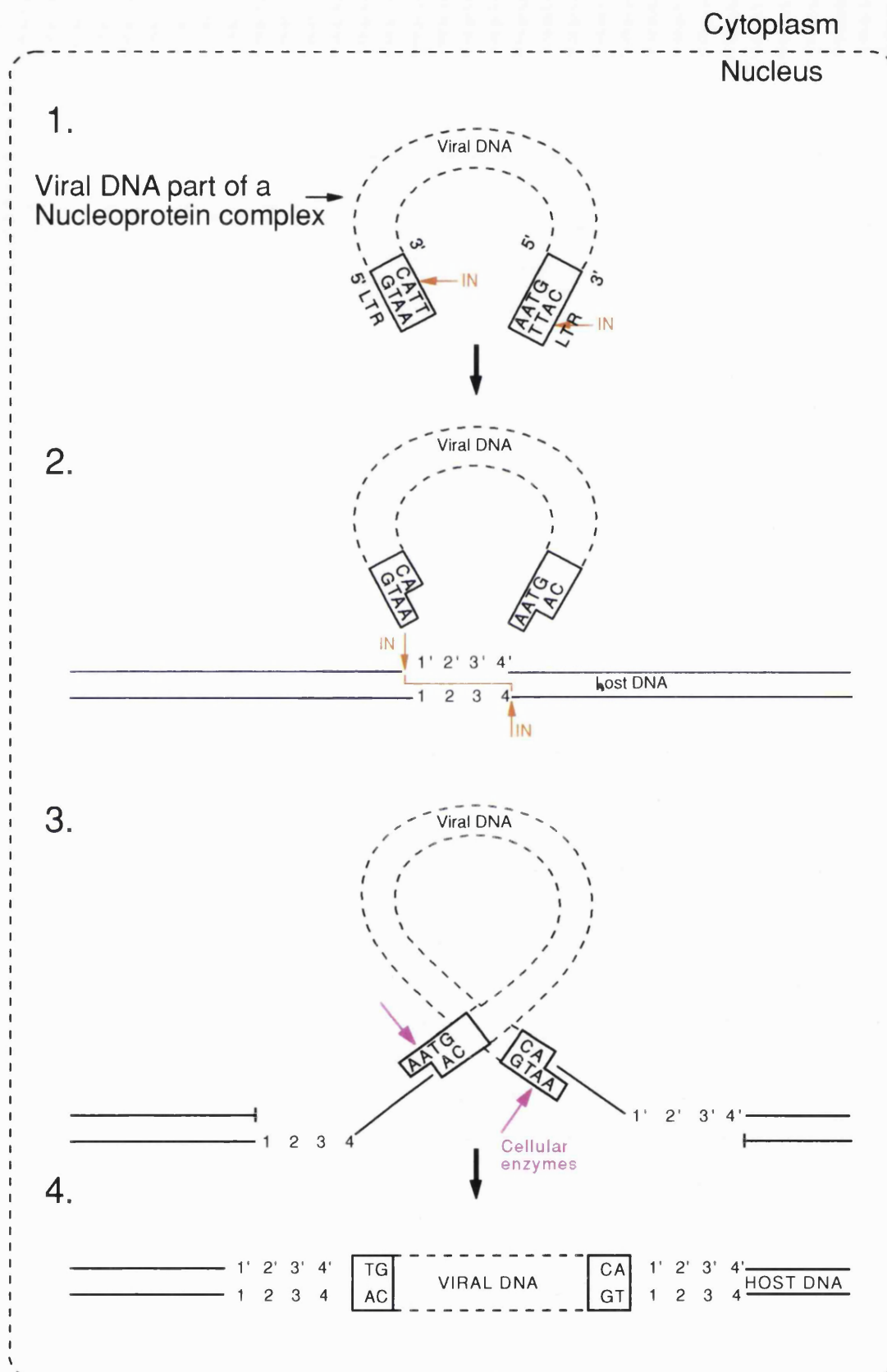
host protein synthesis (Brown et al., 1987). Noteworthy here is that a strain of mice, carrying the Fv-1 gene allele, are found to be resistant to MLV 'infection' (Jolicoeur, 1979). Although cells of such mice allow binding, entry and synthesis of viral DNA by MLV, such DNAs fail to integrate into the host genome. It may be that the correct preintegration viral DNA precursors are not made in these animals since viral DNAs isolated from their cells are mostly of the circularised form. In any event, these mice may provide a good model for investigating whether any host-derived factors can limit viral integration.

1.8.6 Generation of replication-incompetent retroviruses

A replication-defective retrovirus is one that is able to infect, integrate and express its genes in a cell but is unable to produce new viral particles. Such viruses have been used to deliver specific cDNAs that encode either therapeutic (e.g. Drumm et al., 1990) or histochemical marker genes (Price et al., 1987; Fileds-Berry et al., 1992) into single cells without any deleterious effect on the infected cell or horizontal spread of virus to other cells.

Production of such viruses requires two fundamental steps: first, production of suitable vectors that are efficiently expressed in infected cells, and second, production of viable virions capable of carrying such vectors. Retroviruses *per se*, pose a special problem as far as the first of these steps is concerned since there is a limit on the number of kilobases that a retroviral vector can carry. In the case of MLV-based vectors, this is about 6-7 Kb and although larger vectors can be produced, they will not be packaged efficiently into virions. In contrast to other vectors, however, retroviral vectors do not have a minimum vector size requirement. Successful MLV-based vector have been as small as 2.8 kb (Miller and Temin, 1986).

During the construction of a replication defective retroviral vector, *trans* acting but not *cis* acting genes are replaced by genes of interest. In the BAG vector, for example, *gag, pol* and *env* of the Mo-MLV have been replaced by E.Coli *Lac-Z* gene (encoding enzyme β-galactosidase) and the selectable marker, *neo* (encoding antibiotic neomycin). In the same vector, the transcription of the *Lac-Z* gene, is promoted by promoter sequences within the viral LTRs, and that of *neo* by a non-viral promoter, the SV-40 early promoter. This second 'internal' promoter is required because promoters of the viral LTRs can sometimes inhibit expression of some inter-LTR sequences.

1.8.6.1 Packaging cell lines: a strategy for generating recombinant virions.

Generation of virions that can carry recombinant vectors is achieved by use of what are termed packaging cell lines (figure 1.10). These are essentially transformed murine or avian fibroblasts that have been transfected with a distinct viral vector. This vector, in contrast to the replication-defective vector, carries all but one sequence of the wild type

Figure 1.10 Strategies for generating replication-defective retroviral vectors. Replication-defective retroviruses are made by transfecting or infecting packaging cells that lack the Ψ sequence and hence can not package viral RNA into virions, with a viral vector that carries the Ψ sequence upstream of genes of interest. In the case of BAG virus these genes are E.coli *Lac Z* and *neo*. In this way, the Ψ bearing vector RNA is packaged in to virions whose structural proteins are provided in *trans* by the packaging cell.

Infection of an ordinary cell (shown in the bottom diagram) with the recombinant virus results in expression of recombinant RNA and protein within that cell. However, because such a virus does not carry the viral structural genes, *gag*, *pol* and *env*, no new virions are produced within the infected cell, thus the recombinant retroviral vector is said to be 'replication-defective'.

Production of Replication-defective Retrovirus (BAG)

Ψ^+

| | Lac Z Neo | | ② |

Ψ^-

| ① | gag | Pol | env |

Transfection or Infection

Transfection Packaging all line

Nucleus

Ψ^- Integration

gag | Pol | env

Ψ^+ Integration

Lac Z Neo

RNA + Proteins
Ψ^+ Lacz Neo

RNA + Proteins
Ψ^- gag pol env
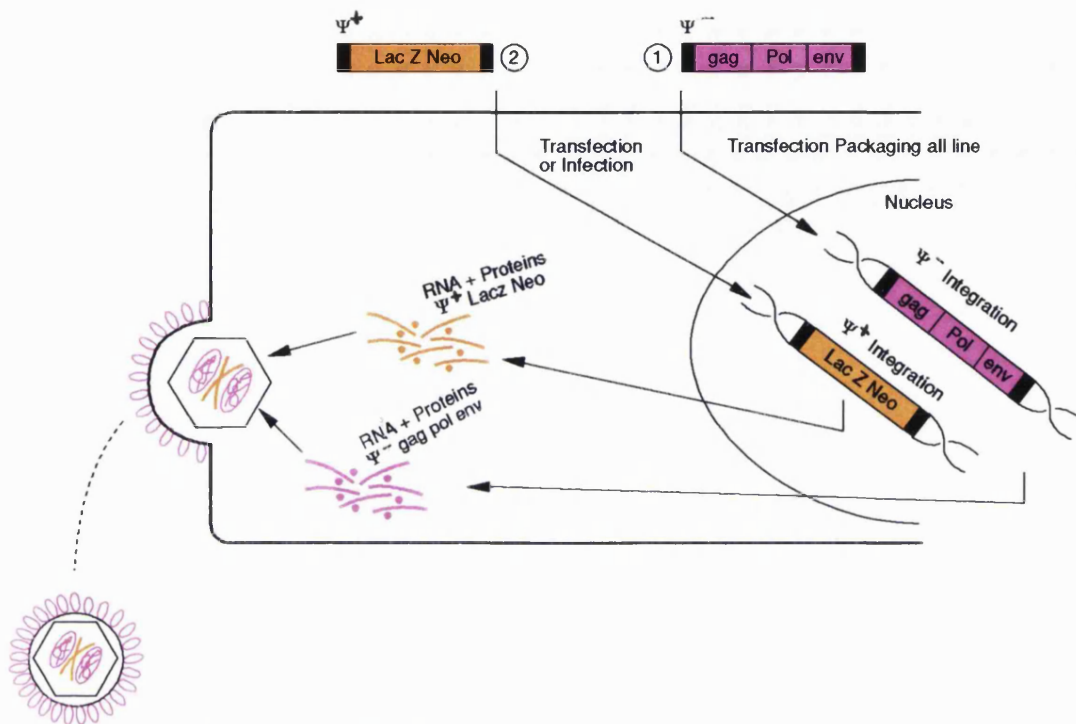
Infection of cells with replication-defective retrovirus (BAG)

cytoplasm

Nucleus

Uncoating

Reverse Transcription

Viral DNA

Ψ^+ Integration

Lac Z | Neo

β-galactosidase

Transcription and Translation

No packaging

X

RNA
Ψ^+ Lacz Neo

Neo
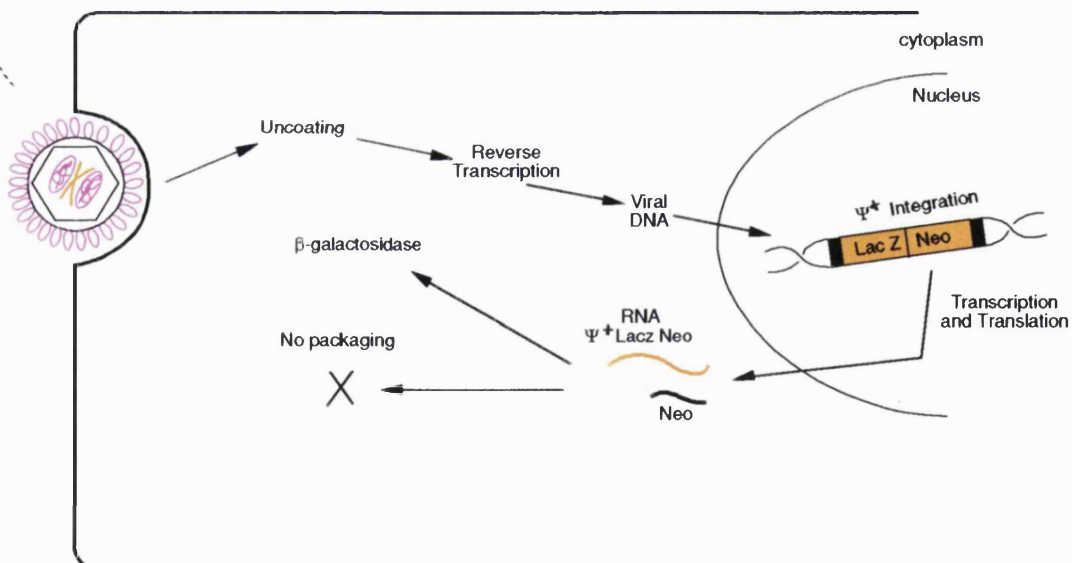
viral genes. The excluded sequence is psi (Ψ), the signal responsible for packaging viral RNA into virions. In this way, packaging cells would transcribe and translate viral structural proteins encoded by *gag*, *pol* and *env* from genomic and subgenomic RNAs respectively (see section), but in the absence of the psi sequence on the genomic RNAs produced in these cells, these RNAs do not become efficiently packaged into virions. This is not to say that virions are not normally produced by these cells, rather, they will be devoid of viral RNA or may even contain randomly packaged cellular RNAs.

In order to generate virions that carry the recombinant vector of choice, packaging cells are transfected (or infected) by the recombinant vectors (figure 1.10). This vector integrates in the genome of packaging cells and is translated into RNA and protein. In the case of BAG, packaging cells incorporating this vector stain positive for the β-galactosidase protein and can be selected by their expression of neo. Because a recombinant vector carries the psi sequence, it is packaged efficiently into virions whose structural proteins are provided in *trans* by the packaging cell line (figure 1.10).

Packaging cell lines are important in one other respect; the *env* gene products that are inserted into the membrane of the packaging cell and therefore the envelope of a replication-defective virus, will determine the host range of infection by these particles. Ecotropic retroviruses generated from Ψ2 or ΨCRE cells (Mann et al, 1983, Danos and Milligan, 1988), for example, can only infect mouse and rat cells, while this host range is extended to some other mammalian species by amphotropic viruses produced from Ψam, PA12 or PA317 cells (Cone and Mulligan, 1984, Miller et al, 1985, Miller and Bultimore, 1986).

However, recently the host range of MLV-derived viral vectors has been extended by using what is called a pseudotype virus (Burns et al., 1993). The *env* genes in packaging cells that produce this virus have been replaced by the vesicular somatitis virus envelope protein, G (VSV-G), which does not bind to specific receptors but interacts directly with lipoprotein membrane of cels. When packaging cells carrying the VSV-G env gene are transfected with an MLV-derived vector, a pseudotype virus is generated in which the core is MLV-derived but whose envelope carries VSV-G. This virus can, therefore, deliver MLV vectors to species not normally permissive to infection by MLVs.

1.8.6.2 Viral titres and helper virus

Production of replication defective retroviruses has posed two problems. The first, is production of adequate titres with which in vivo experiments can be carried out; low titers of such viruses would be inefficient in delivering genes into target cells as they are readily diluted by host fluids. Although many viral particles may be produced by packaging cell lines, most of these are lost during collection and concentrating of viral stocks. This problem again seems to have been partially resolve by production of the VSV-G pseudotype virus (Burns et al., 1993).

The second and more serious problem, however, is the possible generation of a wild type or 'helper' virus by a packaging cell. Helper virus is generated when a packaging cell line encoding viral structural genes but deficient in psi sequence (figure 1.10), acquires this sequence. This can occur in several ways:

(a) During continuous growth and expansion (by serial passaging) of a packaging cell line, there is a chance of genomic recombination between the vector that encodes viral structural genes and the replication-defective vector that carries the psi sequence, or (b) if a packaging cell line were to become infected with a wild type virus it would acquire the psi sequence. Should either of these events occur, wild type viral RNA will be packaged in preference to the replication-defective vector RNA.

More commonly, however, helper virus is produced by co-packaging of two different RNA molecules into the same virion; one derived from the replication defective vector carrying the Ψ sequence, and the other RNA encoding viral structural genes, deficient in this sequence. Should this virion infect a second packaging cell, a recombinant DNA will be produced during the intermolecular mode of reverse transcription. This DNA would then encode both the viral structural genes (derived from one RNA strand) and the psi sequence (obtained from the replication defective RNA). With subsequent integration of this recombinant helper DNA into the genome of a cell, helper particles will be produced. If these cells were to be packaging cells, then the helper virus will spread to other packaging cells. Interestingly though, this spread occurs very slowly since adjacent packaging cells, which express viral structural proteins in their cytoplasm, are resistant to superinfection by virus of the same envelope prototype.

Several approaches have been taken to reduce the chances of helper virus production. One is to reduce the homology between sequences in the vector encoding viral structural genes, and the replication-defective vector, thereby reducing the chances of recombination between these genes. The second and more successful approach, however, relies on producing packaging cell lines in which the viral structural genes are introduced into packaging cells as independent vectors. e.g. by transfecting packaging cells with two separate vectors, one encoding gag-pol, and the other env only. Although gag-pol and env will still be expressed in these cells, the chances of co-packaging RNAs encoding both these vectors with that of a replication-defective RNA into the same virion becomes infetesimally small. Indeed, no helper viral particles have been generated thus far from such packaging cell lines, examples of which includes the amphotropic packaging cell line, ΨCRIP (Danos and Mulligan, 1988).

1.9 Outline of the present study

Work presented in this thesis concerns three main lines of investigation. The first of these, presented in chapter II, asks what is developmental potential of cultured cortical precursor cells, in particular, whether cortical precursor cells are specified in their phenotypic potential as suggested by cell lineage studies of cerebral cortex in vivo. Further to this, studies presented in chapter IV investigate the effects of basic fibroblast growth factor (bFGF) on cultured cortical precursor cells in vitro with a view to understanding what role may bFGF play in the development of such precursors in vivo. In both these investigations, the emphasis will be on neuronal precursors. Moreover, both such investigations are carried out by analysing the fate of single retrovirally-labelled cortical precursor cells. In this regard, the third line of investigation, presented in chapter III, analyses an important aspect of viral integration which may have profound consequences for any study of retrovirus-mediated gene transfer. Work presented in chapter III asks whether retroviral DNA can integrate into post replication host DNA, as a consequence of which only some of the progeny of a virally-infected cell would inherit the viral genes. This question is addressed by analysing the distribution of viral genes amongst the progeny of single NIH-3T3 cells.

Chapter II

An analysis of the developmental potential
of rat cortical precursor cells

2.1 Introduction

Studies of cortical cell lineage in vivo (reviewed in section 1.4.4.5 of chapter I), in which the fate of retrovirally-labelled cortical precursors have been analysed, show that most such precursors give rise to clones composed of only a single cell type, either neurones, oligodendrocytes or astrocytes (Luskin et al., 1988, 1993; Price and Thurlow, 1988). This suggests that cortical precursors are specified in their phenotypic potential at early stages of development. This conclusion remained questionable, however, for two reasons. First, recent findings that some cortical cell types can migrate for hundreds of micrometers in vivo suggested that distant retrovirally-labelled cell clusters, previously defined as the progeny of separate precursors, may infact be related; i.e.may have been generated by a common precursor. These findings, therefore, cast doubt on the phenotypic uniformity of clone types in vivo, and hence on the early specification hypothesis (see review by Guthrie, 1992). The second reason was that cell lineage studies in situ only analysed the presumptive cell fate and not the developmental potential of cortical precursor cells. To test developmental potential, Williams et al. (1991) carried out a clonal analysis in vitro, and discovered that the majority of retrovirally-labelled E16 precursor cells give rise to clones that are composed of only one cell type. Moreover, the clone type restrictions obtained in vitro were identical to those found in vivo. Williams et al. (1991), therefore, provided evidence that E16 cortical precursors were specified in their phenotype. However, Williams et al. (1991) did not provide an accurate measure of the proportions of different clone types, particularly that of neuronal clones. Moreover, these authors did not analyse the proliferative potential of cultured E16 precursors in vitro. Several factors may have contributed to these shortcomings. First, Williams et al. (1991) could identify only a subpopulation of neurones; those that were immunoreactive for anti-neurofilament or anti-MAP-2 antibodies. While the former labels mature neurones, the latter is known to label only a subpopulation of neurones in vivo (Huber and Matus, 1984) and in vitro (own observations). Second, the same authors labelled cortical precursor cells some 24 hours after their isolation from the embryonic cortex, a time gap during which the behaviour of cortical precursors may have changed in vitro.

As a prelude to studying factors that may influence the development of neuronal precursors in vitro, this study, like that of Williams et al. (1991), has aimed to test the developmental potential of cortical precursor cells. By labelling precursors immediately after their isolation from the embryonic cortex, and by identifying all neurones with a novel neurone-specific antibody, this study asks two particular questions: what proportion of cortical precursor cells generate neurones, and what is the proliferative potential of neuronal precursor cells in cultures of E16 and E14 rat cortex?

2.2 Material and methods

2.2.1 Cell cultures: culture medium

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, pH 7.0, Northumbria Biologicals) supplemented with: penicillin (0.06 g/l), streptomycin (0.1g/l) and L-glutamine (0.3g/l). According to which cell type was cultured, this medium was supplemented further with serum, buffers or special substances. For NIH-3T3 cells and Ψ2 BAG producer cells, this medium was supplemented with 10% Normal Calf Serum (NCS, Gibco). For cortical astrocytes, the supplement was 10% Foetal Calf Serum (FCS, Globepharm). Dissociated embryonic cortical cells, by contrast, were cultured in Sato's medium (Bottenstein and Satos, 1979) using the modified recipe of ffrench-Constant and Raff (1986). The ingredients of this medium were: Bovine Serum Albumin (BSA, 100μg/ml), glucose (5.6mg/ml), insulin (5μg/ml), progesterone (60ng/ml), putrescine (16μg/ml), selenium (30nM), thyroxine (2 mg/ml), transferrin (100 μg/ml), triiodothyronine (0.3μg/ml) and 0.5% heat-inactivated FCS. Some of these ingredients were added in a step wise process; insulin was first dissolved in 1 ml of distilled water by addition of a few drops of concentrated hydrochloric acid (HCl); selenium was added from a 1mg/ml solution of sodium selenite; progesterone was added from a 12 mg/ml stock solution dissolved in 95% ethanol/ 5% distilled water; thyroxine and triiodothyronine were first dissolved in a small (1to 2ml) volume of ethanol to a concentration of 10 mg/ml and 7.5 mg/ml, respectively, after which few drops of sodium hydroxide were added and the solution was diluted in 5 ml of distilled water; and finally, transferrin was always added last.

All media, whether supplemented or not, were kept at 4°C and warmed to 37°C prior to use.

2.2.2 Maintenance of NIH-3T3 and Ψ2 cells in culture

Cultures of NIH-3T3 or Ψ2 cells were set up using cells previously frozen and kept at -190° C (in liquid nitrogen), in 10% DMSO/90% DMEM. 1ml of frozen-cell aliquots were rapidly thawed at 37° C and mixed with 9 ml of prewarmed 10% NCS/DMEM. This cell suspension was then centrifuged for 10 minutes at 1000 rpm. The resulting supernatant was discarded and the remaining pellet of cells was resuspended in fresh 10% NCS/DMEM and plated onto a 9 cm Falcon plate.. 24 hours later, the cells were inspected under an inverted microscope, and if found to be healthy, were passaged onto fresh 9 cm plates.

2.2.3 Passaging (subculturing) of NIH-3T3 and Ψ2 cells

When confluent, NIH-3T3 or Ψ2 cells were routinely subcultured onto fresh 9 cm plates. For this, medium bathing the cells was removed and plates were rinsed once with 8 ml of prewarmed 0.1% trypsin-versene solution (containing 0.1 g/l EDTA, NIMR media).

Cells were then incubated for 5 minutes at 37° C with 2 ml of fresh prewarmed trypsin-versene solution and detached from the plates by gentle shaking. Trypsinisation was stopped by adding 8 ml of fresh 10% NCS/DMEM and the resulting cell suspension was diluted 1:10, 1:20 or 1:40 in 10% NCS/DMEM and plated onto fresh 9 cm plates and transferred to a 37° C, humidified, 5% CO_2 incubator. Thereafter, cultured cells were inspected and fed every 48 hours by replacing the medium bathing them with fresh 10% NCS/DMEM.

Plates of NIH-3T3 or Ψ2 cells passaged at 1:40 dilution, were found to reach confluency after 4 and 5 days in culture, respectively.

2.2.4 Cryopreservation of NIH-3T3 and Ψ2 cells

In order to maintain a constant source, NIH-3T3 or Ψ2 cells were occasionally frozen down and stored at -190° C (in liquid nitrogen). Normally, ten 90%-confluent plates were used for this purpose; cells were removed from these plates by trypsinisation (described above), pooled together, and centrifuged for 10 minutes at 1000rpm. The resulting supernatant was discarded and the pellet of cells resuspended in freshly prepared 10% DMSO/ 10% NCS/80% DMEM. This suspension was then aliquoted into plastic cryotubes (Nunc) in a ratio of 1 ml per original plate, then frozen for 30 minutes at -20° C followed by 24 hours at -70° C, before finally being transferred to -190°C (liquid nitrogen).

2.2.5 Preparation of BAG viral stocks
2.2.5.1 Collection of BAG virus from supernatants of Ψ2 producer cells

BAG virus was collected from surpernatants of Ψ2 cells. For this, Ψ2 cells were plated onto 7-10 falcon plates, as described above. When plates had reached 90% confluency, the medium bathing the cells was changed from 10 ml 10% NCS /DMEM, to 5 ml of 10% FCS/ DMEM. 16 hours later, medium from all plates was pooled and passed through an analytical filter unit (0.45 μm pore size) to remove any floating cells but not viral particles. The filterate was then divided into 1 ml aliquots in cryotubes and kept for 30 minutes at -20°C before being transferred to - 70°C.

2.2.5.2 Titration of viral stocks

The viral titre was determined by infecting NIH-3T3 cells. For this purpose, NIH-3T3 cells were removed from confluent plates and plated in 2ml of medium onto grided 3.5 cm falcon plates, to a final density of 1.0 x 10^5 cells per plate. Ten to twelve hours later when the cells had attached, the medium was removed and cells were infected overnight in 1ml of medium with different dilutions of either the virus under test or virus of known titre, in the presence of 8 μg/ml polybrene (Sigma). The following day, the infection cocktail was replaced by 2 ml of fresh medium and cells were thereafter fed every 48 hours with fresh

10% NCS/DMEM. Three to four days after infection when the plates had become confluent, cells were fixed for 15 minutes with 0.5% glutaraldehyde (BDH, made by 1:50 dilution of a 25% solution in 1x PBS), and stained with the chromogenic substrate X-gal (Diagnostic Chemicals, Charlottetown, Canada). Titrations were done in triplicates, the results of which were used to estimate the titre of virus in the original suspension expresses as colony forming units (cfu) per ml.

2.2.6 Detection of BAG-infected NIH-3T3 cells by substrate X-gal

BAG-infected NIH-3T3 cells, which would express the enzyme β-galactosidase, were detected by incubating plates of cells exposed to the BAG virus with a mixture of X-gal solution and buffer. X-gal solution was prepared in dimethyl formamide (DMF) at 40 mg/ml. Buffer (pH 7.4) was prepared in 1x PBS from the following substances/ solutions (final buffer concentrations): 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$; 2mM $MgCl_2$; 0.01% NaOH and 0.02% Nonident P-40 (NP40). Before use, X-gal was added to the buffer at a concentration of 1 mg/ml. Glutaraldehyde-fixed cells were then incubated with this mixture for 1 hour at 37°C. Once blue cells became visible, X-gal mixture was removed and cells were rinsed once with, and later kept in 1x PBS. No blue staining was observed when uninfected plates of NIH-3T3 cells were incubated with X-gal.

2.2.7 Screening viral stocks for 'helper virus'

As explained in section 1.8.6.2, with successive passages of Ψ2 cells, the replication-defective BAG vector may acquire the Ψ sequence thereby generating a replication-competent vector and eventually a 'helper virus'. Cultured and frozen stocks of Ψ2 cells were, therefore, routinely tested for the presence or the production of helper viruses. This was assayed by determining BAG-infected NIH-3T3 cells were capable of producing new viral particles. For this test, NIH-3T3 cells grown on 9 cm plates were infected with undiluted stocks (containing approximately 6.0×10^4 cfu) of BAG virus in the presence of 8 µg/ml polybrene. 12 to 15 hours after infection, the plates were rinsed once with fresh medium, then incubated in 10% NCS/DMEM for 48 hours, at which point this conditioned medium was collected and added to fresh uninfected, low density plates of NIH-3T3 cells, in presence of 8 µg/ml polybrene. These NIH-3T3 cells were again grown to confluency and stained with substrate X-gal. The presence of blue colonies among these NIH-3T3 cells was taken as evidence of helper virus production. In no case, however, did these plates show blue colonies, indicating the absence of helper virus.

As control for this analysis, BAG-producer Ψ2 cells were stained with the same X-gal solution; every cell on these plates stained blue.

2.2.8 Preparation of primary cultures of cortical astrocytes

Primary cultures of cortical astrocytes were prepared according to the protocol of Noble and Murray (1984). Brains of 1-2 day old rats were removed after decapitation and placed in 9cm falcon petridishes containing 0.02M Hepes-buffered medium. The cerebral hemispheres were dissected free of olfactory bulbs, the hippocampal formation, diencephalic structures and the meninges, and pooled in 5 ml of Hepes-buffered medium. Dissected cortices were then minced by trituration in a glass pasteur pipette and incubated with 0.025% trypsin (Sigma) for 30-40 minutes at 37°C. Trypsinization was stopped with 5 ml of DMEM containing 10% FCS. Tissues were drawn three to five times into and out of a syringe through hypodermic needles, first using a 0.8 mm, and then a 0.6 mm needle. In order to remove clumped cells and debris, the resulting homogenate was passed through a piece of sterile nylon gause with grid size of 200 µm. The resulting filterate was centrifuged (Centaur 2) for 5-8 minutes at 1000 rpm, and the pellet resuspended in 8-10 ml of fresh 10% FCS/ DMEM. The cells were counted in a heamocytometer; on average, 3-4 $\times 10^5$ cells were obtained from two cortical hemispheres of each neonatal animal.

Dissociated cortical cells were plated in 10% FCS/ DMEM at a density of 1.0 x 10^7 per ml onto poly-D-lysine-coated 80 cm^2 flasks (Nunc). Flasks were then transferred to a 37° C, humidified, aerated (5% Carbon dioxide, 95% air) incubator. The day after plating and every subsequent 48 hours, cultures were fed with fresh 10% FCS/ DMEM until they were confluent. Usually two layers of cells were visible in such confluent cultures; cells in the lower layer were flat and resembled astrocytes or fibroblasts, while those of the 'top' layer resembled oligodendrocytes. Occasionally, however, a pure monolayer of flat cells only could be seen in such cultures. In order to detach the top cells, flasks containing confluent cultures of new-born cortical cells were wrapped in aluminium foil and placed overnight on a horizontal shaker (200 rpm), in a 37° C warm room. The following day, the detached cells were removed by aspirating the media in the flasks. The adherent cells were then detached from the flasks by a 5 minute treatment with prewarmed 0.1% trypsin (in 0.02% EDTA) solution and transferred to fresh PDL-coated 80 cm^2 flasks. The subcultures were treated for 12 hours with the mitotic inhibitor, cytosine arabinoside (Ara-c, 0.1mM Sigma) to kill rapidly dividing cells. Following this treatment and thereafter, the purified subcultures of cortical astrocytes were fed every 48 hours by replacing the medium bathing the cells with fresh 10% FCS/ DMEM.

2.2.8.1 Transfer and subculturing of astrocytes onto PDL-coated coverslips

Purified cortical astrocytes were plated onto PDL-coated coverslips. For this, the medium in each flask was removed and cells were rinsed once with 8 ml of prewarmed trypsin-versene solution. The cells were then incubated for 5 minutes at 37° C with 2 ml of fresh trypsin solution and detached by a sharp tap to the side of the flasks. Trypsinisation was then stopped by addition of 8 ml of fresh 10% FCS/DMEM. The cells

were then counted and plated at a density of 2 x 10^5 cells/ml onto PDL-coated glass coverslips placed in the wells of 24-multiwell plates. 24 hours later, and every subsequent 48 hours, these purified astrocytes were fed with fresh 10% FCS/DMEM, replacing half the medium bathing the cells. Astrocytes were then allowed to form a confluent monolayer on glass coverslips. To test the purity of these astrocyte monolayers some coverslips were stained with antibodies against the glial fibrillary acidic protein (GFAP). 95% of cells in these cultures were found to be positive for this antibody.

2.2.9 Preparation of cultures of embryonic cerebral cortical cells

Timed pregnant rats were acquired from the Biological Services at NIMR. On their 14th or 16th day of pregnancy, plug date being day zero, rats were asphyxiated with CO_2 and the embryos delivered by caeserian section. Embryos were then cut out of the uterine and amniotic sacs, placed in a 9 cm petri dish containing Hepes-buffered medium, and transferred to a sterile hood cabinet. They were aged by measuring their crown-rump length according to the measurements of Hebel and Stromberg (1986). However, since these authors time embryonic development from the pro-oestrus stage (rather than the appearance of the cervical plug a day later), there would have been a one day disparity between their staging and that of this study. Accordingly, therefore, their crown-rump length of 20.5 mms for 17-day-old embryos (E17), was used as a measurement for E16 embryos.

Cerebral cortices of the staged embryos were dissected free of non-cortical structures and the meninges, pooled, trypsinised and dissociated as described for preparation of cortical astrocytes (section 2.2.8). Cells were counted with a haemocytometer; typically, 1.0 x 10^6 cells were obtained from each embryonic cortex. Embryonic cells were then plated in 0.5 ml of 10% FCS/DMEM onto monolayers of cortical astrocytes that had been grown on glass coverslips placed inside the wells of 24 multiwell plates (figure 2.1). Normally, 2.0-2.5 x 10^5 cells were added to each well. Prior to plating, however, embryonic cells were mixed in suspension with a small volume of supernatants collected from Ψ2 BAG producer cells, allowing 15 μl of supernatant per well. This volume, which delivered approximately 900-1000 cfus of BAG virus to each well, was shown in preliminary experiments to yield a low number of infections (see below).

Once cells were plated, plates were transferred to and kept at 37° C in a humidifed incubator, containing 5% Co_2. This was noted as day 1 of culture. Twenty hours after plating, all of the medium bathing the cells was replaced with 0.5 ml of Sato's defined medium and thereafter cultures were fed every 48 hours, by replacing only half of the volume of medium in each well with fresh, prewarmed Satos/ DMEM.

In a few experiments, cells were infected with BAG at different time points after plating e.g. on days 2, 3 or 4 of the culture. This was done by exposing cultured cells to 900-

Figure 2.1 Diagram of the protocol followed for culturing embryonic cortical cells on confluent monolayers of cortical astrocytes. Purified monolayers of cortical astrocytes derived from the cerebral hemispheres of newborn rats were prepared as described in text. These astrocytes were grown to confluency on 35 mm PDL-coated glass coverslips placed inside the wells of multi-well plates. Embryonic cortical cells were obtained by microdissection and subsequent dissociation of embryonic cerebral cortices, and plated on confluent monolayers of cortical astrocytes at which point a low titre of BAG virus was also added to cultures inorder to infect a small number of embryonic cortical cells. 24 hours after plating, cultures were switched from the plating medium that contained serum to Sato's medium (Bottenstein and Sato, 1979; see text for ingredients).

After seven days in cultures, during which period cultures were routinely inspected and cells were fed every 48 hours with fresh medium, glass coverslips were recovered and cultures were fixed and stained with anti-β galactosidase as well as a combination of two other cell type-specific antibodies.

Preparation of Embryonic Cortex

Embryonic
Day 16

Cerebral
Hemispheres

Preparation of Cortical Astrocytes

Postnatal
Day 1-2
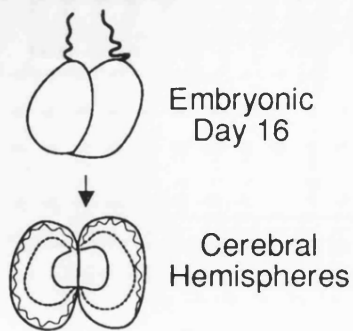
Cerebral
Hemispheres
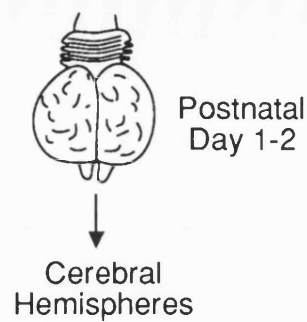
95% pure preparation
of cortical astrocytes

Poly-D-lysine

35mm
Glass Cover
Slip

Coverslip recovered
and stained
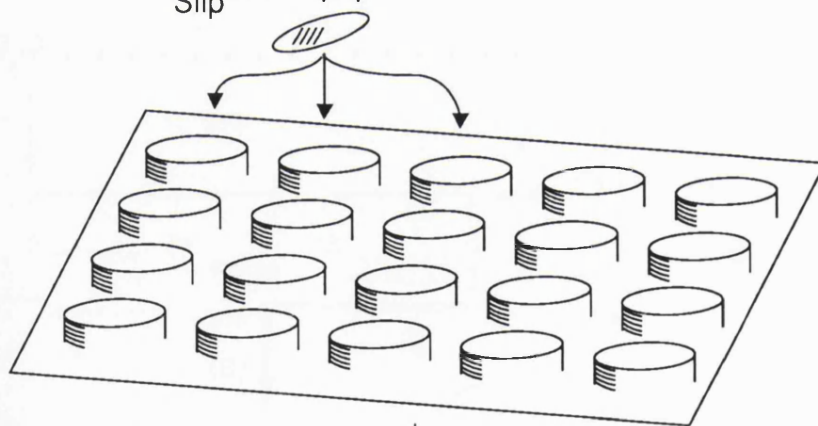
Confluent Monolayer
of Astrocytes

+BAG Virus

Culture switched to
defined medium

Day 0*   1   2   3   4   5   6   7

Days in culture

1000 cfu of BAG per well, overnight. 20 hours after the exposure, the entire medium bathing the infected cells was replaced with 1ml of fresh Sato's medium.

All embryonic cultures were grown for a minimum of seven days, a period during which cultures were examined briefly every other day under an inverted microscope.

2.2.10 Detection of BAG-labelled cells and identification of their phenotype in cultures of embryonic cortical cells

The aim of this work was to identify the cell types that single BAG-labelled cortical cells produce after seven days in culture (method outlined in figure 2.1). This was achieved by first detecting the BAG-labelled cells and then identifying the phenotype of such cells with cell type-specific antibodies. BAG-labelled cortical cells could be distinguished by their expression of the enzyme β-galactosidase, the protein product of the BAG reporter gene, *Lac-Z*. This protein could be detected in one of two ways: either by antibodies to the protein, or by incubation of cells with the chromogenic substrate, X-gal. For this study, however, immunofluorescence was preferred. Nonetheless, the steps involved in either methods of staining is described below.

2.2.10.1 Fixation and preparation of cultured cells for immunofluorescence staining

After seven (or in a few experiments 10 or 13) days in culture, all medium in the wells was gently removed and cells on coverslips were fixed for 15 minutes with 4% paraformaldehyde in 1x PBS (pH 7.4). After this, paraformaldehyde solution was removed and cells were rinsed gently with 1x PBS solution. Coverslips were then removed from the wells using a curved needle tip and rinsed several times with 1x PBS buffer before being incubated with primary antibody.

2.2.10.2 Staining of fixed cells with anti β-galactosidase and cell type-specific antibodies (combined triple immunofluorescence)

2.2.10.2.1 Type and dilution of antibodies used

All primary and/ or cell-type specific antibodies that were used in this study, are shown in Table 2.1. Unless otherwise stated, antibodies were diluted to the appropriate concentration (also show in Table 2.1) in a solution of 10% Normal Goat Serum (NGS) made in 1x PBS. Moreover, prior to staining with primary antibodies, all fixed cells were treated once for 10-15 minutes with 10% NGS/ PBS solution inorder to block any non-specific binding sites that may have been present on cortical cells, or the Fc bindings sites on macrophages that occasionally developed in the cortical cultures.

Coverslips stained with anti-β gal antibody were treated for 5 minutes with 1% triton-X100 solution (made in 1 x PBS). Also, when staining with anti-MAP-2, MAP-5, or GFAP antibodies, cells required a 15 minute pretreatment with 5% glacial ethanoic acid/95% absolute ethanol (acid-alcohol) solution at -20°C.

Cell type recognised	Antibody or antibody against	Class	Dilution used	Source/ References
Neurones	Neurofilament (NF)	IgG_{2a}	1: 50	Wood and Anderton (1981)
	Microtubule associated protein-2 (MAP-2)	IgG_1	1: 20	Sigma/ Binder et al. (1986)
	Neuron-specific Class III, β-Tubulin (TuJ1)	IgG_{2a}	1: 1000	Geisert and Frankfurter (1989)
Neural cells (including neurones)	Microtubule associated protein-5 (MAP-5)	IgG_1	1: 50	Sigma/ Riederer et al. (1986); Tohyama et al. (1991)
Oligodendrocytes	O4	IgM	1: 3	Sommer and Schachner (1981)
Astrocytes	Glial fibrillary acidic protein (GFAP)	IgG_1	1: 100	Boheringer- Manneheim/ Bignami et al. (1972)
Others antibodies	β-galactosidase	Poly- clonal	1: 1000	Generated in J. Price laboratory

Table 2.1 Primary/ cell type-specific antibodies used in this study. All antibodies were diluted in 1 x PBS, containing 10 % Normal Goat Serum (NGS). During the initial experiments, TuJ1 was not available and thus neurones were identified with NF or MAP-2. Only a subpopulation of β-gal positive cells that resembled neurones, however, were found to label with MAP-2; the neural identity of the remainder could be verified by staining with MAP-5. However, MAP-5 was also found to label some oligodendrocyte precursors. Subsequent availability and staining with TuJ1 showed that all neurone-resembling β-gal positive cells were positive for this antibody. Moreover, double labelling with TuJ1 and MAP-2 showed that indeed MAP-2 labels only a subpopulation of mature neurones as has been reported in vivo.

2.2.10.3 Order and combinations of antibody staining

All coverslips were stained with anti-β gal antibody as well as a combination of two cell-type specific antibodies shown in Table 2.1. Combinations used were: 1 = {O4, β-gal, MAP-2 or MAP-5, or NF}; 2 = {O4, β-gal, TuJ1}; 3 = {O4, β-gal, GFAP}; or 4 = {TuJ1, β-gal, GFAP}, respectively. As routine, cells were incubated with primary antibodies for 30-40 minutes at room temperature (19-21° C). However, some primary antibodies, such as anti-β gal and anti- MAP-2 antibodies, required an overnight incubation, in which case cells were incubated at 4° C.

In between each each primary antibody, the relevant fluorescently-tagged secondary antibody (all obtained from Bionuclear Services) was applied. Staining with O4 antibody was normally visualised by tagging with rhodamine-coupled goat anti-mouse IgM immunoglobulins, and β-galactosidase with fluorescene-coupled goat anti-rabbit immunoglobulin. Staining with anti-GFAP, TuJ1, MAP-2 and Neurofilament were visualised by a two step tagging with secondary antibodies; first the relevant class specific biotinylated goat anti-mouse antibody was applied, after which cells were incubated with streptavidin-coupled 7-amino-4 methyl-coumarin-3-acetic acid, which appears blue under the blue/coumarine filter. These secondary antibodies were always applied for 30 minutes at room temperature.

2.2.10.4 Controls

In the initial experiments, both positive and negative controls were carried out by omitting one or more of the primary or the relevant fluorescently-tagged secondary antibodies, respectively. In both cases, no staining of the fixed cortical cells was observed.

2.2.10.5 Identification of BAG-labelled cells by combined X-gal/ immunocytochemistry

Some cultures of BAG-infected cortical cells were stained with X-gal. For this, cells were fixed for 15 minutes at room temperature with 0.5% glutaraldehyde in PBS solution. Fixed cells were then incubated with X-gal in a procedure identical to that described for detection of BAG-infected NIH-3T3 cells (section 2.2.6).

In some experiment, X-gal-stained coverslips were also stained with one cell type-specific antibody; anti-TuJ1 antibody, for example, was used to identify neurones on such coverslips. For this purpose, X-gal stained coverslips were first treated with 1% triton for 5 minutes, followed by a one hour incubation with TuJ1. TuJ1 was then tagged with horse radish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin, followed by a 5-10 minute incubation with a 0.5 mg/ml solution of Di-aminobenzedene (DAB; made in 0.05M Tris buffer), in the presence of 0.01% hydrogen peroxide solution. When stained in this way, a brown colour developed over TuJ1-positive cells and their processes.

2.2.10.6 Analysis of stained coverslips

2.2.10.6.1 Mounting of stained coverslips

Coverslips stained either with the triple combination of antibodies or with X-gal were washed thoroughly in 1x PBS solution and placed onto a drop of glycerol/PBS (Citiflour), placed on rectangular glass slides. Coverslips were then gently pressed and excess glycerol/ PBS was wiped off their edges with absorbent tissue. Coverslips were further immobilised on glass slides by applying a thin film of clear nail varnish to their rims.

2.2.10.6.2 Scanning stained coverslip: definition of a clone

The entire surface of each stained coverslip was scanned under the x25 objective of a Zeiss microscope which was equipped with an ultra violet light source and three different excitation filters: fluorescene, rhodamine and coumarine (blue). During this scan, clusters or colonies of β-gal positive cells could be seen scattered randomly over each coverslip surface. Two clusters of β-gal positive cells separated by at least 300 μm, were regarded as arising from infection of two distinct precursor cells, and therefore two separate 'clones'. This distance was initially determined using a graticule mounted in the microscope eye piece, although it could also be readily judged using the diameter of one circular field of view under the x25 objective, which, corresponds to approximately 590-600 μm on the coverslip surface.

2.2.10.6.3 Identification of clone types and numbers

The phenotype of cells within each clone was determined by visualising β-gal positive cells under a filter corresponding to a particular fluorescently-tagged cell type specific antibody. Neurones, for example, were usually seen under the blue filter because, neurone-specific antibodies were normally tagged by a two step process to strepavidin-coupled coumarine which appears blue under the blue excitation filter.

As well determining the phenotypic composition of each cell cluster, the number of cells in each cluster were also noted. Whenever necessary, cells were examined further at higher power, typically under the x63 objective.

Photographs were taken using Fujichrome 1600 colour or black and white films, either under the x25, or x63 objective. Exposed films were processed and printed by NIMR's photographic section.

2.2.10.7 Calculating the probability of superimposition of two separate clones

As noted in section 2.2.6.2.7.2, two clusters of β-galactosidase positive cells separated by at least 300 μm were considered as two separate clones; i.e the progeny of two separate virally-labelled precursor cells. However, because precursors were infected at random and independent of each other, and that the original position of such infected precursor cells could not be determined, the possibility that occasionally two precursors separated by

less than 300 μm became infected must also be considered. If this were to happen, the progeny of two different precursors would have been counted as a single clone; the so-called superimposition of clones or 'lumping error'. Naturally, the probability of such an error increases with the number of infections (clones) obtained per coverslip; in the experiments described in this thesis, between 14 to 32 clones could be found per coverslip. Thus, in order to calculate the probability of clone super-impositions with these clone numbers, the following argument was developed.

In these experiments, precursor cells were infected while cultured on circular 13 mm-wide glass coverslips, the area of which would be approximately 132.7 mm^2 according to the formula, πr^2, where r is the radius (6.5 mm). Furthermore, if 300 μm were to be the criteria by which two neighbouring cell clusters are regarded as two separate clones (clones A and B below), the area allocated to each clone would be approximately 0.28 mm^2 according to the formula πr^2, where r is 300 μm or equal to the distance between two neighbouring cell clusters.
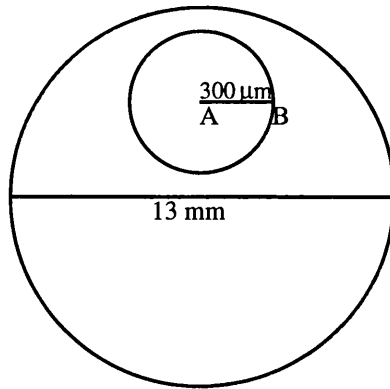
Thus in calculating the probability of clone super-impositions, one can ask what is the probability that after the first infection has taken place, for the second infection not to fall within the presumptive clonal area of the first infection. Similarly, for the third infection, one may ask what is the probability of the third infection not taking place within the presumptive clonal areas of the first two infections, and so on.

In this regard, the probability of superimposition for the first infection is 1, since this is a unique infection; i.e. P = 1. However, the probability that the second infection would fall outside the clonal area of the first would be given by:

$$P = 1 - \left(\frac{0.28}{132.7} \right) = 0.9978$$

where 1 - (0.28/132.7) is the area in which infection of the second precursor would not lead to super-imposition of clones. For the third infection, this probability would be:

$$P = 0.9978 \times \left(1 - \frac{0.28 \times 2}{132.7 - 0.28} \right) = 0.9936$$

and, for the fourth:

$$P = 0.9936 \times \left(1 - \frac{0.28 \times 3}{132.7 - 0.56} \right) = 0.9872$$

According to this progression, the value obtained for the 14th, 24th and 32nd infections are: 0.8338, 0.5504 and 0.3309, respectively. This is to say that with 24 infections per coverslip, 45 (from 1 - 0.5504) out of every 100 coverslips would have a superimposed clone, or, 45 out of every 2400 clones arise from clone super-imposition, a ratio approximately equal to 1 out of every 53 clones. Similarly, for 14 clones per coverslip, 1 out of every 82 clones, and for 32 clones per coverslip, 1 out of every 67 clones would be the result of clone superimposition.

Thus, with increasing clone numbers per coverslip the chances of clone superimpositions increases dramatically. It should be noted, however, that most experiments analysed in this study contained between 14 and 24 clones per coverslip.

However, there is the possibility of another error, the so-called 'splitting error'. In this senario, if members of the same clone were to migrate away from each other by more than 300 μm, they would be counted as two separate clones. Without knowing how far such cells migrate, however, it is difficult to calculate this probability.

2.3 Results

BAG-labelled E16 or E14 cortical precursor cells were grown for seven days amongst unlabelled cortical precursors on monolayers of cortical astrocytes. The first question was whether each cultured cortical precursor cell was capable of generating one, or several of the cell types found in the adult cortex in vivo. To answer this question, the progeny of each BAG-labelled precursor, defined as a clones of β-galactosidase positive cells, were stained with a combination of cell type-specific antibodies so as to identify the phenotype of cells within each clone.

Neurones were identified using either TuJ1, an antibody against a neurone-specific form of tubulin (Geisert and Frankfurter, 1989), or anti-neurofilament, or anti-microtubule associated protein (MAP-2) antibodies. Oligodendrocytes and astrocytes were identified using O4 and GFAP antibodies, respectively. It was discovered that each of these cell types demonstrates a characteristic morphology in vitro. Mature oligodendrocytes usually had a stellate to chandellier-like appearance (figure 2.2), while astrocytes appeared as flat or fibrous cells, distinct from the cortical astrocytes of the monolayer (figure 2.3). Neurones, by contrast, usually possessed small, round to elipsoid cell bodies from which only a few processes emanated. Moreover, a perinuclear spot was consistently visible in β-gal positive neurones only (figure 2.4), a phenomenon that has also been observed in X-gal positive neurones in vitro, and in vivo (Price and Thurlow, 1988).

Cells within each clone could therefore be identified, not only by their positive immunoreactivity with a phenotypic marker, but also by their characteristic morphology.

2.3.1 Type and frequency of clones found in cultures derived from E16 and E14 cortical precursors

When clones derived from E16 or E14 precursors were analysed, it was discovered that the majority of clones were made up of only a single cell type (Table 2.2). Four such clone types could be found; these were, clones made up entirely of neurones, oligodendrocytes, astrocytes, or cells which neither stained with any of the phenotypic markers, nor showed any characteristic morphology. The only exception to this clonal homogeneity was the discovery of a rare clone of neurones together with astrocytes in E14 cultures.

Each of the described clone types occurred at a different frequency, with neuronal clones being the most frequent (Table 2.2). In E16 cultures, for example, 87% of all clones were neuronal; the remaining 13% accounting for the combined frequencies of oligodendrocyte, astrocytes and unidentified clones. A similar proportion of neuronal to non-neuronal clones was also observed in E14 cultures (Table 2.2). These proportions were obtained, however, only if cortical cells were infected within 48 hours of plating. When cultures (E16) were infected with BAG at successively later time points, dramatically fewer number of clones were obtained, most of which were non-neuronal (Table 2.3).

Progressively fewer clones were also obtained the longer the cells were cultured. In one experiment, an average of 15 clones per coverslip were found after 7 days in culture. When two sister coverslips of the same experiment were analysed 6 days later, however, only 3 clones could be found per coverslip (Table 2.4), suggesting that as much as 80% of clones had disappeared between days 7 and 13 of culture.

3.

2.2. Size range of different clone types

As well as recording the frequency, the number of cells in each clone was also recorded. The size of non-neuronal clones was found to show extreme variations both between and within experiments. In one experiment, for example, four oligodendrocyte clones were recorded; three of these contained between 2 and 5 cells while the fourth contained 100 cells. Noteworthy, however, is that such 100-cell oligodendrocyte clones were rare. Also, clones of astrocytes or unidentified cells rarely exceeded 20 cells.

In view of their low frequency of occurrence, therefore, it seemed as though there was no obvious correlation between the size of non-neuronal clones and a particular culture condition or age; i.e. similar variations were found in E14 as well as E16 cultures. However, a closer examination would be required to categorically rule out any such correlation.

2.3.3 The size of neuronal clones: a comparison of E16 and E14 derived cultures

Neuronal clones detected in both E16 and E14 cultures could be divided into those containing only one cell (i.e. single-cell clones), and those containing 2 or more cells. The majority of neuronal clones found in both E16 and E14 cultures fell into the first category; in E16 cultures, just over 90% of all neuronal clones were single-cell clones, a proportion that was close to 75% in E14 cultures (figure 2.5)

E16 and E14 cultures, however, differed dramatically in their frequency and size range of the second category of neuronal clones. In E16 cultures, the 2-cell and greater neuronal clones accounted for only 9% of all neuronal clones, and the largest such clone contained only 5 cells. In E14 cultures, by contrast, these larger neuronal clones made up 25% of all neuronal clones. Moreover, neuronal clones as large as 20 or even 50 cells were detectable (figure 2.5 and 2.6). In terms of morphology, it was possible to divide these larger E14-derived neuronal clones into two groups. Cells in the first group, were more dispersed, had a uniform appearance and resembled E16-derived neurones. Typically, 2-10 cell neuronal clones fell into this group. Those in the second group, that contained 20-50+ cells, however, appeared as tight clusters of round and process bearing cells. Cells in this group were so tightly clustered that it was often impossible to obtain an accurate count of the number of cells within the clone. Moreover, it was also difficult to determine whether all cells in such clones were positive for the neuronal marker, TuJ1 (figure 2.6). Nonetheless, no member of these larger neuronal clones was found to be positive for any

of the non-neuronal markers. In addition, these clones were distinct from the large, rare clone of neurones and astrocytes found in E14 cultures (figure 2.7). In these clone mixed clones, astrocytes were found to be the dominant cell type.

Figure 2.2 Clones of BAG-labelled oligodendrocytes. Frame A shows a three cell clone of β-gal positive cells visualised with fluorescene-conjugated goat anti-rabbit antibody. These cells have a chandelier-like morphology typical of mature oligodendrocytes. O4 staining in frame B, as visualised with rhodamine-conjugated goat anti-mouse anti-serum, shows that indeed these cells are oligodendrocytes. Frame C shows a large clone of oligodendrocyte-like cells as revealed by X-gal staining of a 7-day cultures of E16 cortical cells. Note how members of an oligodendrocyte clone are dispersed relative to each other. Scale bar in A and B= 10 μm, and in C= 30 μm.
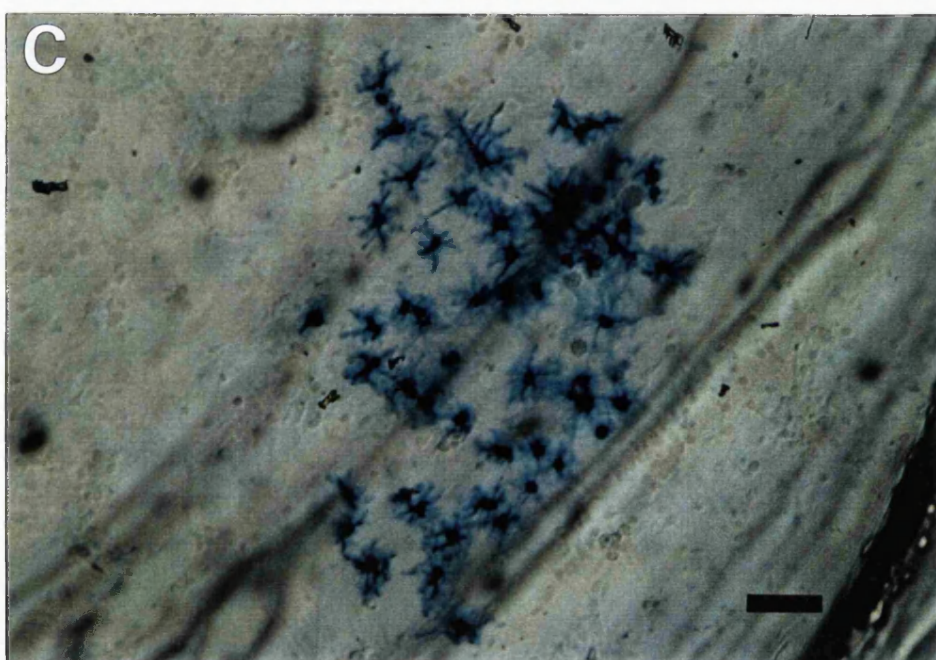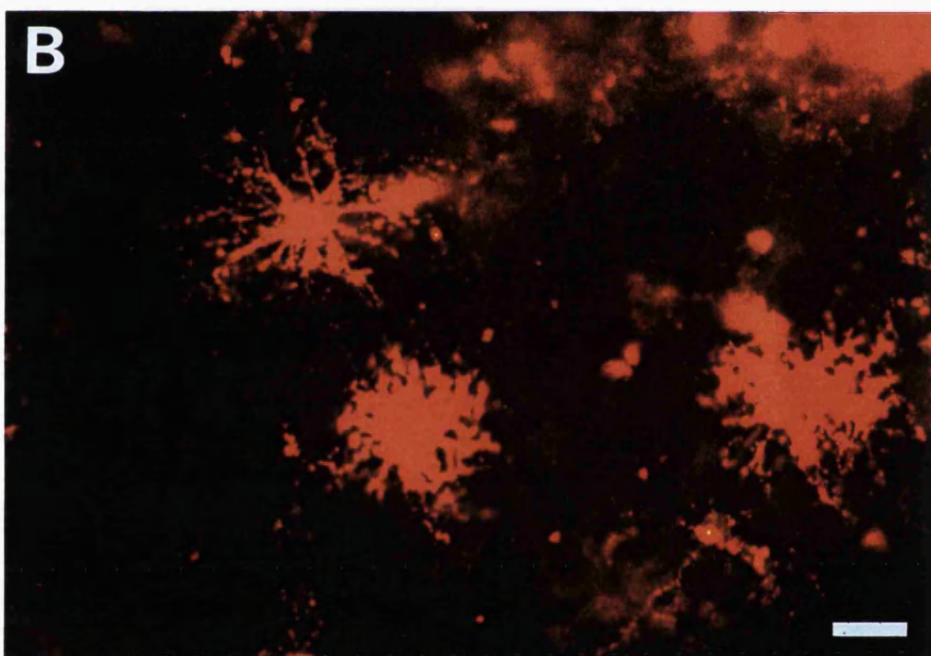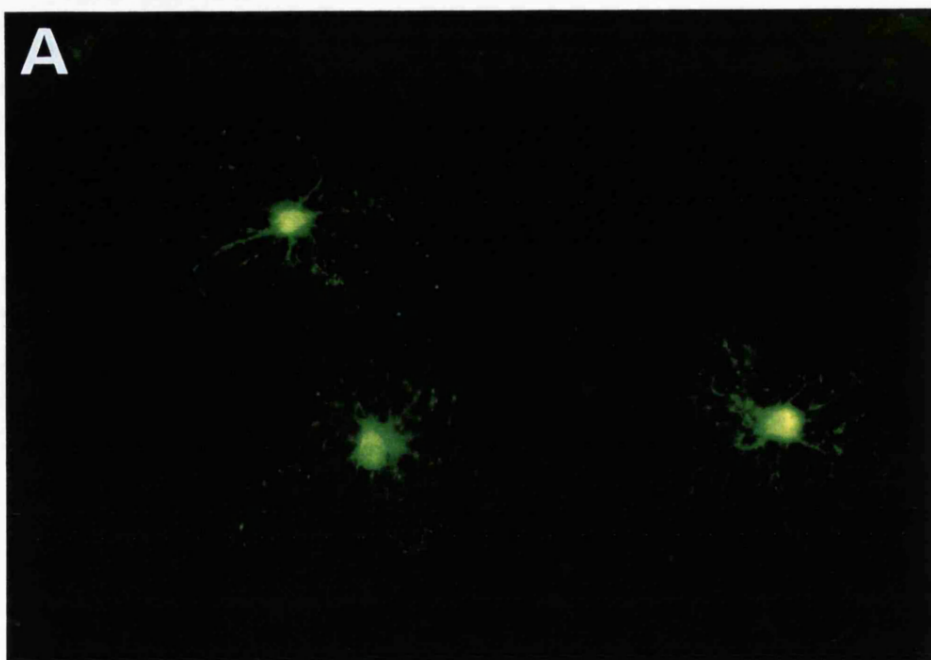
Figure 2.3 A clone of astrocytes. A 7 cell-clone of BAG-labelled astrocytes detected with anti-β gal antibody, visualised with fluorescene-conjugated goat anti-rabbit anti serum is shown in frame A. Astrocyte clones were usually rare in 7 day cultures of E16 cortical cells. However, in order to show pattern of GFAP staining (in B) this clone was selected from cultures of E16 precursors that had been grown on poly-D-lysine coated coverslips, under which condition more astrocyte clones were usually obtained (see Chapter IV). Note how members of an astrocyte clone become dispersed with respect to each other. Scale bar= 10 μm.
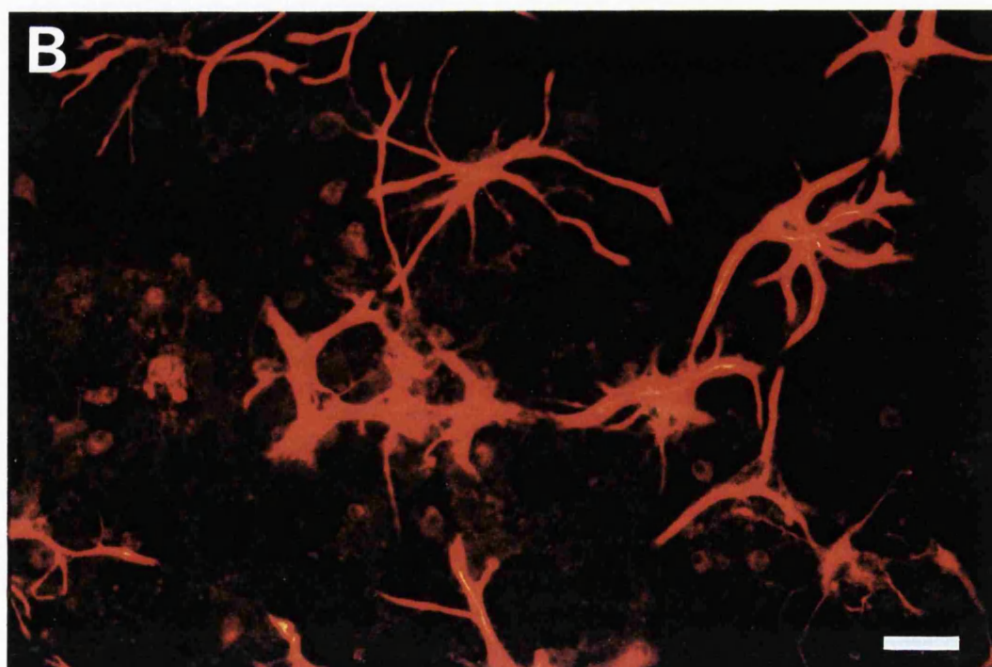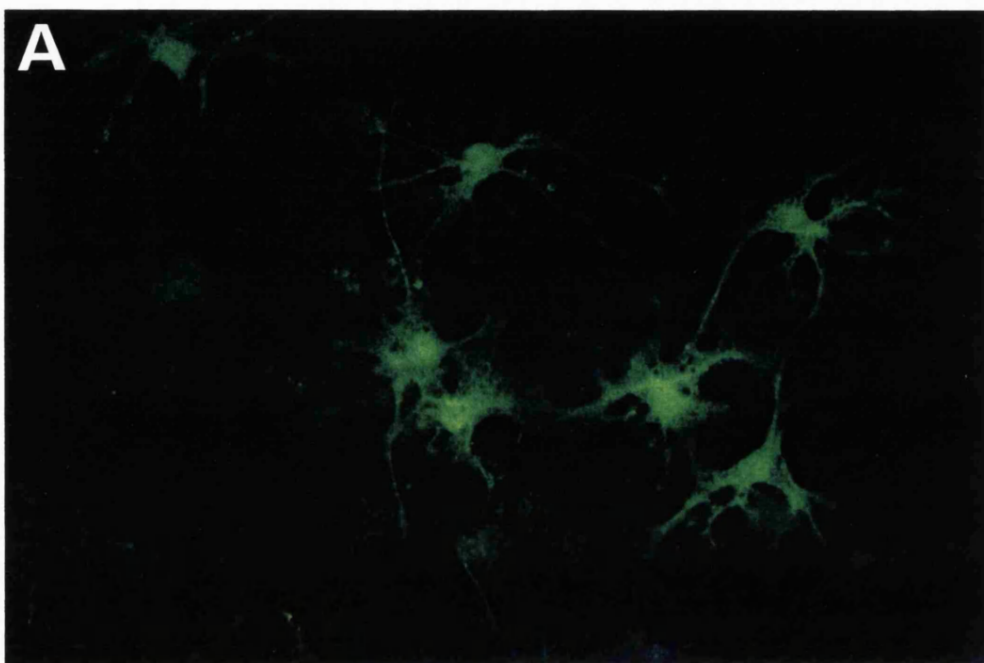
Figure 2.4 Neuronal clones. This figure shows a single-cell (frame A) and a 2-cell clone (frame C) clone of BAG-labelled neurones, as detected with anti-β gal antibody, visualised with fluorescene-conjugated goat anti-rabbit anti serum. These β-gal positive cells were identified as neurones by staining with TUJ1 (frame B) or anti-MAP 2 antibody (frame D). Staining with both these antibodies were visualised by biotinylated goat anti-mouse antibody linked to streptavidin coupled-coumarine. In both cases, neurones show a round to ellipsoid cell body and anti-β gal staining reveals a spec of fluorescence (arrow heads) found specifically in β-gal positive neurones. Such a spec can also be found in X-gal positive neurones in vitro and in vivo (Price and Thurlow, 1988).

Arrows in frame D show that although MAP-2 staining of the neuronal cell-bodies found in C is not clear, the processes of both cells are labelled with this antibody. Scale bar in A and B= 7μm; in C and D= 10μm.
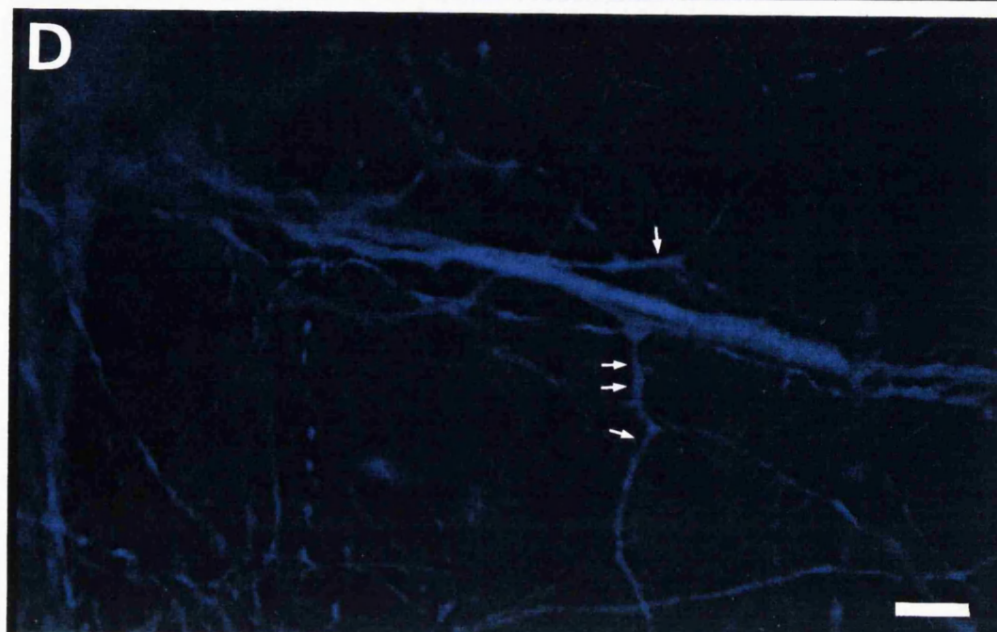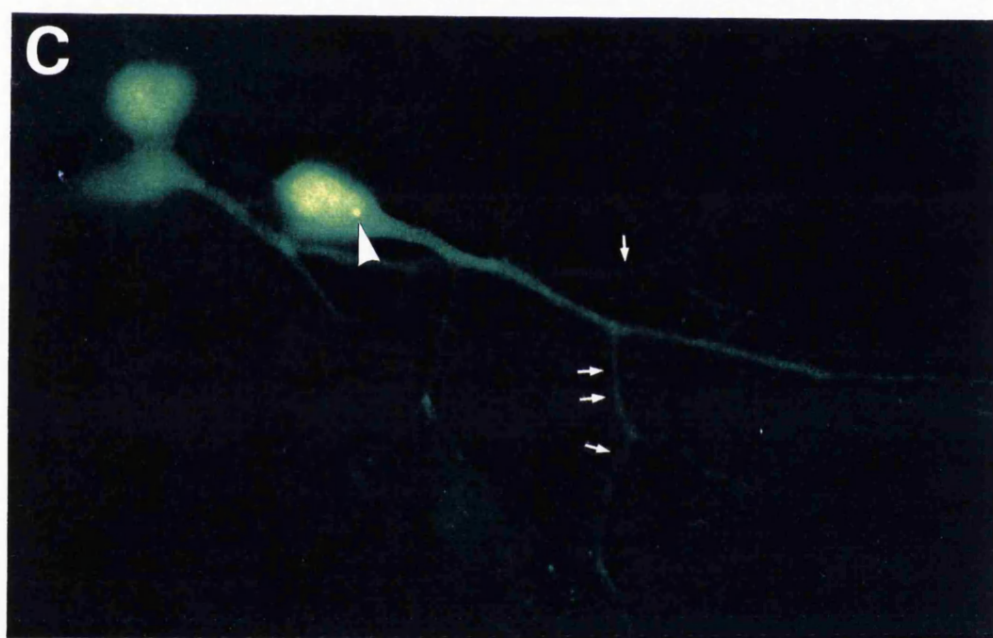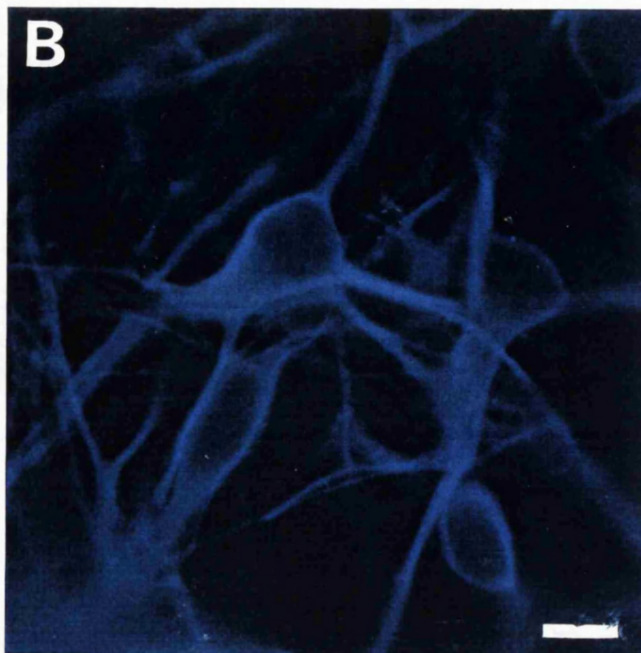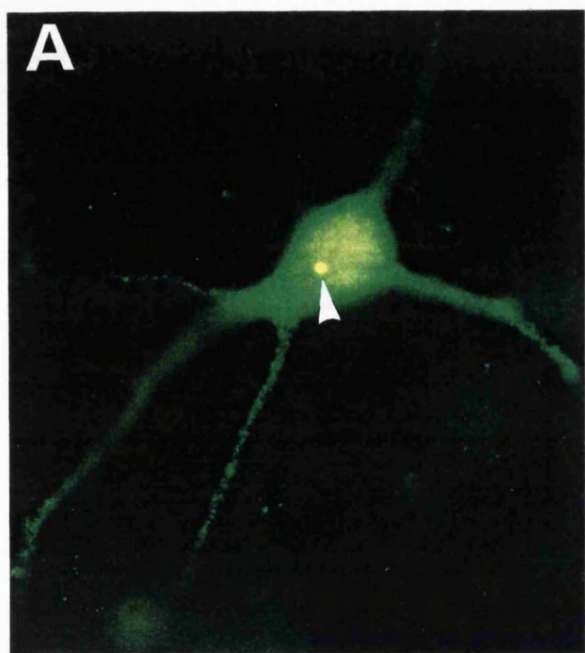
Precursors derived from:

Clone type	E16	E14
Neurones	654 (87%)	103 (90%)*
Oligodendrocytes	42 (5%)	7 (6%)
Astrocytes	1 (neg.)	1(~1%)*
Unidentified cells	59 (8%)	4 (3%)
Total	759	115

Table 2.2 Type and frequency of clones found in cultures of E16 and E14 cortical precursors infected with BAG virus at the time of plating and analysed seven days later.

Numbers outside the brackets represent the total number of clones of a particular type found in E16 or E14 cultures. The total numbers in E16-derived cultures is from a total of 17 experiments, while that of E14 is from 2 experiments. Moreover, on average, 14 clones per coverslip were found in E16, and 19 clones in E14 cultures.

'neg.' denotes negligible. As GFAP staining was not routinely included in the combinations of triple-immunostaining (see methods), the actual frequency of astrocyte clones shown above may have been under-estimated; i.e., in the absence of GFAP staining, astrocytes would have been recorded as unidentified cells.

* The only mixed neurone-astrocyte clone found in E14 cultures, is not represented in this table.

Experiment 1

Clone type	Point of infection (+Hours in culture)			
	0	+24	+48	+72
Neurone	8.3 (97%)	nd	0.3 (7%)	0
Oligodendrocytes	0.3 (3%)	nd	4.3 (93%)	4.3 (100%)
Astrocytes	0	nd	0	0
Unidentified cells	0	nd	0	0
Total	8.6	nd	4.6	4.3

Experiment 2

Clone type	0	+24	+48	+72
Neurones	18 (92%)	17 (94%)	nd	1 (25%)
Oligodendrocytes	1 (5%)	0	nd	0
Astrocytes	0	0	nd	0
Unidentified cells	0.6 (3%)	1 (6%)	nd	3 (75%)
Total	19.6	18	nd	4

Table 2.3 Type and relative proportions of clones obtained in E16 cultures infected with BAG virus at different time points after plating. '0 hours' denotes infection at the time of plating dissociated cell suspension of E16 cortical cells.

Numbers (outside bracket) are expressed as average number of clones per coverslip, i.e. average of three coverslips per condition per experiment. All cultures were analysed seven days after plating.

	---------------- Days in culture ------------	
Clone type	+ 7	+ 13
Neurones	12 (82%)	2 (66%)
Oligodendrocytes	2 (13%)	0.5 (17%)
Astrocytes	0	0
Unidentified cells	0.6 (4%)	0.5 (17%)
Total	14.6	3.0

Table 2.4 Number and proportions of clone types found in cultures of E16 cortical cells infected with BAG at the time of plating and analysed either 7, or 13 days later. Numbers are expressed as average number of clones per coverslip; i.e. average of three coverslips per condition.

Figure 2.5 Comparison of neuronal clone size distribution between E16 and E14 cultures. Two important points can be noted from this comparison. First, the majority of neuronal clones derived from precursors of either age, contained only a single cell; more so in E16 than in E14 cultures. However, clones containing 2 or more neurones were more frequent in E14 cultures where a range of clones sizes some containing as many as 50 cells were evident. This is in marked contrast to E16 derived 2-cell and greater neuronal clones which were restricted in their size, rarely containing more than 2 cells.

It should be noted that although this graph represents a comparison of pooled data from all E16 versus all E14 experiments, the marked difference in neuronal clone sizes was also evident in parallel E16 and E14 experiments.

Comparison of neuronal clone size distribution between E16 and E14 cultures
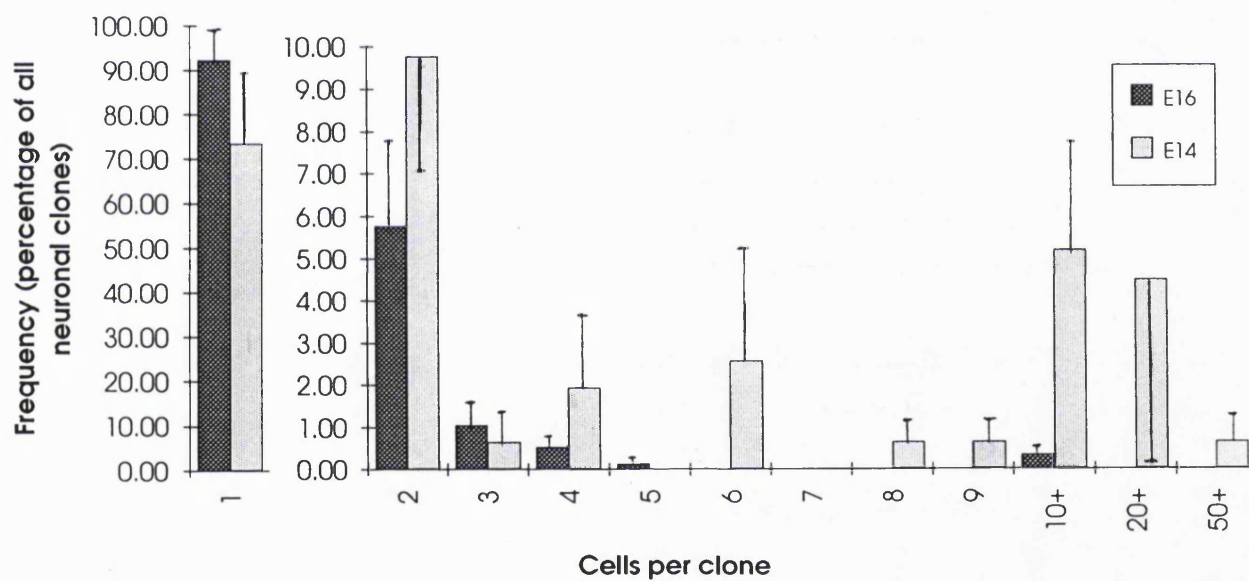
Figure 2.6 Two examples of large neuronal clones found in 7-day cultures of E14 cortical cells. Frames A and C shows staining with anti-β gal antibody, while frames B and D show staining with TUJ1. In both cases the tight clustering of clonally-related cells made it difficult to obtain an accurate count of clone numbers. Nonetheless an inspection at higher power showed that clone A contained 22-23 cells while clone C contained 18-20 cells. Moreover, with this tight apposition of cells it was difficult to determine if all members of both clones were TUJ1 positive; examples of cells that were clearly TUJ1-positive are indicated with small arrows. No member of either clone, however, stained with non-neuronal markers.

Also noteworthy is that the characteristic neuronal spec is visible in some cells of these clones, especially in frame C. Scale bar in all frames= 30μm.
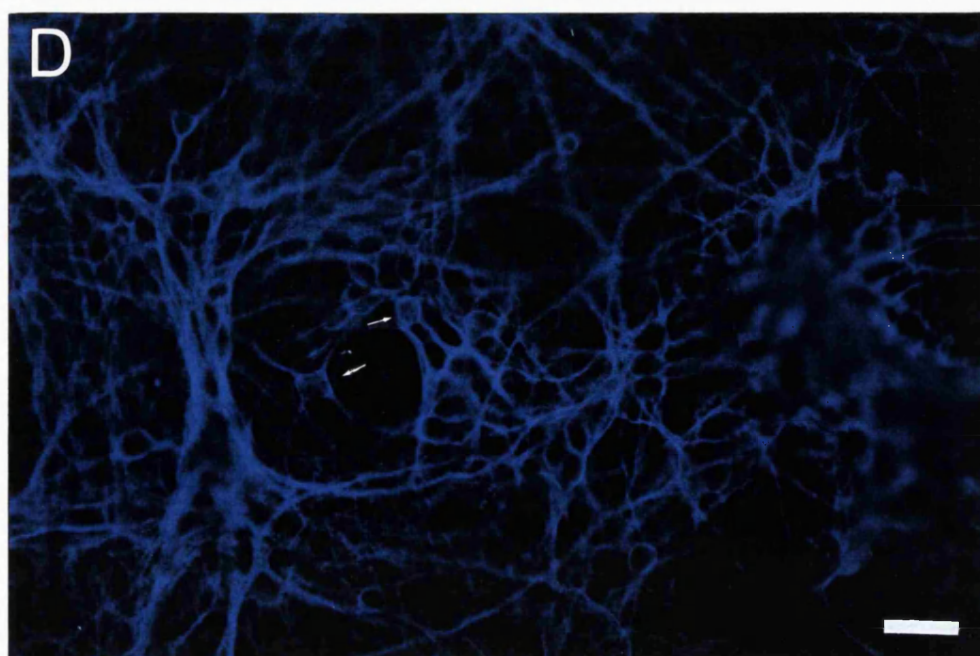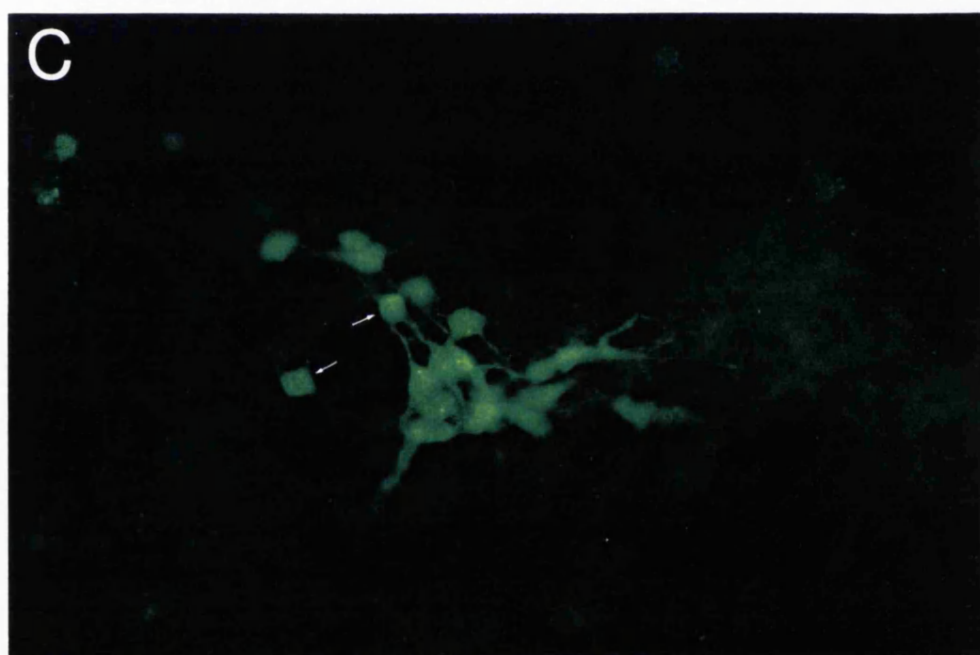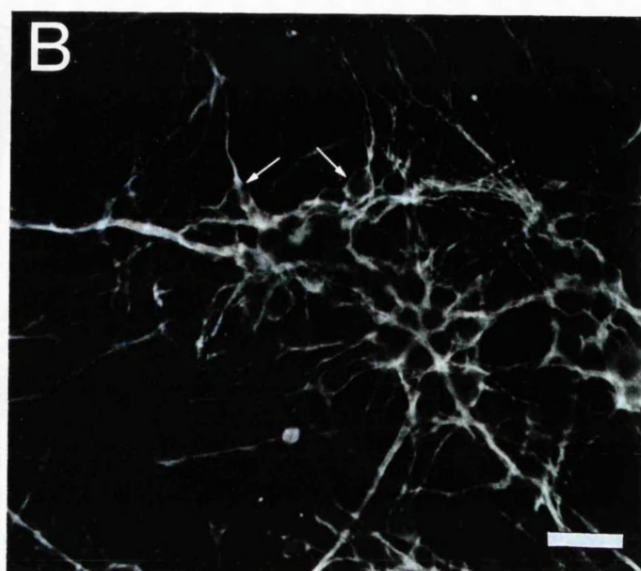
Figure 2.7   A mixed clone composed of both neurones and astrocytes, found in 7-day cultures of E14 cortical cells. The entire clone as revealed by anti-β gal staining is shown in frame A. This clone contained 20 astrocytes and 4 neurones. The neuronal members are shown at higher magnification in frame B; TUJ1 staining shown in frame C confirms their neuronal identity. Frame D focuses on another 4 β gal positive members of this clone that have a flat astrocytic morphology, an identity confirmed by GFAP staining shown in frame E; arrows in frame D and E point to three such cells.

Noteworthy, however, is that such phenotypically-heterogenuous clones were rare; i.e. as in E16 cultures, most clones found in E14 cultures were composed only of a single cell type. In this regard, this clone may be the result of clone superimposition since it came from experiments which contained as many as 19 clones per coverslip (see section 2.2.10.7). However, it is interesting to note that the neurones in the clone opposite have an atypical neuronal morphology although they are clearly TuJ1-positive. This may suggest that these neurones are derived from an 'atypical' precursor, in this case a bipotential precursor that would have also generated astrocytes.

Scale bar in A= 20μm and in frames B to E= 10μm.
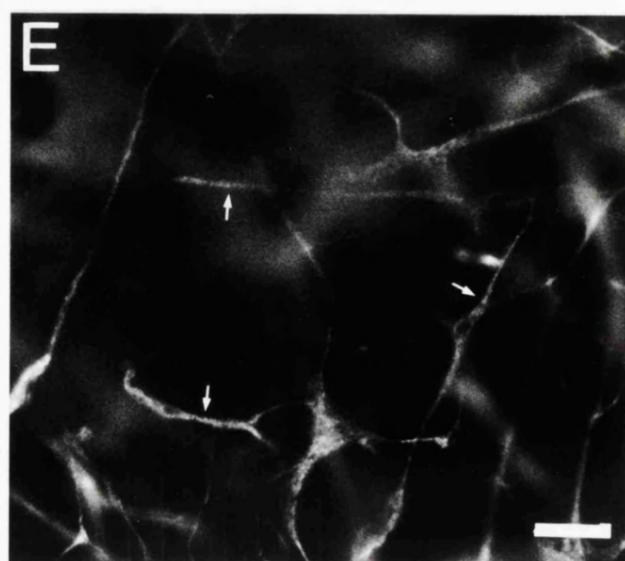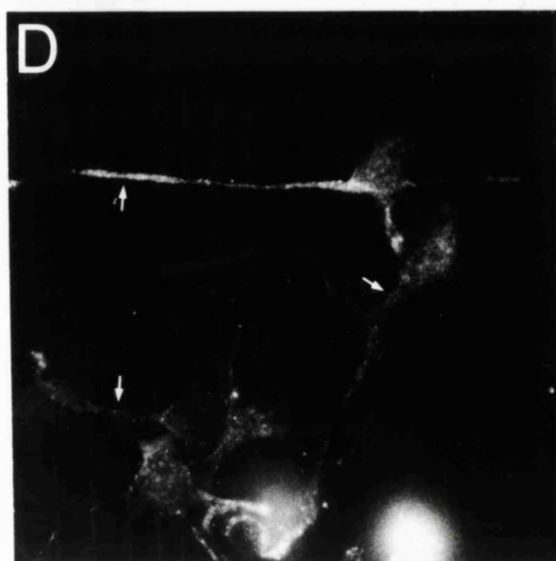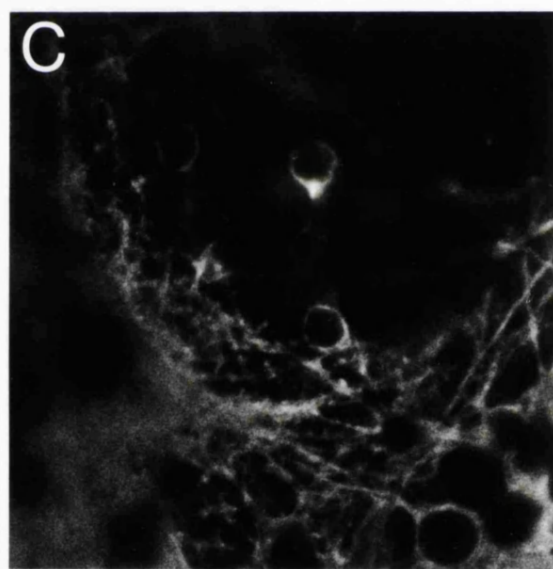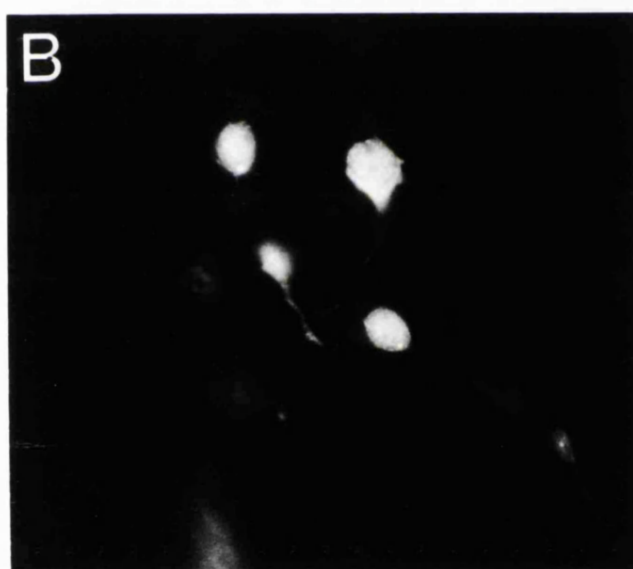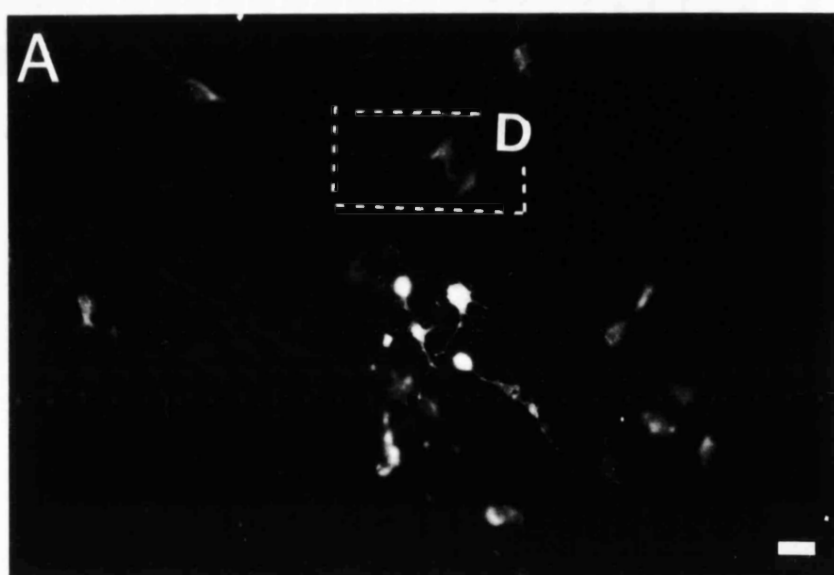
2.4 Discussion

In this study, retrovirus-mediated gene transfer was used in order to carry out a clonal analysis of embryonic cortical cells in bulk cultures of E16 and E14 cortical precursors. At least three important findings could be noted from this type of fate analysis. First, the majority of E16 or E14 precursors were found to give rise to clones that were uniform in cell type, namely, clones of neurones, oilgodendrocytes, astrocytes, or clones whose entire members were unidentifiable, but were similar in morphology. Second, the majority of clones derived from E16 or E14 precursors were neuronal. Finally, most E16 and E14 derived neuronal clones contained only a single-cell, and 2-cell or greater neuronal clones were more frequent in E14 than in E16 cultures. The significance of each of these findings is discussed below. In this discussion, the emphasis will be on E16 precursors as more experiments were conducted with precursors derived form this embryonic age; whenever E14 cultures are discussed, this will be indicated.

2.4.1 Homogeneity of E16 and E14 derived clones: evidence for the existence of specified cortical precursors

Infection of a low number of cortical precursor cells as well as the clonal probability calculations (see section 2.2.8.3) indicates that in these cultures, cells in each homogeneous clone are the likely decendant of a single cortical precursor cell. Detection of homogenuous clones, however, could be explained in one of two ways, the first of which would be by cell death. It could be argued, for example, that homogeneous clones arise by selective death of a particular cell type from an otherwise heterogeneous clone; as most clones were found to be neuronal, this death would have to have been biased towards glial cells. Although cell death was observed in these cultures, it was not accurately quantified within the first week of culture. Nonetheless, no evidence could be found for selective death of glial cells. *P.T.o.

The second and more likely explanation, however, is that homogenuous clones reflect the true developmental potential of cortical precursors in culture. Moreover, the high frequency of homogenuous clones suggests that most cultured cortical precursors were somehow restricted in their developmental potential, a restriction that could have been inherent to the precursor cells at the time they were labelled, or that could have been imposed by the condition under which they were cultured. The latter of these two possibilities is unlikely; E16 precursors cultured under a variety of conditions, such as in the presence of serum or at different cell densities (Williams et al., 1991), or on variety of substrates (see chapter IV) continue to generate clones that are uniform in cell type. This suggests, therefore, that at the time they were isolated from the embryonic cortices, the majority of embryonic cortical precursors were already restricted in their developmental potential. In turn, this observation supports the hypothesis that as early as E16 in vivo, cortical precursors are specified in their phenotypic potential. To emphasise the points

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The relevance of discussing selective death of glial cells in sections 2.4.1 and 2.4.2 are two fold. First, these sections ask whether homogeneous clones, the majority of which were neuronal, are indicative of the presence of specified precursors, or whether homogeneous clones arise because a particular cell type, in this case glial cells, are selectively removed from otherwise heterogeneous clones. Second, these sections ask whether detection of a low frequency of glial clones is a reflection of selective death of glial cells or wether this low frequency represents the actual frequency of *dividing* glial precursors present when cells were infected with BAG immediately after their isolation from the embryonic rat cortex. In this regard this study has not accurately quantified the rate of cell death, nor whether particular cell types were more prone to death under the particular culture conditions. Nonetheless, even in the absence of an accurate measure of cell death in these cultures, several observations and circumstantial lines of evidences suggest that *selective* death of glial cells in these cultures is an unlikely explanation for the occurrence of a high frequency of homogeneous neuronal clones. These are discussed below.

1. Visual examination of cultures at 24 hour intervals revealed that cells that resemble glia (mainly oligodendrocytes) appeared only after 48-72 hours in culture, and glial cells (mainly oligodendrocyte-resembling cells) became were more numerous the later the cultures were examined. Moreover, the later the cultures were infected with BAG, the more glial clones were obtained (Table 2.3). These observations suggests that atleast 48-hour and older cultures provide conditions under which glial cells can survive and proliferate. Therefore, drastic and *selective* death of glial cells could not have occured during this period and hence must, if at all, have occured within the first 24-48 hours of culture. This calls for an analysis of cell death during the first 24-48 hours in culture (see below).

2. In these experiments cortical precursor cells were routenly cultured on a monolayer of cortical type-1 astrocytes in the absence of serum. Under such culture conditions, however, type-1 astrocytes remained as healthy viable cells throughout the seven day period of the experiment. Thus, the culture conditions were not such that would eliminate 'glial' cells *per se* and favour neuronal survival.

3. It is remarkable that not only did clones remain homogeneous but also the frequency of glial clones remained remarkably constant when cortical cells were cultured on a variety of substrates: cortical type-1 astrocytes or their matrix, PDL-coated coverslips or NIH-3T3 cell matrix. Moreover, it is known that the frequency and proportion of different clone types in E16 cultures does not change in the presence of serum (Brenda Williams, personal communication) or several defined growth factors (results presented in chapter IV).

4. It is also highly relevant that almost all studies of E16 cortical cell lineage in vivo (Price and Thurlow, 1988; Moore and Price, 1991; Luskin et al., 1993; Mione et al., 1994) also report finding a low frequency of glial clones, with most clones being homogeneous neuronal clones. Although there is no data on the rate of glial cell death in the rat cortex in vivo, E16 is unlikely to be a major period of glial cell death considering that gliogenesis (e.g. generation of oligodendroctyes) does not get underway until the first post-natal week of rat development. Collectively, these observations suggest that the detection of homogeneous clones as well as the low proportion of glial clones in these cultures is a likely reflection of the presence of specified precursors in E16 (or E14) rat cortex, only a small proportion of which are glial precursors. Nonetheless, this conclusion would be further supported by an accurate measure of cell death, in particular whether glial precursors were selectively lost from the cultures during the first 48 hours of culture as described above. This, however, may prove difficult. First, due to low levels of β-galactosidase expression, it may not be possible to analyse the progeny of BAG-labelled precursors within the first 24-48 hours after infection. Second, it is not certain that one will be able to identify the different precursor types during this preiod; i.e.during the first 24-48 hours of culture, cortical glial precursors may not have expressed the glial markers used in identifying O-2A lineage cells for example. Similarly, there are at present no definite markers of cortical neuronal precursors available.

discussed in section 1.4.1, however, these precursors cannot be regarded as 'committed', since as yet unidentified factors may exist that could alter their developmental potential.

2.4.2 Frequency of different clone types

The detection of a disproportionately higher number of neuronal clones in these cultures could be explained in several ways. The first of these could again be through selective cell death; it would be conceivable that although an equal proportion of neuronal and non-neuronal precursors were originally labelled, non-neuronal cells - oligodendrocytes and astrocytes - or their precursors may have been more prone to cell death. As already discussed, however, no evidence for specific death of either of these cell types could be found.

The second possible explanation may relate to the retroviral labelling itself which would have been biased toward actively dividing cells, since retroviruses integrate only into dividing cells. It is possible, for example, that when cultures were exposed to virus, non-neuronal precursors were either quiescent, or had longer cell cycle times than neuronal precursors. However, it is also possible that since precursors were infected at random, the final clone proportions could be a true reflection of the proportions of neuronal and non-neuronal precursors present when exposed to BAG. The finding that relatively more glial, and fewer neuronal clones are obtained the later the (E16) cultures are infected (Table 2.3), however, does not resolve these two alternatives. These glial clones could have been derived from earlier quiescent precursors, or from an expanding population of mitotic glial cells.

In any event, detection of fewer neuronal clones with later infection protocols, is significant in itself. As BAG labels only dividing cells, this observation suggests that the proportion of dividing neuronal precursors falls rapidly during the first 48 hours of culture. In other words, it appears as though soon after being cultured most neuronal precursors become post-mitotic.

Although these parameters were not tested in E14 cultures, a similar explanation may also account for the high ratio of neuronal to glial clones in these cultures. Moreover, whatever the explanation, it is remarkable that the proportion of neuronal and glial clones found in these cultures is similar to those found in vivo; for example, neuronal clones also account for 60-80% of all clones derived from BAG-infected E16 or E14 cortical precursors, in vivo (Price and Thurlow, 1988; Moore and Price, 1991; Mione et al., 1994).

2.4.3 A comparison of the proliferative potential of E16 and E14 neuronal precursors

Results of this study demonstrate that by and large E14 neuronal precursors have a greater proliferative potential than E16 neuronal precursors, in vitro; under identical culture conditions, E14 neuronal precursors produced clones containing as many as 20 or even 50 cells, while E16 precursors were restricted to a maximum clone size of 3 or 5 cells (figure

2.5). Moreover, clones containing 2 or more neurones were more frequent in E14 than in E16 cultures.

Detection of 50-cell neuronal clones in E14 cultures, however, outlines another important difference between E14 and E16 neuronal precursors, which relates to the mode of division of a neuronal precursor. In theory, at every division, a neuronal precursor cell could divide either symmetrically, so as to produce two daughters of equal developmental potential, either both mitotic or post-mitotic, or, asymmetrically (stem cell-like) so as to generate a developmentally-restricted daughter, such as a post-mitotic neurone, and a stem cell-like daughter that may go on to produce more neurones. The studies of McConnell (1989; also reviewed in 1992) suggests that as far as cortical lamina fate is concerned, some neuronal precursors of the VZ divide asymmetrically; they produce one post-mitotic daughter that migrates along radial glial cells to reside in a particular cortical laminae, and, a second daughter that re-enters the cell cycle and serves as a precursor for neurones of later-generated cortical lamina. However, this is not say that all neuronal precursors divide asymmetrically; rapid growth and expansion of the developing cortex may require some neuronal precursors to show no such 'commitment' and undergo symmetrical division, simply to add to the pool of cortical precursors. In this respect, the terms asymmetrical and symmetrical describe two quantitatively different modes of division, an 'arithmetic' and a 'geometric' mode, respectively.

Given that both E16 and E14 precursors were cultured for seven days, and that E14 and E16 cortical precursors have a cell cycle time of approximately 13-17 hours in vivo (Waechter and Jaensch, 1972), it becomes clear that 50-cell clones in E14 cultures could not have been generated by arithmetically dividing precursors, because, such a precursor would generate a maximum of 10-12 cells during this period. It may be argued that as a result of culturing the cell cycle time of arithmetically-dividing precursors becomes shortened. However, even at the shortest known mammalian (ectodermal) cell cycle time of 7 hours (MacAuley et al., 1993) an arithmetically dividing precursor could not generate a 50-cell clone; i.e. with a cell cycle time of 7 hours, only a maximum of 25 cells would be produced by such precursors during 7 days of culture. Thus, 50-cell clones in E14 cultures were the result of geometric division. Obviously, it would be difficult to determine whether or not geometrically dividing neuronal precursors were present in E16 cultures; clones smaller than 50 cells could have arisen from geometrically or arithmetically dividing precursor cells. A 4-cell neuronal clone, for example, could be the result of four rounds of arithmetic, or two rounds of geometric division.

2.4. The enigma of single-cell neuronal clones

Despite their greater proliferative potential, over 70% of E14 neuronal precursors gave rise to clones that contained only a single cell. These single-cell clones were even more frequent in E16 cultures, where they made up over 90% of all neuronal clones (figure

2.5). Single-cell clones are not a peculiarity of these culture conditions, however, since studies of cortical cell lineage in vivo have also found that a substantial number of neuronal clones are single-cell clones (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Austin and Cepko, 1990; Moore and Price, 1991; Mione et al., 1994).

The fact that BAG virus labels only dividing cells suggests that single-cell clones are derivatives of multi-cellular (2 or greater) clones. What then, has happened to the siblings of these single cells? Authors of cell lineage studies have put forward a number of explanations as to how single-cell clones arise in vivo. First, it has been suggested that one or more members of a multi-cellular clone may either not express, or, with time turn off expression of the viral genes. Although some preimplantation embryonic cells are known to present a block to viral expression (Savatier et al., 1990), there is no evidence that cells derived from later stages of development may present such a block. Moreover, the only evidence for down-regulation of viral gene expression comes from virally-infected fibroblasts (Palmer et al., 1991), a phenomenon that was observed a month after infection of such cells. In cortical cell lineage studies, however, single-cell clones can be found both in short and long-term analyses of virally-labelled precursor cells. Mione et al. (1994), for example, could detect single-cell clones only three days after the infection of E16 cortical precursors in vivo. Thus, down-regulation of viral gene expression is not a satisfactory explanation of single-cell clones. *ᴀᴜ ᴀˡˢᴏ ᴜ₃ᴸ (P.T.O.)

The second suggestion is that single-cell clones may be the result of cell dispersion. In the developing cortex, for example, clonally-related cells could be separated through active migration or three dimensional growth and expansion of cortex. In this regard, Walsh and cepko (1988; 1992) have provided evidence for widespread dispersion of some clonally-related cells in the cerebral cortex. These authors, who infected cortical precursors with a cocktail of 100 genetically different viruses, have PCR amplified the viral genes from two remote single-cell clones to show that both were the result of infection by one viral prototype, suggesting that they were the descendants of a single precursor cell. However, these authors also report a number of single-cell clones for which no siblings could be found. Moreover, single-cell clones have also been found in retroviral lineage studies of retina (Turner and Cepko, 1987; Turner et al., 1990), a structure in which cell migration is far less extensive than in cerebral cortex. In cultures, cell dispersal is also an unlikely explanation of single-cell neuronal clones, because, multi-cellular neuronal clones, form where single-cells would have presumably originated, were consistently seen as groups of well-clustered cells (figure 2.6).

The third possible explanation for the occurrence of single-cell clones is cell death. It is conceivable that with increasing time in vivo, or in culture, some progeny of a virally-labelled cells may die leaving a single-cell clone. Although it is unreasonable to suggest that cell death should always affect an entire clone in culture, it is equally difficult to reason why all except one member of a clone should die, unless single-cell clones come from

<u>Single-cell clones may represent terminal division of precursors</u>

The interpretation of single-cell clones relies heavily in the first instance on the reasons for their occurrence. Out of the possibilities discussed in section 2.4.5, cell death and retroviral labelling were favoured as two possible ways by which single-cell neuronal clones could arise. Of these, retroviral labelling was put forward as a better explanation because results of chapter IV show that when the survival of neuronal precursors is enhanced by as much as 50%, single-cell clones still make up the majority of neuronal clones, although, cell death as a cause for the occurrence of *some* single-cell clones could not be ruled out. In was further suggested that if retroviral labelling were to be the major cause of single-cell clones, it can be deduced that single-cell clones arise from precursors that were undergoing their terminal round of division. Whether this cessation of division is the consequence of culturing or is inherent to the precursors, however, is highly debatable. Detection of single-cell clones by all cortical cell lineage studies, both by short-term (Mione et al., 1994) and long-term (Price and Thurlow, 1988; Moore and Price, 1991; Mione et al., 1994) studies, in vivo suggests that this may be an inherent property of cortical neuronal precursors, although it is not known to what degree cell death is a cause of single-cell clones in vivo.

Therefore, if cessation of division by cortical precursors in culture were to be a premature one, it would certainly place a limitation on the way the developmental potential of cortical precursors is analysed; a cultured precursor that undergoes premature division so as to produce a neurone, may, if it were to divide further, have generated glial cells. If, on the other hand, the cessation of division was to reflect an inherent proliferative potential of a specified precursor, then there would be no such limitation on the conclusions reached in discussion sections to this chapter (chapter three) and that in chapter four.

Naturally, one way of resolving these issues would be to analyse the fate of single cortical cells by methods other than retroviral labelling. One may carry out a short term analysis of the fate of a small population of dye (fluorescent)-labelled precursors grown amongst a bulk population of unlabelled cells, in vitro. In such an analysis one may ask how frequently do dye-labelled cells give rise to single-cell clones? If single-cell clones are found to be widespread, then clearly cell death and not retroviral labelling is a major cause for their occurrence. Crucial to such an analysis also is to know what proportion of such single-cell clones are neuronal. This may prove difficult, however, since some neurones may not have fully differentiated during the period of analysis.

As for such an analysis in vivo, to date it has not been possible to analyse the fate of embryonic cortical precursors by methods other than retroviral labelling.

small clones of two or three cells. In any evemt, results presented in chapter IV of this work suggest that cell death alone can not be the only reason for the occurrence of single-cell clones. These results show that when the survival of cultured neuronal precursors is enhanced by as much as two fold, single-cell clones still make up the large majority of neuronal clones.

However, there may be a fourth possibility, which may relate to the integration of retroviruses. Studies of retroviral integration show (see next chapter) that progression through S phase, the DNA synthesis phase of the cell cycle, is important to retroviral integration. This has been taken as evidence that integration itself may take place during S phase. If this were the case, then the retroviral DNA could in theory integrate either into a section of S-phase DNA that has not replicated, the so called pre-replication DNA, or a section that has undergone replication, the post-replication DNA. Obviously, as far as inheritance of viral genes are concerned, these two possibilities would have difference consequences. If BAG viral genes were to integrate into pre-replication host DNA, then duplication and subsequent inheritance of viral genes would result in both daughters of an infected cell becoming labelled. If BAG viral genes were to integrate into post-replication DNA, however, then only one daughter of an infected precursor cell would inherit viral genes at the subsequent mitosis and thus express the viral marker genes. Thus, if a neuronal precursor undergoing its final round of division were to incorporate viral genes into its post-replication DNA , it will produce a single cell-clone. In this respect, the following chapter is devoted to investigating whether retroviruses can integrate into post-replication host DNA.

Chapter III

A study of modes of retroviral integration

3.1 Introduction

Integration is an important phase of the retroviral life cycle. It is the process by which a DNA copy of the viral genome becomes incorporated into the chromosomal DNA of infected host cells, as a consequence of which, viral genes are efficiently transcribed along with cellular genes and translated into protein. New viral particles are assembled from these proteins and after release from the infected cells go on to infect other cells, leading to propagation of retroviruses (reviewed in detail in chapter I).

However, integration of viral DNA has consequences for host cells too. Stable incorporation of viral DNA means that the viral genes become inherited by the progeny of an infected cell. If viral DNA were to integrate into DNA of germ cells, then viral genes can be passed on to future generations of a particular species. In this respect, it has been shown that the genome of most species contain some viral-resembling gene (Finnegan, 1989). In mouse Mus Muscus, for example, these genes account for one percent of the total genome (Coffin, 1982). Retention of these genes may have conferred some evolutionary advantages to these species. For example, a strain of mice has been discovered that carry env-resembling viral genes and are therefore resistant to infection by murine retroviruses (Gardner et al., 1991; see also section 1.8.4.3 , chapter I).

Sites of viral DNA integration are also important. Retroviruses have powerful enhancer/promoter elements in their genome which can up or down regulate expression of flanking host genes (see Varmus and Brown, 1989). If these host genes were to encode proto-oncogenes, then their upregulation could induce abnormal cellular growth and hyperplasia (Dickson et al., 1984; Nusse et al., 1984). Mammary tumors in mice, for example, are caused by proviral activation of proto-oncogene int-2, by mammary tumour viruses (Dickson et al., 1984). Derailed cellular growth could also result from direct introduction of oncogenes into host cells by retroviruses.

These aspects of viral integration have encouraged many workers to use replication-defective retroviruses as vehicles of gene transfer into cells with a view to gene therapy (Williams et al., 1986; Friedmann et al., 1989; Drumm et al., 1990; VanBeusechem et al., 1992), to study the development of genetically-labelled cells (see lineage studies in Chapter I), or to study normal cellular gene function and its regulatory elements (Stuhlmann et al., 1981). However, despite this wide application of retroviruses, some aspects of viral integration remain obscure. It is not known, for example, how host cell-derived factors may influence integration of viral DNA, or indeed at what precise stage of the cell cycle does retroviral integration take place. The molecular details of integration, reviewed in chapter I, suggests that at least on a molecular level integration can take place independent of host cell-derived factors. It has long been known, however, that cell cycle plays an important part in integration, since some retroviruses can not become established in non-dividing cells arrested in G1. Initially, it was thought that non-dividing cells may present a block to viral infection per se, or that viral DNA could not be formed in these cells

(Fritsch and Temin, 1977). Subsequent studies, however, showed this not to be the case. For example, Harel et al. (1981) were able to find Brdu-labelled viral DNA in growth-arrested NIH-3T3 cells infected with MoMLV. Several workers suggested that viral DNA generated in quiescent cells may not be of the correct configuration for integration (Varmus et al., 1977; Chen and Temin, 1982). Roe et al. (1993), however, have shown that viral DNA isolated from acutely infected quiescent cells could successfully take part in integration reactions in vitro (Brown et al., 1987).

These observations together with the finding that infected quiescent cells could integrate viral DNA once they were allowed to resume cycling, suggested that other phases of the cell cycle may be important to viral integration, in particular, S phase, the DNA synthesising phase of the cell cycle. Humphries et al. (1981), for example, discovered that the appearance of integrated provirus in cells released from block in G1 co-incided with S phase. Similarly, Miller et al. (1990) have shown that successful integration of a replication-defective retrovirus into rat fibroblasts, requires their passage through S phase, suggesting that S phase may be the phase of viral integration. A recent study by Roe et al. (1993), however, suggests that integration may take place subsequent to S phase, possibly during Mitosis.

These observations notwithstanding, the timing of viral integration is of particular importance to some applications of retovirus-mediated gene transfer, such as cell-lineage studies by retroviral labelling of precursor cells. As already alluded to in the previous chapter, all or only some of the progeny of an infected precursor cell may become labelled with a retrovirus depending on whether viral integration occurs during S phase, G2 or M; during S phase, viral DNA may integrate into either pre- or post-replication DNA, where as if integration were to occur during G2 or M, only post-replication host DNA would incorporate viral genes. It follows that if an infected precursor cell were to incorporate viral DNA into its post-replication DNA, then only one of its daughters would inherit the viral genes, as a result of which only half the progeny would become labelled. Thus, if an infected precursor cell were to produce two phenotypically-distinct daughters, then detecting only one of these may portray a wrong impression of the developmental potential of that precursor cell.

Work presented in this chapter, therefore, asks whether viral DNA can integrate into post-replication host DNA. This question is addressed by asking whether one or both daughters of single NIH-3T3 cells infected with BAG inherit the viral genes.

3.2 Material and Methods

3.2.1 Outline: cells and medium used

The aim of the experiments described below, was to follow and analyse the progeny of single retrovirally-labelled NIH-3T3 cells. Single cells derived from bulk cultures of NIH-3T3 cells were plated into the wells of multiwell plates and infected with BAG (Price et al., 1987) and/ or DAP (Fields-Berry et al., 1992) viruses (figure 3.1). For this, bulk NIH-3T3 cells were grown and maintained in culture as described in the previous chapter, section 2.2.2. Moreover, BAG virus used for infection of single NIH-3T3 cells was prepared, titrated and screened for helper virus as described in section 2.2.5. DAP viral stocks were prepared from amphotropic DAP producer cells (Fields-Berry et al., 1992), in a way similar to BAG.

As with bulk cultures, single NIH-3T3 cells (see below) were routinely grown in Dulbecco's modified Eagle's medium (DMEM, pH 7.0, Northumbria Biologicals), supplemented with antibiotics: penicillin (0.06 g/l); streptomycin (0.1 g/l) and L-glutamine (0.3 g/l). This medium was supplemented further with 10% (final concentration) New-born Calf Serum (NCS, Gibco) as well as NIH-3T3 cell conditioned medium (see below).

Both supplemented and unsupplemented medium were normally stored at 4° C and warmed to 37° C prior to use.

3.2.2 Analysing the progeny of single virally-infected NIH-3T3 cells

3.2.2.1 Culturing single NIH-3T3 cells: optimising the culture conditions

A crucial step in these experiments was to ensure that only the progeny of a single cell is being analysed; i.e. to be able to identify wells that contained only a single cell. In this regard, it was discovered that fluoresecently-labelled cells could be detected more readily and accurately than unlabelled cells. This method had another advantage; because detection of fluoresecently-labelled cells was quicker, cells spent less time outside the incubator, thereby improving their subsequent chances of survival. Preliminary experiments showed that the survival of cultured single NIH-3T3 cells could also be enhanced by the presence of 50% NIH-3T3 cell conditioned medium. This conditioned-medium was collected from confluent plates of uninfected NIH-3T3 cells. In order to prevent transfer of cells, however, the conditioned medium was first passed through an analytical filter unit with a 0.2 μm pore size.

3.2.2.2 Fluorescent labelling of NIH 3T3 cells

NIH-3T3 cells were fluorescently-labelled in suspension. Cells were detached from semi-confluent plates using trypsin-versene solution as described in section 2.2.3. 5 mls of the resulting cell suspension was then incubated for 30-40 minutes at 37° C with 30 μM 5-carboxyfluorescein diacetate, succinidimyl ester, CFSE (Molecular probes). This was done by diluting a 100mM stock CFSE solution, previously prepared in dimethyl

Figure 3.1 Experimental protocol for analysing the distribution of viral genes amongst the progeny of single virally-infected NIH-3T3 cells. Fluorescently labelled NIH-3T3 cells were plated at clonal densities into the wells of 72-well Terasaki plates. 12-15 hours after plating wells containing only a single cell were noted. These were infected with a low titre of BAG or DAP viruses for 3.5-4 hours, after which cells were grown for 3-4 days and either (A) stained with substrate X-gal (to detect β-galactosidase) or X-phos (to detected alkaline phosphatase), or (B) subcloned into fresh Terasaki plates, grown for 3-4 or sometimes 7 days, then stained with X-gal. DNA from stained and unstained subclones was collected and subjected to PCR inorder to detect BAG viral genes amongst the stained and unstained progeny of single infected NIH-3T3 cells.

NIH 3T3 Cells

+ CFSE

Serial dilution to 1cell/well

Terasaki plates

+ 12- 15hrs

Wells inspected for single cells

+ BAG (DAP) virus
3.5 - 4hrs

(A) → Fix + stain with Xgal → Clones noted

0   24   48   72

Hours

(B)

Subcloning

3-4 days

Fix + stain
with Xgal.
DNA prepared
for PCR
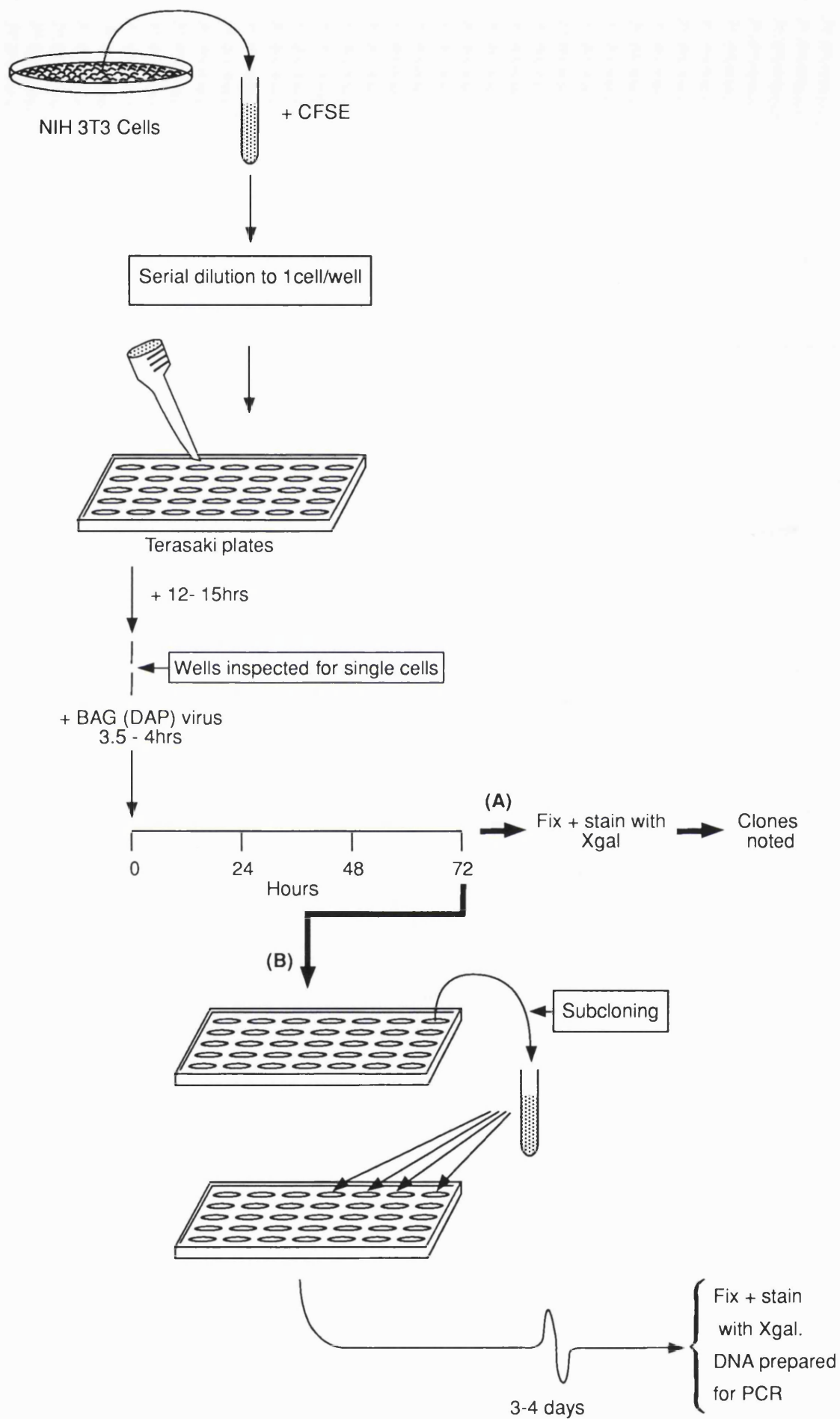
sulphoxide (DMSO), in the NIH-3T3 cell suspension. CFSE was then by adding 5 more mls of fresh 10% NCS/DMEM to the cell suspension. This suspension was then thoroughly mixed, centrifuged for 10 minutes at 1000 rpm and pellet of NIH-3T3 cells was resuspended in 5 mls of fresh medium. This method was shown to label every cell in the suspension. This was determined by plating some labelled cells onto 9 cm falcon plates and examining them at a later time point under an epifluorsecent microscope equipped with bright field and UV light source; every cell seen under the phase/ bright field filter, also fluoresced under the fluorescence filter. Furthermore, this fluorescence was retained by the labelled cells for at least 72 hours post-labelling.

3.2.2.3 Culturing single fluorescently-labelled NIH-3T3 cells into the wells of Terasaki plates

The number of cells present in a suspension of CFSE-labelled NIH-3T3 cells was determined using a heamocytometer (Weber, England). 2 mls of this suspension was then serially diluted with 5% NCS/ 45% DMEM/ 50% NIH-3T3 conditioned medium, down to a density of 200 cells per ml, a cell density which corresponds to 1 cell per 5 μl medium. This suspension was then diluted further to a density of 1/2 and 1/4 cell per 5μl medium and plated as 5μl aliquots into the wells of 72-well Terasaki plates using an automised microdispencer (EDP-plus). Although the wells of a Terasaki plate have a capacity of 10μl, by plating in 5μl volumes, cells were encouraged to settle at the bottom of each well rather than adhere to the walls of the wells. Typically, 4 Terasaki plates were allowed for each dilution, i.e. 4 at 1 cell per well, 4 at 1/2 and 4 at 1/4.

Soon after all wells of a plate had been filled, two drops of sterile distilled water were placed at the corners of each plate, the plate lids were tightly closed and plates were transferred to a 37° C incubator and kept in a humidified atmosphere containing 5% CO_2.

3.2.2.4 Screening wells of Terasaki plates for the presence of single cells

12 to 15 hours after plating serially diluted cell suspensions of NIH-3T3 cells, each well of Terasaki plates was scanned under the 10x objective of an epifluorescent (Axiovert) microscope; under the fluorescence filter, labelled cells appeared green. It was discovered that the presence of a spec of fluorescene, even when out of the plane of focus, corresponded to the presence of a single cell. This could be verified by focusing on the spec, both under U/V light and the bright field, either under the x10 objective or at higher power, under the x20 objective. In this way, both the number and position of cells in wells that contained one or more cells was noted and recorded in the relevant grids of a previously prepared chart.

3.2.2.5 Infecting single NIH-3T3 cells with BAG or DAP viruses

Immediately after scanning all wells of Terasaki plates, an infection cocktail composed of

1x 10^5 cfu/ml of BAG or DAP, 8 μg/ml polybrene and 10% NCS/DMEM was prepared. Cells were then infected by adding 4μl of this prewarmed cocktail to each well noted to contain a single NIH-3T3 cell, a volume that delivered approximately 400 cfus of virus to each well. After addition of virus, plates were swirled gently and returned to the incubator. 3.5 to 4 hours later, all the medium in each infected well was gently removed using a sterile hypodermic needle tip fitted to the end of an aspirator tube. The wells were then filled with 10μl of fresh 5% NCS/ 45% DMEM/ 50% NIH-3T3 cell-conditioned medium.

24 hours after infection, each infected well was again scanned under an epifluorescent microscope, noting particularly whether the infected cell was still present or dying, or whether it had divided. Moreover, where possible, the position of the cell or its progeny was also noted. Following this scan, more distilled water was placed at the corners of the inspected plates to prevent cells from drying, and plates were returned to the incubator. This procedure was repeated 24 hours later, except that infected wells were now fed with fresh 5%NCS/ 45% DMEM/50% NIH-3T3 cell conditioned medium. This was done by carefully aspirating half of the medium in each well and replacing it with fresh medium.

48-72 hours later, cells in the infected wells were either subcloned or fixed with fixative and processed for X-gal/ X-phos staining.

3.2.3 Subcloning the progeny of single infected NIH-3T3 cells

In several experiments, clones of NIH-3T3 cells that had been exposed to virus were allowed to reach about 25-30 cells in size (figure 3.1). Medium in the wells harbouring such progeny was replaced carefully with 12 μl of prewarmed tyrpsin-versene solution. The plates were then kept for 2-3 minutes at 37° C before being inspected under an inverted microscope; trypsinised cells could be seen as round cells still attached to base or walls of the wells. These cells were mechanically detached by gently triturating the 12 μl volume up and down a fine finn pippette tip. This volume was then collected and added to 500μl of prewarmed 10% NCS/ DMEM. Trypsinised wells were inspected once more and if a considerable number of cells were left behind, the procedure was repeated once again.

Once all cells had been collected, 500μl more 10%NCS/ DMEM was added to the cell suspension and after a gentle, but thorough mixing, the diluted suspension was plated as 10 μl aliquots into the wells of fresh Terasaki plates. A few drops of sterile distilled water were placed at the corners of each plate and plates were transferred to the 37° C incubator. 3-4 hours later, each well of these Terasaki plates was inspected and the presence of subclones was noted on the relevant charts. Thereafter, subcloned cells were fed every 48 hours with fresh 10% NCS/DMEM supplemented with NIH-3T3 conditioned medium.

3-4 days after subcloning, each well was again scanned under a inverted microscope, noting the presence (or absence) as well as the number of cells within each subclone. Following this scan, cells were fixed with 0.5% glutaraldehyde and stained with X-gal and/ or X-phos.

3.2.4 Staining the progeny of single infected NIH-3T3 cells with X-gal
3.2.4.1 Preparation of X-gal and X-phos staining reagents:

BAG infected cells were detected by X-gal staining as described in section 2.2.6. DAP-infected cells were detected by incubation with substrate X-phos. As for X-gal, the final X-phos mixture was made by dilution of X-phos reagent in a reaction buffer. The components of this reaction buffer, which was prepared in 1x PBS (final pH 7.5), were as follows: 100 mM Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$ and 0.1% Tween 20. Just before staining cells, the final X-phos mixture was prepared by adding the following to the reaction buffer: X-phos (0.1mg/ml), NBT (0.2 mg/ml) and levamisole (0.7 mg/ml).

3.2.4.2 Incubation of cells with X-gal or X-phos mixtures

The medium in wells harbouring the progeny of single infected NIH-3T3 cells or that of the subcloned progeny, was carefully removed using a needle tip attached to the end of an aspirator. In order to prevent transfer of cells from one well to another (as in the feeding stage), in between each aspiration, the needle tip was dipped in absolute alcohol and flamed. Cells were then fixed for 15 minutes by addition of 10-12 µl of 0.5% glutaraldehyde to each well. Subsequently, glutaraldehyde solution was gently removed and cells were incubated with X-gal from one to three hours at 37° C, or overnight at 4° C. As soon as blue cells were seen in some wells, X-gal solution in all wells was replaced by 1x PBS solution.

Wells noted to have contained single cells at the time of plating, were all scanned for presence or absence of blue cells, noting the total number, as well as the proportion of blue to unstained cells. In the case of subcloned progeny, all wells noted to have contained any cells prior to fixation were scanned, and the number of such progeny, whether blue or unstained, was noted.

In experiments in which both X-gal and X-phos staining was required, cells were first stained with X-gal and wells containing blue cells were noted. All wells containing blue or unstained cells were then stained with X-phos, and the number of purple cells was determined.

3.2.5 Detection of viral genes amongst the subcloned progeny of single BAG-infected NIH-3T3 cells
3.2.5.1 Collection of DNA for polymerase chain reaction (PCR) from the subclones

After staining the subcloned progeny of single NIH-3T3 cells with X-gal, wells containing stained (blue) or unstained progeny were identified and DNA from both the stained and unstained cells was collected and amplified for BAG viral genes in the following way.

After the X-gal staining, the sublcones were washed with 1 x PBS. While still in the wells of Terasaki plates and in 10 µl of PBS, cells were frozen and thawed twice in order

to breakup the cell membranes, after which, the 10 µl volume from each well was transferred to a corresponding 400 µl autoclaved plastic eppendorf tube and 10 µl of 1mM EDTA added. This solution was then overlaid with 20 µl of light mineral oil and heated for 30 minutes at 93° C, a treatment that is thought to inactivate cellular enzymes and denature cellular DNA and its associated proteins (Handyside et al., 1992). This solution was then heated for a further 5 minutes at 95° C in the presence of 30 µl of 1 x PCR reaction buffer (50 mM KCl; 10mM Tris-HCl, pH 8.3; 0.1% Triton X-100, Promega); 2 mM $MgCl_2$; and dinucleotides (ATP, GTP, CTP and TTP), before being cooled to 16° C.

3.2.5.2 PCR reaction

'Nested' PCR was carried out on DNA collected from subclones, according to the standard Perkin-Elmer protocol. For this, two sets of primers were used; an outer set for the first round of the PCR reaction and an inner set for the second round. The outer set, described in 5' to 3' direction, were: L4 from the *lac Z* region (GAA GAA GGC ACA TGG CTG AAT ATC GAC GGT), and the N7 from the *Neo* region (GTC CAG ATA GCC CAG TAG CTG ACA TTC ATC) of the proviral DNA (figure 3.2). The inner set, also described in 5' to 3' direction, were: L3 (TTC CAT ATG GGG ATT GGT GGC GAC GAC TCC), and N6 (CGG ACT GGC TTT CTA CGT GTT CCG CTT CCT) (figure 3.2). All primers were synthesised by NIMR synthetic laboratory on an Applied Biosystem 's instrument and were purified on 10% acrylamide gel by electrophoresis.

The first round of PCR reaction was carried in the presence of the first set of primers (L4 and N7); Promega Taq DNA polymerase (2.5 U final conc.); BIND-AID Amplification enhancer (0.5 µg/ml E. coli single-strand DNA binding protein (SSB); 0.5 µg/ml acetylated BSA in 10 mM Tris-chloride, pH 8.3; 1.5 mM $MgCl_2$; and 50 mM KCl) to the mixture obtained at the end of section 3.2.5.1. This entire mixture was spun at 10,000 rpm for one second and heated for 2 mins at 95° C. Subsequently, the mixture was subjected to 70 PCR cycles, each cycle consisting of: 45 seconds at 95° C, followed by 45 seconds at 52° C, and finally 90 seconds at 72° C. After this, 30 µl of the resulting reaction mixture was removed and brought up to 100 µl by adding more Taq polymerase, PCR buffer, sterile distilled water as well as the second set of primers (L3 and N6). As with the first round of reactions, this solution was also subjected to 70 PCR cycles, again, for 45 seconds at 95° C, followed by 45 seconds at 52° C, and finally 90 seconds at 72° C. 50 µl of each reaction product was then separated by electrophoresis on 1% agarose gel, stained with ethidium bromide and visualised with Ultra Violet light.

Figure 3.2 The BAG vector and PCR primers. Two sets of primers were used in a nested PCR reaction inorder to amplify BAG viral genes from DNA collected from X-gal positive or X-gal negative progeny of single BAG-infected NIH-3T3 cells. This figure shows the position of these primers whose sequences are described in the text, along the BAG vector (Price et al., 1987). L4 and N7 constitute the outer, while L3 and N6 represent the inner set.
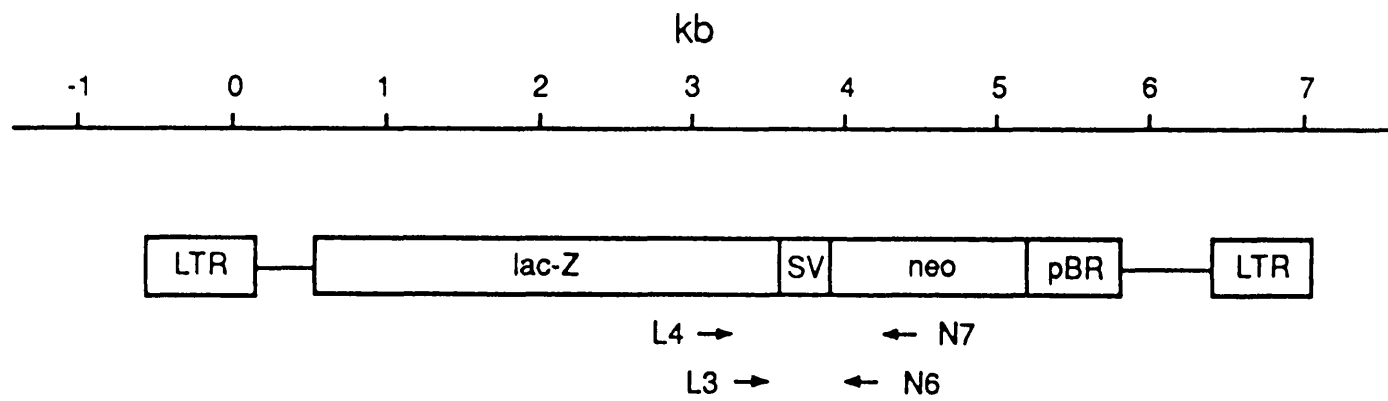
3.3 Results

In this study, single NIH-3T3 cells were plated at single cell density into the wells of 72 well Terasaki plates and exposed briefly to BAG virus, 12 to 15 hours after plating. Thereafter, the fate of single retrovirally-labelled cells was followed over a 48 hour time period, noting whether such cells or their progeny had died, divided, or failed to divide. 72 hours after plating, cells were fixed and stained with X-gal in order to the detect the product of BAG viral transgene, *Lac-Z*, in cells that had become infected with this virus.

An over all analysis of the progeny of single cells in 15 separate experiments showed that of 1767 single NIH-3T3 cells exposed to virus, only 53 gave rise to stained progeny, representing an approximate infection rate of 3% (Table 3.1). Noteworthy was that single cells that failed to divide did not produce any stained progeny, supporting current notions that retroviruses integrate only into dividing cells.

It was noted that 4 of the 53 stained clones contained only a single cell, the rest being multicellular clones. History of the single-cell clones, however, showed that they were the only survivors of a multicellular clone. These were, therefore, eliminated from further analysis.

When the progeny of the remaining 49 clones, were analysed, it was discovered that 39 (80%) of such clones had produced progeny that were composed of both stained and unstained cells, an example of which is shown in figure 3.3. Moreover, in most such clones, stained and unstained progeny occurred at an approximate ratio of 1:1, although a few gave a disproportinately higher number of unstained cells (e.g. clones 2.1 and 10.4 in Table 3.1). In the remaining 10 (20%) clones, only stained cells could be detected. These 'all-stained' clones varied in size from 2 to 10 cells (Table 3.1).

3.3.1 Analysis and interpretation of the mixed stained/unstained progeny

The observation that single BAG-infected NIH-3T3 cells could give rise to both stained and unstained progeny could be interpreted in two different ways: (a) either viral genes were inherited by only half the progeny of a single infected cell, or (b) all the progeny inherited the viral genes but half subsequently turned off expression of these genes. To resolve between these possibilities, DNA from the stained and unstained progeny of single NIH-3T3 cells was amplified for BAG viral genes. For this purpose, the progeny of single NIH-3T3 cells that had been exposed to BAG were separated after 3 to 7 days in culture and grown as subclones for a further 3 to 4 days, at which stage, cells were fixed and stained with X-gal.

Most sublcones either died or simply did not give rise to any stained progeny. The last of these was not particularly surprising, since it was not known a priori whether the progeny to be subcloned were derived from an infected or an uninfected single cell. In two separate experiments, however, subclones survived and also gave rise to blue progeny (Tables 3.1 and 3.2 and 3.3). With only one exception, each of these subclones was found to contain

Table 3.1 Clones of X-gal stained cells obtained from single BAG-infected NIH-3T3 cells. Results of 15 experiments are presented in this table. Column 2 shows the number of single-cells plated, while column 3 shows the number of X-gal positive clones obtained from each experiment. The size and the proportion of stained to unstained progeny within such clones are shown in the fourth and fifth columns, respectively. Column 6 indicates whether the plated single cells divided within the first (1D) or the second (2D) hours after plating.

Note that in several experiments, the progeny of single BAG-infected NIH-3T3 cells were subcloned; only in 2 experiments (Exp. 4 and 8), however, did such subclones survive the experimental period. These subclones are shown in Table 3.2.

In one experiment, progeny of a single BAG-infected NIH-3T3 cell was subcloned onto a 90mm petridish (clones 11.2). When grown to approximately 500 cells in size, this clone was stained with X-gal; although the number of stained to unstained cells within this clone was not accurately determined, it was found to be of the order of 1:1.

In experiments 13 and 14, single NIH-3T3 cells were infected with a cocktail containing equal titres of BAG and DAP viruses (see methods). Purple or purple and unstained cells were noted in 5 clones; these were, clones 13.3, 14.4, 14.5, 14.8 nad 14.10. The remainig clones contained blue and/or unstained cells in the ratios/ numbers indicated.

Exp. No	Single Cells Plated	Clone No.	Clone Size	Blue:Unstained	1D/2D
Exp. 1	62	1.1	9	4:5	1D
		1.2	4	2:2	1D
		1.3	4	1:3	1D
		1.4	2	2:0	2D
		1.5	6	2:4	2D
		1.6	9	4:5	1D
Exp.2	120	2.1	14	4:10	1D
		2.2	12	2:10	2D
		2.3	10	10:0	1D
Exp. 3	64	3.1	35	17:18	2D
		3.2	32	16:16	1D
		3.3	9	7:2	1D
Exp.4	82	4.1	10	6:4	n.d.
		4.2	3	2:1	1D
		4.3	12	6:6	n.d.
		4.4	subcloned		1D
		4.5	subcloned		2D
Exp.5	86	5.1	3	3:0	1D
		5.2	6	2:4	2D
		5.3	16	11:5	1D
Exp.6	150	6.1	4	4:0	1D
		6.2	5	3:2	2D
Exp.7	110	7.1	5	3:2	1D
		7.2	2	1:1	1D
Exp.8	124	8.1	subcloned		1D
		8.2	6	6:0	n.d.
Exp.9	159	9.1	1	1:0	2D
Exp. 10	109	10.1	3	2:1	1D
		10.2	3	3:0	1D
		10.3	26	14:12	1D
		10.4	59	13:46	n.d.
Exp.11	141	11.1	7	7:0	2D
		11.2	500	ca 1:1	2D
Exp. 12	155	12.1	5	3:2	2D
Exp.13	175	13.1	2	2:0	1D
		13.2	6	4:2	1D
		13.3	1	1:0	1D
		13.4	18	9:9	2D
		13.5	23	11:12	1D
		13.6	5	1:4	1D
Exp. 14	178	14.1	2	1:1	n.d.
		14.2	4	2:2	n.d.
		14.3	7	3:4	1D
		14.4	7	7:0	2D
		14.5	1	1:0	1D
		14.6	9	3:6	1D
		14.7	3	2:1	1D
		14.8	2	1:1	n.d.
		14.9	8	7:1	1D
		14.10	12	4:8	1D
		14.11	1	1:0	n.d.
		14.12	3	3:0	2D
Exp15	57	15.1	7	3:4	1D
			Total B:U	177:217	

Figure 3.3 A clone of BAG-infected NIH-3T3 cells. The photomicrograph shows a clone of four cells derived from a single NIH-3T3 cell infected with BAG. Incubation with this clone with substrate X-gal revealed two positively stained (blue, thick arrows) and two unstained (open arrows) progeny. Scale bar= 50 μm.
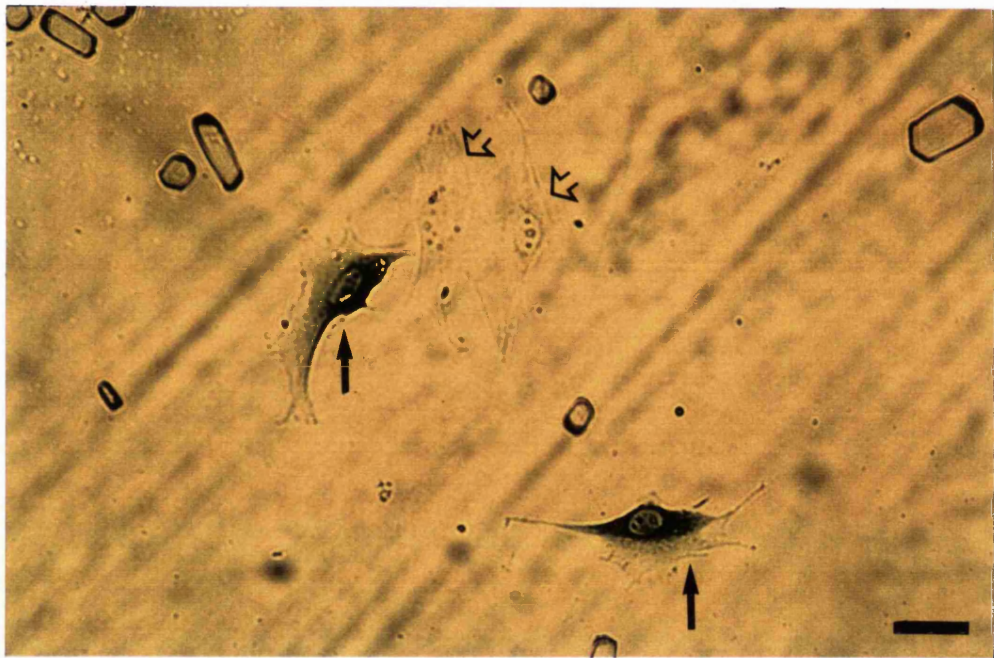
only stained or unstained cells; clone 4.4, for example, gave rise to 5 all-stained and 5 unstained subclones (Table 3.2). The only mixed subclone observed (subclone 8.1.18 in Table 3.3) could have arisen from co-plating of two cells in one a single well at the subcloning stage. This would have been possible, because, after subcloning, determination of cell numbers in each well relied on bright field rather than fluorescence examination; at the subcloning stage cells were not labelled with CFSE, in case they were lost or damaged in the process.

3.3.1.1 Analysis of sublcones using PCR

In order to determine whether unstained subclones carried a copy of the BAG provirus, DNA from both stained and unstained subclones was collected and PCR-amplified for proviral genes. In this respect, a positive PCR signal was obtained from every subclone that had stained positive (blue) with X-gal, verifying that such subcloned cells were carrying viral genes. Unstained subclones, by contrast, never gave a positive PCR signal (Tables 3.2 and 3.3). This observation was not due to the sensitivity of the PCR assay since a positive signal could be detected from even a single stained cell (e.g. lane 8 in Table 3.2).

These results showed that there is a prefect correlation between positive X-gal staining and presence of viral genes within any stained cell. In other terms, unstained progeny of single infected NIH-3T3 cells had not inherited the viral genes and therefore integration must have been into post-replication DNA. This conclusion, however, could not be carried over directly to all clones, since, a sizable (20%) proportion of clones derived form single infected NIH-3T3 cells gave rise to all-stained progeny.

3.3.2 Analysis of all-stained progeny; lack of evidence for integration into pre-replication host DNA

Primary clones in which all members were stained could not be taken as absolute evidence for integration of viral DNA into pre-replication host DNA, since, these clones could arise by several other possibilities. The first of these would be by infection of single NIH-3T3 cells with more than one viral particle. In this senario, several viral DNA molecules (one per virion) would be generated in the infected cell, each integrating independently into random sites within the host genome. Unless all such viral DNAs were to integrate into the same post-replication host DNA strand, then as a consequence of super-infection, both daughters of an infected cell would inherit the viral genes at mitosis thus giving rise to an 'all-stained' clone.

Superinfection in these experiments, however, is highly unlikely because single cells were exposed for only 3-4 hours to a low titre of virus, as the result of which only 3% of the cells gave rise to stained progeny. Nonetheless, in an attempt to investigate whether superinfections were occuring, single NIH-3T3 cells were infected with equal titres of two

Table 3.3 Analysis of subclones I. Subcloned progeny of single BAG-infected NIH-3T3 cells (indicated in Table 3.1) were stained with X-gal, to discover that each subclone was invariably composed of stained or unstained cells (column 3). DNA from some stained and unstained subclones was collected as described in text and PCR amplified inorder to detect the presence of BAG viral genes. In each case, a positive PCR signal at the expected 559 bp band was obtained from stained subclones even when this was only a single cell (lane 8 corresponding to subclone 4.5.1). Unstained subclones, by contrast, did not produce such a signal (lanes 5 and 11, corresponding to subclones 4.4.4 and 4.5.8, respectively).

1 2 3 4 5 6 7 8 9 1011121314151617

← 559
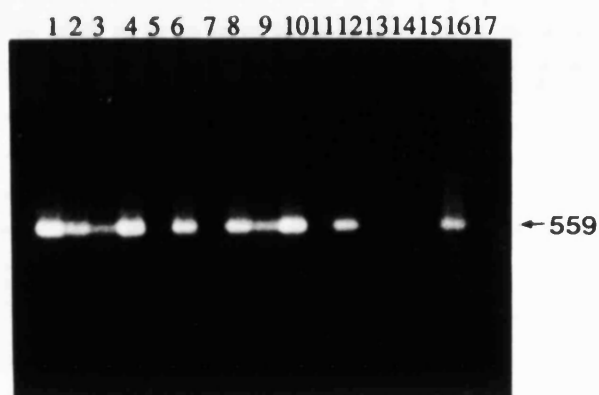
Clone Number	Subclone Number	Blue: Unstained	PCR Lane
4.4	4.4.1	0:1	
	4.4.2	0:1	
	4.4.3	0:1	
	4.4.4	0:2	5
	4.4.5	0:9	7
	4.4.6	2:0	6
	4.4.7	18:0	4
	4.4.8	2:0	3
	4.4.9	3:0	2
	4.4.10	2:0	1

4.5	4.5.1	1:0	8
	4.5.2	6:0	
	4.5.3	21:0	9
	4.5.4	1:0	
	4.5.5	14:0	10
	4.5.6	3:0	
	4.5.7	11:0	12
	4.5.8	0:1	11

Other PCR lanes:

Lane 13   Uninfected cells plus first primer set 3/6
Lane 14   Uninfected cells plus second primer set 4/7
Lane 15   No DNA
Lane 16   Infected cell DNA
Lane 17   ΦX DNA + Hae III

Table 3.3 Analysis of subclones II. Results of a second experiment in which DNA from stained or unstained subcloned progeny of single NIH-3T3 cell was PCR amplified in order to detect the presence of BAG viral genes. As with the results of the first experiment, presented in Table 3.2A, a 559 bp band was detected from stained (lanes 2-6), but not unstained (lanes 7-10) subclones.

One of the subclones in this experiment (subclone 8.1.18) was composed of both stained and unstained cells. This is thought to have arisen by co-plating two subclones into the same well of a Terasaki plate (see text, section 3.3.1).

1 2 3 4 5 6 7 8 9 10111213141516

←559
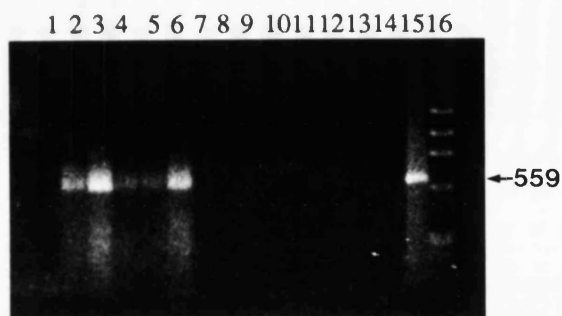
Clone Number	Subclone Number	Blue: Unstained	PCR Lane
8.1	8.1.1	0:5	10
	8.1.2	2:0	
	8.1.3	0:1	
	8.1.4	0:13	9
	8.1.5	2:0	
	8.1.6	1:0	
	8.1.7	0:3	7
	8.1.8	7:0	6
	8.1.9	2:0	5
	8.1.10	2:0	4
	8.1.11	0:3	8
	8.1.12	12:0	3
	8.1.13	0:3	5
	8.1.14	1:0	
	8.1.15	2:0	
	8.1.16	5:0	2
	8.1.17	0:4	1
	8.1.18	3:3	
	8.1.19	1:0	
	8.1.20	2:0	

Other PCR lanes:

Lane 11   DNA from X-gal-negative cells exposed to BAG
Lane 12   Uninfected cells plus first primer set 3/6
Lane 13   Uninfected cells plus second primer set 4/7
Lane 14   No DNA
Lane 15   Infected cell DNA
Lane 16   ΦX DNA + Hae III

✳ Nonetheless, the number of experiments carried out with the two viral prototypes is not sufficient to exclude the possibility that all stained clones arise from superinfection of a single cell with more than one viral particle.

different viruses, each carrying a different histochemical marker. If superinfections were occurring, by chance it would have been expected that progeny of single infected cells would stain positive for both markers.

3.3.2.1 Infection of single NIH-3T3 cells with BAG and DAP: analysis of their progeny

In 2 experiments, the progeny of single NIH-3T3 cells that had been exposed to equal cfus of BAG and DAP, were stained first for X-gal and then for X-phos. When the progeny of such cells were analysed, clones could be found that were composed of blue and unstained cells, or, purple and unstained cells. Moreover, in a few cases, the progeny were 'all-stained'; either all blue or all purple. However, no clone, whether mixed (stained and unstained) or all-stained contained blue together with purple cells. This result strongly suggested that each clone arises from infection of single cells with only one retroviral type and therefore all-stained clones can not be explained wholly interms of superinfection. Furthermore, this experiment demonstrated that detection of stained and unstained cells among the progeny of single BAG-infected NIH-3T3 cells was not a peculiarity of this virus, since DAP virus could also produce such mixed clones. *See opposit.

3.3.2.2 Cell death: a possible explanation of all-stained clones

A second way by which all-stained clones may have arisen is by cell death, since cell death was an unavoidable factor in these experiments. In this case, an all-stained clone could arise if after integration of viral DNA into post-replication DNA, the daughter/s that did not inherit the viral genes die. This possibility was investigated by calculating the probabilities of cell death amongst the progeny of single infected NIH-3T3 cells.

3.3.2.2.1 Calculation of the rate of cell death amongst the progeny of single BAG-infected NIH-3T3 cells

Because the progeny of single NIH-3T3 cells were monitored during the first 48-72 hours of culture, it was possible to determine the rate of cell death within each clone. At each inspection time-point (24 and 48 hours post-plating) cells were seen to adopt one of three fates; cells either died, remained undivided, or divided (figure 3.4). It was discovered, for example, that within the first 24 hours after infection, 31% of original plated single cells had died or disappeared, 46% had remained as viable single cells, and 23% had given rise to two daughter cells. In the subsequent 24 hours, 43% of the remaining single cells had died (or were not visible), 34% again remained as single cells and 23% had divided. More important to this analysis was the fate adopted by cells that divided within the first 24 hours (figure 3.4). In the subsequent 24 hours, some of these 2-cell clones remained as 2-cell progeny while others divided further. Significantly though, in a small proportion, either the entire 2-cell progeny disappeared or the clone was reduced to one cell. A few of the all-stained clones were seen to arise from clones that had

Figure 3.4 Quantitative analysis of the fate of single NIH-3T3 cells; evaluation of the rate of cell death. The fate of single NIH-3T3 cells was followed over a 48 to 72 hour time period before staining with X-gal. At each inspection time point, either 24 or 48 hours after plating, NIH-3T3 cells were seen to adopt one of three fates as shown in the diagram; cells had either died or were not detectable (assumed dead), or divided, or remained as viable single cells. At the first inspection time point, these fates were denoted as a, b or c respectively. These numbers are given in the third, fourth and fifth columns of the lower table, respectively. Thus the rate of death during the first 24 hours is given by a/A,where A is the total number of single cells originally plated. This value was found to be approximately 31 ± 12 %.

The rate of death during the second 24 hours was calculated by analysing the progeny of single cells that had not divided during the first 24 hours in culture (denoted b). This rate, expressed as d/b, was found to be 37 ± 15%. The significance of this rate of death to possible interpretation of all-stained clones is explained in text (section 3.3.2.2.1).

Single cells plated
A

First 24 hours

Died or not visible (a) Remained as single cell (b) Divided (c)

2nd 24 hours

Died or not visible (d) Remained as single cell (e) Divided (f) One or both progeny died No further division Divided further (g)
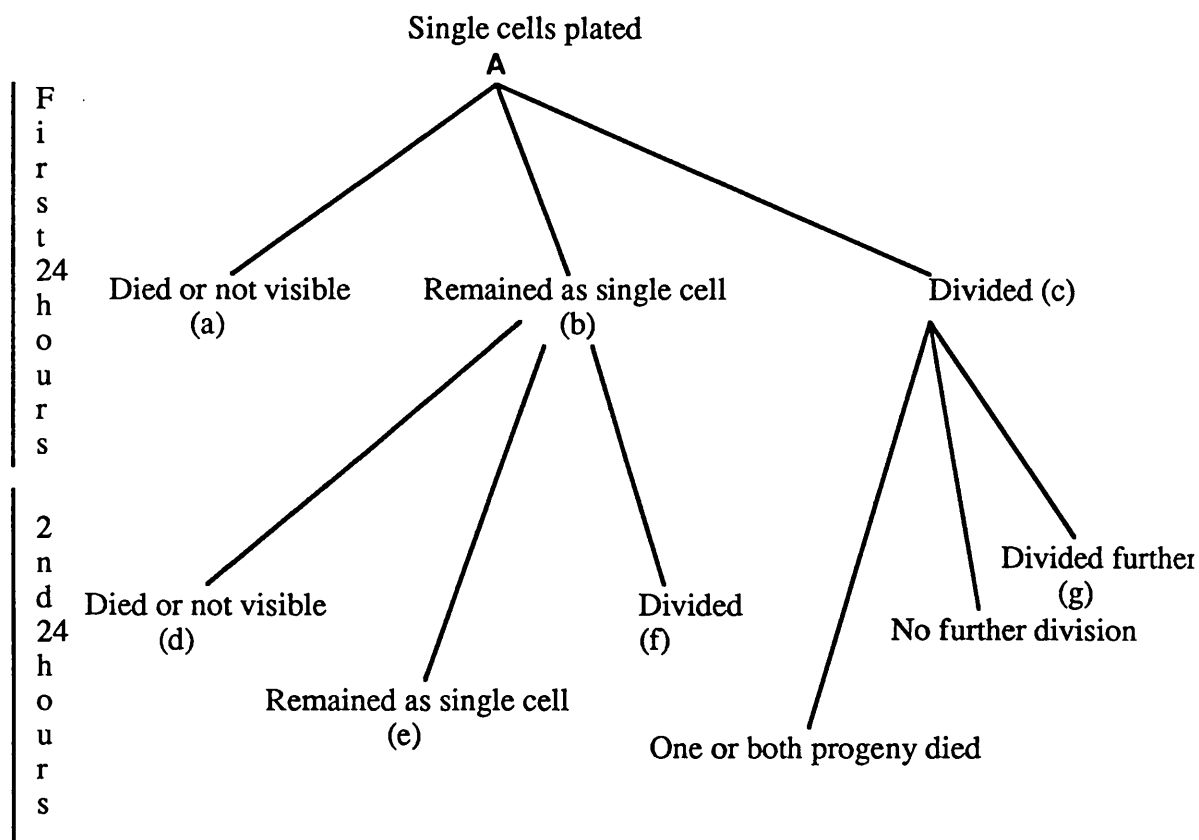
Exp. Number	Number of single cells plated A	a	b	c	Percentage of cell death during the first 24 hours (a/A)	d	e	f	g	Percentage of cell death during the second 24 hours (d/b)
1	62	nd	23	nd	nd	nd	nd	15	nd	nd
2	120	19	74	27	16	18	27	29	11	24
3	64	nd	13	nd	nd	nd	nd	12	nd	nd
4	82	16	44	22	19	9	11	24	11	20
5	86	26	41	19	30	18	14	9	nd	39
6	150	31	105	14	21	20	60	25	8	19
7	110	nd	nd	nd	nd	nd	nd	nd	nd	nd
8	124	nd	24	nd	nd	nd	nd	24	nd	nd
9	159	73	60	26	46	21	25	14	6	35
10	109	56	39	14	51	23	9	7	6	60
11	141	51	63	12	36	14	26	23	7	22
12	155	39	74	42	25	39	15	20	22	52
13	175	42	101	32	24	61	25	15	13	60
14	178	77	74	27	43	31	32	11	13	42
15	57	nd	nd	nd	nd	nd	nd	nd	nd	nd
				Total	31% ± 12				Total	37% ± 15

Figure 3.5 Consequences of labelling symmetrically and asymmetrically-dividing precursor cells with a retrovirus. Integration of retroviral DNA into host post-replication DNA means that following mitosis, only one daughter of an infected precursor cell would inherit the viral genes. However, as far as detecting the progeny of infected precursor cells are concerned, the consequence of labelling a symmetrically dividing precursor cell (A) would be quite different from that of labelling an asymmetrically-dividing cell (B).

Because both daughters of a symmetrically-dividing cell are equal in their developmental potential (A), detecting the progeny of only one of these with a retrovirus is like labelling the lineage of a symmetrically-dividing cell one cell cycle later than otherwise desired. However, labelling an asymmetrically dividing precursor (B) that at each division gives rise to two daughters of different developmental potentials, e.g. a post-mitotic cell as well as a stem cell, would have a number of outcomes. If the post-mitotic cell were to inherit the viral genes then a single-cell clone would result, no matter how late or early in development was the asymmetrically-dividing cell labelled with a retrovirus. This would naturally give a false impression of the full developmental potential of such a precursor.

However, a single-cell clone would always result when an infected precursor cell undergoes terminal division, whether it was dividing symmetrically or asymmetrically. Thus, one can not be certain that single-cell clones are always derived from asymmetrially as opposed to symmetrially-dividing precursors.

Consequences of labelling symmetrically and asymmetrically-dividing precursor cells with a retrovirus.

A Infection of a symmetrically dividing precursor cell (undergoing three rounds of division)
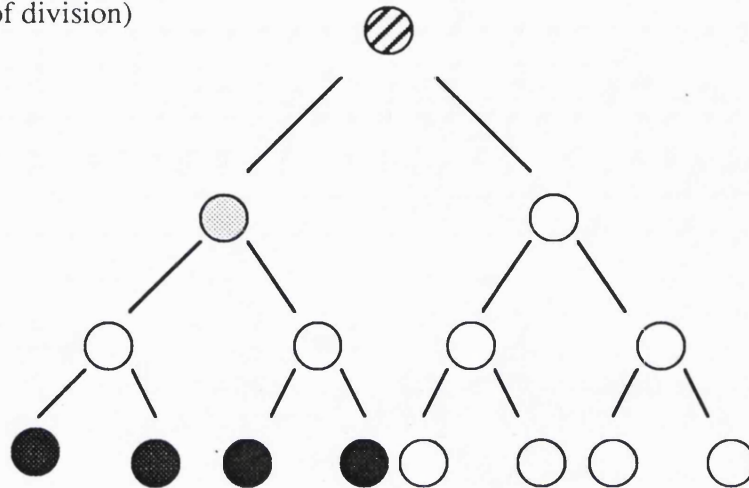
B Infection of an asymmetrically dividing precursor cell (undergoing two rounds of division)

Asymmetrically dividing precursor undergoing terminal division
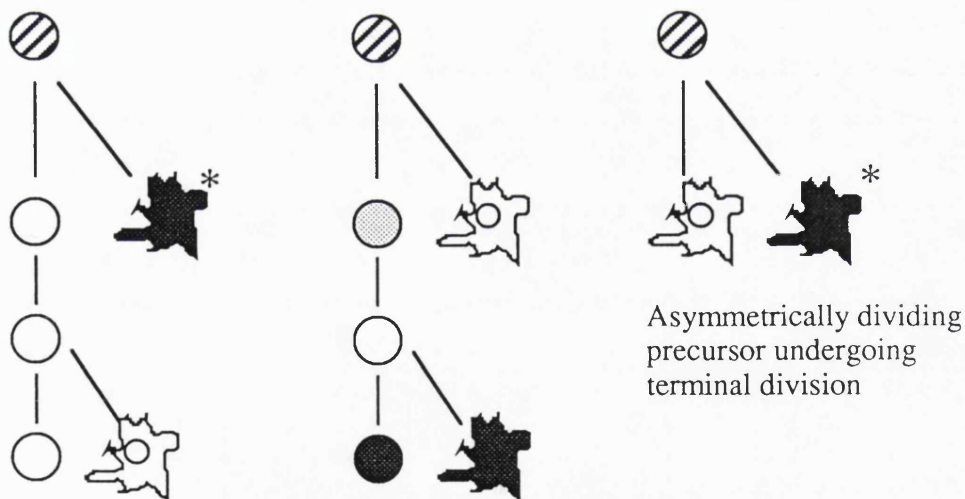
Key:

⊘ Cell exposed to virus

▢ Daughter inheriting the provirus

▪ Progeny expressing viral genes

* Single-cell clone

lost some of their progeny during this time period.

However, a standard rate of cell death during the second 24 hours was calculated by analysing the fate of single-cells that had failed to divide during the first 24 hours (b in figure 3.4). It was discovered that 37% of such cells had died during the second 24 hours in culture, thus suggesting that during this period any given single cell had a 37% chance of death. If this rate of death were to be translated across to single-cells that had divided during the first 24 hours, it would mean that almost a third of 2-cell clones could have lost one of their daughters within the second 24 hours in culture. Accordingly, if viral DNA were to integrate into post-replication DNA, then in half of these cases ($\approx$15 %), the daughter that inherited the viral genes would die, leaving a wholly unstained, hence undetected clone. In the other half ($\approx$15 %), however, the daughter that did not inherit the provirus would have died, leaving an all-stained clone. In this respect, the observed frequency of all-stained clones (20%) seemed to accord well with this expected occurrence of all-stained clones. It is likely, therefore, that all-stained progeny arise from death of daughters not carrying a copy of viral genes, and hence by themselves can not be taken as evidence for integration of viral DNA into pre-replication host DNA.

3.4 Discussion

Results presented in the previous section show that on the whole, infection of a single NIH-3T3 cell results in only half of its progeny carrying the viral genes. This suggests that retroviruses integrate predominantly, if not exclusively, into post-replication DNA of infected cells. Exclusive integration of viral DNAs into post-replication host could be explained in at least three possible ways. First, it may be that viral integration takes place during S phase and is directed to post- as opposed to pre-replication host DNA. Second, integration may not occur during S phase *per se*, but during G2 or M, when all of the host DNA has replicated. Third and finally, integration may be delayed until the next cell cycle, i.e.Viral DNA may remain unintegrated in the cytoplasm of a cell, be inherited by only one daughter after mitosis, and then integrate.

The last of these possibilities would require persistence of a stable viral (DNA) intermediate in the cytoplasm. In this respect, Miller et al. (1990) failed to find such a stable intermediate; these authors observed very little integrated provirus when they allowed growth-arrested infected cells to resume cycling as early as 6 hours after infection. Results of the present study, by contrast, may argue for existence of stable viral intermediate; a third of the stained clones in the present study were derived from single cells that had not divided for at least 24 hours after infection with BAG (Table 3.1, column 6). However, because the exact point of viral infection in this study is unknown, it is difficult to know if such stable intermediates exist; i.e. it is merely known that cells were exposed to virus for a period of 3-4 hours. In any event, the time course of viral life cycle in replicating cells argues against delayed integration. It is known that reverse transcription of the viral genome can occur at a rate of reverse transcription at 2000 nucleotides per hour (Varmus and Brown, 1989) and thus viral DNA can be detected in the nucleus of infected cells as early as 7 hours of infection (Roe et al., 1993).

Thus, delayed integration is not a satisfactory explanation for predominant integration into post-replication DNA.

3.4.1 Do viral DNAs prefer post-replication to pre-replication S-phase DNA as a substrate for integration?

The issue as to whether retroviruses have preferred sites of integration is still a controversial one in retroviral biology. Several studies report that integration of viral DNA is indeed into particular or preferred sites within the host genome (King et al., 1985; Thomas et al., 1986; Rohdewhold et al., 1987; Shih et al., 1988). Shih et al. (1988), for example, who analysed the flanking host sequences of a random selection of viral integration sites have found that 20% of these sites bear significant sequence homology to each other, suggesting that retroviruses integrate into highly preferred targets within the host genome, the so called 'hot spots'. Similarly, King et al. (1985) who transduced viral genes for the purpose of insertion mutagenesis into teratocarcinoma (EC) cells, report that

viruses integrated more frequently at distinct chromosomal sites than would otherwise be expected by chance.

Several explanations have been put forward for these observations. It has been suggested that integration 'hot spots' may represent distinct sequences that are recognised by, or are more susceptible to the DNAase action of viral integrase enzyme, IN (Rohdewohld et al., 1987) (see section 1.8.5.1 for molecular description of IN's action). Alternatively, the preferred sites of integration may reside in host DNA regions undergoing active transcription (Mooslehner et al., 1990; Scherdin et al., 1990). Interestingly, analysis of bacterial transposable elements that integrate in an analogous fashion to viral DNA fragments, show that they too integrate into specific sites in the host genome (Sandmeyer et al., 1990; Craigie, 1992). However, because these elements do not have an extracellular phase of life cycle and hence rely on host cells for their survival, it is hypothesised that they may have been under evolutionary pressure not to integrate into sites that may perturb their normal host gene function (Craigie, 1992).

In contrast to the above studies, however, several authors have failed to find any evidence for preferred sites of integration. Hughes et al. (1981), for example, who analysed several independent integration sites in rat cell lines transformed by Rous Sarcoma Virus (RSV) found very little sequence similarity between these sites, concluding that integration of viruses occurs at random sites within host DNA. The strongest evidence against preferred integration sites by retroviral DNAs, however, comes from studies of retroviral integration in cell-free systems (Brown et al., 1987; Fujiwara and Craigie, 1989; Ellison et al., 1990; Pryciak et al., 1992). These studies have shown that viral DNA, isolated from acutely infected cells, can integrate into random sites within artifical integration substrates such as phage λ DNA, or short fragments of naked cellular DNA, or DNA associated with histones. Therefore, it seems unlikely that integration of viral DNA is directed toward post-replication S phase host DNA. Perhaps S phase *per se* is not the phase of viral DNA integration since no cellular factors associated with S phase have been identified that could facilitate viral integration; viral integration does not even require ATP or host protein synthesis (Brown et al., 1989). This then leaves the third possible explanation for predominant integration of viral DNAs into post-replication host DNA; i.e. integration could be occuring during G2 or M, when all of host DNA has undergone replication.

3.4.2 Retroviral integration may be occuring subsequent to S phase

A recent study by Roe et al. (1993) has overturned some previously held views about the timing of viral integration, shifting the emphasis from S to the M phase of the cell cycle.

Roe et al. (1993) have investigated the timing of viral integration by infecting cells arrested in different stages of the cell cycle. For example, temperature sensitive mutant cells were arrested in G2, and Rat-1 cells were arrested in G1/S border by serum

starvation or treatment with aphidicolin, or even at the metaphase stage of mitosis by treatment with an inhibitor of microtubule assembly. These authors showed that in each case, these reversibly growth-arrested cells could become infected with a replication-defective murine leukemia virus and were able to support the synthesis of unintegrated viral DNA that could participate successfully in in vitro integration reactions. However, Roe et al. (1993) found that regardless of the phase in which cells were infected, when cells were released from their block, integration always occurred coincidental with the onset of mitosis, in particular its metaphase stage. From these results Roe et al. (1993) drew two conclusions. First, that M rather than S phase is somehow important to viral integration; i.e., importance of S phase to viral integration, as reported previously, could be explained as mere passage of cells through S before reaching M. Second, because viral integration required passage through metaphase, a phase during which nuclear membrane breaks down, it was suggested that the nuclear membrane may pose a physical barrier to the entry of viral DNA into host cell nucleus; i.e., only with its breakdown at metaphase, would cellular DNA become exposed to viral DNA. Studies of form and function of nuclear membrane support this hypothesis.

It is known that nuclear membrane plays a crucial role in cytoplasmic-nuclear protein trafficking and therefore in the normal functioning of a cell. For example, it has been shown that a factor, normally present in the cell cytoplasm, ensures that the DNA of a eukaryotic genome is replicated only once during S phase. It is thought that this so-called 'DNA-replication licensing factor' gains access to nuclear DNA only with the breakdown of nuclear membrane at mitosis (Blow, 1993); this licensing factor binds to nuclear DNA, becomes activated during the next S phase and is rapidly degraded once a single round of DNA synthesis has been completed.

However, nuclear breakdown is not the only route by which distinct molecules gain access to cell nucleus. Morphometric studies show that nuclear membrane possesses pores of 90-110 Å diameter, such that particles with a diameter smaller than this value can freely diffuse across the nuclear membrane (see review of Newmeyer, 1993; Pante and Aebi, 1993). Transport of larger cytoplasmic molecules into the nucleus, however, requires possession of a nuclear localisation signal (NLS) sequence by these molecules. It is thought that NLS sequence is recognised and bound by special carrier proteins, which, by negotiating with the nuclear pores, facilitate the entry of NLS-bearing molecules into the nucleus (Adam and Gerace, 1991; Garcia-Bustos et al, 1991) by a process that requires ATP. On this point, Roe et al.'s hypothesis about the role of nuclear membrane gains further credence. It is known that in contrast to Mo-MLV, the virus on which most replication-defective vectors including BAG are based, the viral DNA of HIV can integrate into genome of some non-dividing cells (Lewis et al., 1992; Bukrinsky et al., 1992), an ability that has been correlated with possession of NLS by this retrovirus. Bukrinsky et al. (1993), who mutated the NLS-bearing region of HIV genome, have shown that such

mutant viruses can not enter the nuclei of G1-arrested cells. No such NLS sequence, however, is found in Mo-MLV genome. Thus, lack of an NLS sequence as well as the large (300 Å) size of an Mo-MLV pre-integration complex may indeed prevent its entry into the nucleus; entry may only take place with the breakdown of nuclear membrane.

However, it is important to note that to date no study has demonstrated directly that an intact nuclear membrane limits viral integration. In this regard, there could be other explanations. For example, the ability to integrate into dividing versus non-dividing cells could be explained in terms of the number or permeability of nuclear pores, since five times as many nuclear pores are found in the nuclear membrane of dividing as compared to differentiated cells (Newmeyer, 1993). Furthermore, Roe et al. (1993) discovered that a proportion of viral DNAs did not integrate for sometime after the break down of the nuclear membrane, suggesting that other factors beside nuclear membrane may be limiting retroviral integration.

These explanations aside, integration of viral DNA into post-replication host DNA is of great importance for practical applications of retroviruses, in particular, for cell lineage studies and prospects of gene therapy.

3.4.3 Viral integration into host post-replication DNA; implications for practical applications of retroviruses

3.4.3.1 Cell lineage studies

Retroviruses carrying detectable marker genes have been widely used in cell lineage studies in order to analyse the presumptive fate or the developmental potential of precursor cells in vivo and in vitro, respectively (see chapter I, section 1.4.4.) Crucial to all such studies, however, is that all the progeny of a labelled precursor cell is analysed. Results presented in this chapter, however, show that because viral DNA integrates into post-replication host DNA, only half the progeny of a virally-infected precursor cell inherit the viral genes and thus become detectable. Naturally, this phenomenon must be accounted for when analysing the fate of single precursor cells. In theory, such precursors could divide either symmetrically or asymmetrically. In a symmetrical division, a precursor cell would generate two daughters of equal developmental potential; two mitotic daughters, for example (figure 3.5). Infection of such a precursor with a retrovirus would mean that only one half of two equal lineages would subsequently be detected, as if the lineage in question was analysed one cell cycle later than otherwise desired (figure 3.5). The consequence of infecting an asymmetrically dividing precursor, however, is more profound, because, at every division an asymmetrically dividing cell generates two daughters of different developmental potentials; one that acts as a self-renewing stem cells, and another that is restricted in its fate and/or proliferative potential (figure 3.5). It is thought, for example, that some cortical neurones are generated by asymmetrically-dividing precursor cells (McConnell, 1988); i.e. at each division, such precursors produce a dividing neuronal

precursor as well as a post-mitotic neurone. Infection of an asymmetrically-dividing neuronal precursor, therefore, would mean that only in 50% of cases will the dividing daughter inherit the viral genes. In the other 50% of cases, the post-mitotic neurone will inherit such genes giving rise to a single-cell neuronal clone (figure 3.5). If the infected neuronal precursor were to undergo its terminal division, then a single cell clone will result no matter which daughter inherits the viral genes. Moreover, some single-cell clones would always be produced no matter how late or early in development an asymmetrically-dividing neuronal precursor is infected.

It is thus tempting to speculate that the high frequency of single-cell clones obtained when cortical precursors are labelled with a retrovirus at different stages of cortical development either in vivo (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Austin and Cepko, 1990; Moore and Price, 1991; Mione et al., 1994) or in vitro (Willimas et al., 1991; results of chapter II), is evidence for presence of asymmterically dividing precursors. However, this is by no means absolute since single-cell clones could also arise from the terminal division of symmetrically-dividing precursors.

3.4.3.2 Prospects of gene therapy

Despite proving to be the safest and most efficient vehicles of gene transfer into cells, the application of retroviruses in current gene therapy protocols is limited by the fact that most retroviruses can not integrate into non-dividing cells (see reviews of Tolstoshev, 1992; Leib and Olivo, 1993). For example, current endeavours in providing gene therapy for Alzhemier's, Huntington' and Parkinson's diseases are focused on delivering genes to post-mitotic neurones of the mature CNS . At present, advenoviral vectors are used for transferring genes into adult CNS cells (Breakefield and Delucia, 1991; Le Gal Le Sal et al., 1993)

If, as suggested by the above findings, the nuclear membrane were to pose a barrier to viral entry, it may now be possible to adopt a new strategy for gene delivery into non-dividing cells. Mo-MLV vectors carrying nuclear localisation sequences could be constructed, thus targeting the viral DNA to the nucleus of the non-dividing cell. Alternatively, it may be possible to use retroviral vectors that already posses nuclear localisation sequences, such as those of lentiviruses, the typical prototype of which is the human immunodeficiency virus (HIV).

Chapter IV

An investigation of the effects of basic fibroblast growth factor
on the development of cortical neuronal precursrors in vitro

4.1 Introduction

Detection of acidic and basic FGF protein and mRNA, as well as that of FGFRs in the developing CNS of mice and rats (reviewed in Chapter I, section 1.7.3.4.3.1) has led many workers to investigate possible roles of these factors in the development of the CNS. In the absence of an in vivo assay, however, such efforts have focused on testing the effects of purified aFGF and bFGF on cultured embryonic cells (reviewed in chapter I). Typically, dissociated embryonic cells derived from a particular region of the developing CNS have been cultured in bulk, in the absence or the presence of these FGFs. The discovery that cultured cells either die in the absence of FGF, or that cultures contain more cells when grown in its presence, has suggested that acidic and basic FGF may be survival factors for CNS neural cells. Furthermore, results of a few workers who could find more thymidine-labelled cells in the presence of FGF, has suggested that FGF may also act as a mitogen for neural precursors.

As discussed in Chapter I (section 1.7.3.4.3.2), however, it is not clear from these studies how FGF may have influenced the behaviour of single precursor cells, in particular whether FGF has any influence on the phenotypic choice of bi- or multipotential precursors that may exist in such cultures. For example, detection of more neurones in 5 day cultures of E13 rat cortex grown in the presence of bFGF, as reported by Gensburger et al. (1987), would raise the following questions: did bFGF act as a survival factor for neuronal precursors or mature neurones,or did bFGF rescue non-neuronal precursors and induce their neuronal differentiation? These questions would be fundamental when extrapolating the in vitro effects of bFGF to its possible roles in vivo. Again as discussed in Chapter I, these questions can only be answered by a clonal analysis in the presence of bFGF.

In this respect, the present study attempts to characterise the effects of bFGF on rat cortical precursor cells using a novel method of clonal analysis. This involves comparing the fate of single retrovirally-labelled precursor cells grown amongst a bulk population of unlabelled cells of the same age and preparation, in the presence or absence of bFGF.

Results presented in Chapter II demonstrated that E16 and E14 rat cortical precursor cells may be specified in their phenotypic potential. Thus, as well as investigating possible survival or mitogenic effects of bFGF on cortical precursors derived from these ages, this study also asks whether bFGF can influence the developmental potential of such precursor cells.

4.2 Material and methods

4.2.1 Procurement and storage of growth factors

The majority of the experiments described in this chapter were carried out using either Xenopus basic fibroblast growth factor (Xen.bFGF, kind gift of Jeremy Green and Jim Smith), or Human recombinant bFGF (British Biotechnologies, Abingdon). Xen.bFGF was supplied as a 60 μg/ml stock solution, and Hu. Rec. bFGF as a 10 μg/ml lypholysed compound, which was reconstituted in 1 ml of normal tissue culture medium (DMEM). Xen. and Hu. Rec. bFGF were diluted in DMEM to stock solutions of 1000 or 100 ng/ml and stored for later use as 0.5 or 1ml aliquots at -20 or -70°C.

In two experiments, FGFs 1 to 7 (kind gift of Dr. Ivor Mason) were also tested for their effects. These factors had been produced by in vitro translation of reticulolysates of cells that had been transfected with cDNA encoding each of these factors (Dr. Ivor Mason, personal communication). The precise concentration of each of these factors was not known and thus they were used at a dilution of 1: 100. Also, to avoid repeated freezing and thawing, these factors were aliquotted and kept at -70°C for later use.

4.2.2 Dilution and storage of heparin

Initially, a 1mg/ml heparin (Sigma, H 3149, tissue culture grade) solution was prepared by dissolving heparin powder in 1xPBS solution. This was diluted further in DMEM to a concentration of 1000 ng/ml and stored at +4° C for later use.

4.2.3 Cell culture

4.2.3.1 Preparation of astrocyte-conditioned medium

Serum-free astrocyte conditioned medium was prepared from medium bathing bulk, purified cultures of cortical astrocytes (described in section 2.2.8). For this purpose, astrocytes that were normally grown in the presence of 10% FCS/DMEM, were rinse twice with Satos medium, and grown in this medium for three days at which point, this medium was collected, centrifuged for 5 mins at 1000 rpm, and passed through a 0.8 μm pore size analytical filter unit. Filtered conditioned-medium was then aliquoted and kept at -20° C for later use.

4.2.3.2 Culturing embryonic cortical cells in the presence or absence of growth factors

Throughout the following experiments, a random population of BAG-labelled E16 or E14 cortical cells were grown among a population unlabelled cells of the same age and preparation on monolayers of cortical astrocytes in the presence or absence of growth factors.

For this purpose, suspensions of dissociated embryonic cortical cells were prepared and plated on monolayers of cortical astrocytes as described in section 2.2.9. Briefly, suspensions of embryonic cortical cells were first diluted in 10%FCS/ DMEM down to a

density of 4-5 x 10^5 cells/ml. BAG virus, in a ratio of 15 μl per well, was then added to this suspension. The suspension was mixed thoroughly and split into volumes receiving bFGF (or other growth factors) and controls. bFGF was usually added to a final concentration of 10 ng/ml and controls received an equal volume of medium. The cells suspensions were again mixed and plated as 0.5 ml aliquots onto monolayers of cortical astrocytes that were grown on coverslips inside placed in the wells of 24 multiwell plates, allowing three wells for each experimental condition. By this procedure, each well received approximately, 2.0-2.5 x 10^5 cells, and 900-1000 cfus of BAG virus.

Soon after cells were plated, plates were transfered to and kept at 37° C in a humidified, aerated (5% Co_2/ 95% air) incubator. For the sake of noting the day on which growth factor were added, this was noted as the first day of culture. 20 hours after plating, the medium in each well was replaced with Sato's medium, at which stage, a second dose of growth factors was added to test wells; again controls received an equal volume of medium. On day 4 of culture (i.e. 72 hours after plating), cells were again examined and fed with fresh Satos medium and in some cases a third dose of growth factors was added. Thereafter, both control and growth factor-supplemented cultures, were examined and fed every 48 hours with Satos.

Occasionally, cells were also grown in the absence of cortical astrocytes. In these experiments, cells were plated either in the presence or the absence of bFGF onto astrocyte or NIH-3T3 matrix or simply on PDL-coated coverslips. Astrocyte and NIH-3T3 cell matrices were prepared by air-drying monolayers of cortical astrocyte or NIH-3T3 cells (that had been rinsed with prewarmed 1xPBS) for one hour inside sterile tissue culture hoods. In some experiments, cells cultured on these matrices were grown in the presence of astrocyte-conditioned medium.

4.2.3.3 Detection of BAG-infected cells and identification of their phenotypes in control and growth factor-treated cultures

Clones of BAG-labelled cells on both control and bFGF-treated cultures were detected in a way identical to that described in chapter II, section 2.2.10. Briefly, after seven days in culture, coverslips on which the cultures were grown were recovered form the wells of 24 multiwell plates, fixed with 4% paraformaldehyde and stained with anti-β galactosidase as well a combination two other cell type specific antibodies, listed in Table 2.1 of chapter II. After this staining, coverslips were mounted in glycerol/PBS (citiflour) and scanned under the x25 objective of a Zeiss microscope equipped with a U/V light source. During this scan, clusters of β-gal positive cells were noted as 'clones' according to the criteria described in chapter II (section 2.2.10.6.2). The number of clones found on both control and bFGF-treated coverslips were thus noted. Moreover, the phenotye and number or cells in each clone was also recorded.

4.2.4 Statistical comparison of the size distribution of neuronal clones found in cultures grown in the absence, compared to the presence of bFGF

The statistical difference in the size distribution of neuronal clones between two different treatments (e.g. size distribution of neuronal clones in E16 cultures grown in the absence compared to the presence of bFGF) were tested using *two-sample test of Kolmogorov-Smirnov* (also called the Smirnov test; Lindgren, 1976) which is based on statistic D, defined as:

$$D= \sup_{all\ x} \left| F_m(x) - G_n(x) \right|$$

where, $F_m(x)$ is the sample distribution function of a sample size m from X and $G_n(x)$ is the sample distribution function of a sample of size n from Y. In our case, Sample X is the total number of neuronal clones obtained for a particular clone size from experiments in which E16 cultures were grown in the absence of bFGF, and Sample Y is the same parameter but for E16 cultures grown in the presence of bFGF.

Thus in order to carry out this test, the total number of clones from all experiments were used from Table 4.1. These data were tabulated as follows:

	E16 cultures (control)			E16 cultures+bFGF			
Clone size	**Sample X**	**Cumulative frequency X**	$F_m(x)$	**Sample Y**	**Cumulative frequency Y**	$G_n(x)$	$F_m(x) - G_n(x)$
1	606	606	0.92	930	930	0.90	0.02
2	38	644	0.98	139	1101	0.90	**0.08**
3	5	649	0.99	32	1121	0.93	0.06
4	3	652	0.99	20	1132	0.95	0.04
5	1	653	0.99	11	1143	0.96	0.03
6	0	653	0.99	11	1147	0.97	0.02
7	0	653	0.99	4	1153	0.97	0.02
8	0	653	0.99	6	1156	0.97	0.02
9	0	653	0.99	3	1170	0.98	0.01
10+	1	654	1	14	1177	0.99	0.01
20+	0	654	1	7	1177	1	0
50+	0	654	1	0	1177	1	0

According to this test, therefore, D= the largest value of $F_m(x) - G_n(x)$, ignoring any negative signs. In this case D= 0.08. This value is then compared with an α value obtained from tables of acceptance limits for the Two-Sample Kolmogorov-Smirnov test. If the value of α is found to be equal to or less than D, then the null hypothesis is rejected

and the two distributions are regarded to be significantly different. However, since this table only accounts for sample sizes up to 15 cells, Kolmogorov-Smirnov suggest a formula for testing significance above this size.

According to this formula, the value $\alpha = 1.36 \sqrt{((n_1+n_2) \div (n_1.n_2))}$ at 5% level of significance, and $\alpha = 1.63 \sqrt{((n_1 + n_2) \div (n_1.n_2))}$ at 1% level of significance, where n_1 and n_2 are the size of the samples; in this example, $n_1 = 654$, and $n_2 = 1177$.

Thus, in this test the value $\alpha = 0.06$ at 5% level and 0.08 at 1% level, according to which the distribution of neuronal clone sizes in E16 cultures grown in the presence of bFGF is significantly different from that grown in its absence.

One drawback of this type of test, however, is that it ignores the variation within experiments since total number of clones from a large number of experiments are analysed, as if they represented a single population. For example, clone numbers for E16 cultures grown in the absence of bFGF was gathered from 17 different experiments.

In any event, similar statistics were carried out for graphs of neuronal clones size distribution presented in the results section, below. The significance values obtained are indicated in the legends to each graph.

4.3 Analysis of Results

In this study, the effects of bFGF on cortical precursor cells were investigated by comparing the fate of BAG-labelled E16 or E14 cortical cells that were grown for a week on monolayers of cortical astrocytes in the presence or absence of bFGF. Two different bFGFs were tested: Xenopus bFGF (Xen. bFGF) and human recombinant bFGF (Hu. Rec. bFGF), both of which were found to have similar effects. In the following description of the effects of bFGF on cortical precursors, however, the emphasis will be on results obtained with Xen. bFGF, simply because three times as many experiments were conducted with this rather than Hu. Rec. bFGF. Moreover, since most experiments were carried out on E16 precursors, the results described below will pertain mostly to precursors derived from this embryonic age; whenever bFGF's effects on E14 precursors are described, this will be indicated.

4.3.1 Effects of bFGF on cultured cortical precursor cells

4.3.1.1 Type and proportion of clones found in E16 and E14 cultures in the presence of bFGF

The types of clones found in the presence of bFGF, both in E16 and E14 cultures, were identical to those found in its absence. These were clones composed of neurones, oligodendrocytes, astrocytes, or clones of cells that did not label with any of the cell type-specific antibodies used, but were homogeneous in appearance. bFGF, therefore, had no apparent effect on the phenotypic composition of clones in both E16 and E14 cultures (Table 4.1). Moreover, bFGF had no apparent effect on the relative proportions of different clone types. The majority of clones found in the presence of bFGF, both in E16 and E14 cultures were neuronal, with non-neuronal clones - clones of oligodendrocytes, astrocytes or unidentified cells - making up only 14% of all clones detected either in the presence or absence of bFGF (Table 4.1). Presence of bFGF did, however, affect the number of clones detected per coverslip, an effect that is described below

4.3.1.2 bFGF's effect on the overall frequency of clones

Consistently more clones were found in bFGF-treated cultures than in controls. When data from all experiments were pooled (Table 4.1) it was discovered that bFGF almost doubled the average number of clones detected per coverslip in E16 cultures. In the presence of Xen. bFGF, for example, on average 24 clones were found per coverslip, compared to an average of 14 clones in control cultures. Similarly, significantly more clones (21 per coverslip) were detected in the presence of Hu. Rec. bFGF than in its absence (12 clones per coverslip).

In E14 cultures, however, in which only the effects of Xen. bFGF were tested, the increase in the number of clones due to the presence of bFGF was less dramatic; on

average 28 clones were detected in the presence, compared to 19 in the absence of Xen. bFGF.

Because control and bFGF-treated cells were cultured at identical densities and were infected with identical concentrations of BAG, detection of more clones in the presence of bFGF suggested that bFGF had acted as a survival factor for E16 (and E14) cortical precursor cells. i.e. a considerable number of BAG-labelled cortical precursors may have normally gone undetected because they did not survive in the absence of bFGF. The question was, however, which type or types of precursor cells had responded to this survival effect? To answer this question, the percentage change in the number of each clone type was analysed. This analysis showed that in the presence of bFGF, there was a 45% increase in the number of neuronal and an 86% increase in the number of astrocytic clones in E16 cultures (Table 4.1). The increase in astrocyte clone numbers may, however, have been underestimated since some unidentified clones, whose numbers themselves showed a 58% increase in the presence of bFGF, were most likely to have been astrocytes (see material and methods). These figures suggested that precursors to neurones, astrocytes and unidentifiable cells, may all have responded to bFGF's survival effect. However, considering that both in the presence and absence of bFGF, astrocytic and unidentified clones together accounted for only 10% of all clones, and that neuronal clones were the most frequent clone type, it was clear that bFGF's survival effect was on neuronal precursors.

Interestingly, however, relatively fewer oligodendrocyte clones were detected in the presence of bFGF; the frequency of oligodendrocyte clones that made up 6-7% of all clones in controls, was 2% in the presence of bFGF (Table 4.1).

4.3.1.3 bFGF's effect on the size of neuronal clones
E16 cultures

As well as more neuronal clones, larger neuronal clones were also detectable in the presence of bFGF. In order to quantify this difference, the size distribution of neuronal clones in control cultures were compared with that of bFGF-treated cultures (figure 4.1). This comparison revealed some similarities and some differences between the two conditions. The two conditions were similar, in that in both cases the majority of neuronal clones were composed of only a single cell; in controls, single-cell clones accounted for over 90% of all neuronal clones, but only 78% in bFGF-treated cultures (figure 4.1). The two conditions differed significantly, however, in their frequency and size of clones containing 2 or more neurones. In control cultures, these 2-cell and greater clones made up only 7% of all neuronal clones and the largest such clone rarely contained more than 2 cells. In the presence of bFGF, by contrast, close to 27% of all neuronal clones contained 2 or more cells. Moreover, even though most of these larger neuronal clone contained only 2-cells, a spectrum of clone sizes ranging from 2 to 20 cells could be found in the presence

of bFGF (figure 4.2). Two interesting properties of these larger neuronal clones were that most remained tightly clustered, and that frequently members of a clone were remarkably similar in morphology (figure 4.2).

E14 cultures

As with E16 cultures, 2-cell and greater neuronal clones in E14 cultures were more frequent in the presence of bFGF (Table 4.1 and figure 4.3). This difference, however, was not as obvious as in E16 cultures, because, even in the absence of bFGF, 2-cell and greater neuronal clones were usually more frequent in E14 than in E16 cultures (for this comparison see results of chapter II). As far as the frequency of single-cell neuronal clones were concerned, however, bFGF produced a different effect in E14 cultures. In contrast to E16 cultures, in which the frequency of single-cell clones remained relatively constant in the presence of bFGF, there was a sharp fall in the frequency of single-cell clones in E14 cultures in the presence of bFGF; single cell clones made up 43% of all neuronal clones in the presence, compared to 73% in the absence of bFGF (figure 4.3).

The question begged by the observed increase in the frequency and size of 2-cell and greater neuronal clones in cultures grown in the presence of bFGF, therefore, was: did bFGF act as a mitogen to increase the size of some neuronal clones, or, did it act simply as a survival factor for precursors of greater proliferative potential that may not have survived in control cultures? Although it was difficult to make such a distinction, some possibilities could be ruled out. bFGF could not have acted solely as a mitogen. Detection of more neuronal clones, a considerable proportion of which were single-cell clones, in its presence was strong evidence for a survival effect. Neither, could bFGF have had acted as a mitogen for *all* E16 neuronal precursors. Had it done so, a significant shift in the size of clones, away from single- and 2-cell clones toward larger neuronal clones would have been expected. This shift, however, was not observed. Therefore, if in addition to it survival effect bFGF had acted as a mitogen, this effect would have been only on a subpopulation of neuronal precursors.

The case for an mitogenic effect by bFGF on E14 precursors, however, may have been stronger, because there was a shift in the frequency of single-cell clones towards that of 3-cell and greater neuronal clones (figure 4.3). However, even this shift could be explained purely as a differential survival effect by bFGF, i.e. in E14 cultures, precursors that gave rise to larger neuronal clones may, and those that produced single-cell or even 2-cell neuronal clones may not, have responded to bFGF's survival effect.

Table 4.1 Type and frequency of clones found in 7-day cultures of dissociated embryonic cortical cells grown on monolayers of cortical astrocytes in the presence or absence of bFGF. The first column of each table shows the types of clones found in cultures of E16 or E14 cortical cells. Neuronal clones in these columns are divided into two groups; those containing only a single-cell (1 Cell N.) and those containing 2 or more cells (2+ N.). The second and third columns show the total number (frequency in brackets) of each clone type found in absence or the presence of bFGF, respectively. The fourth column shows the percentage change in the number of clones due to presence of bFGF. For example, 35% more single-cell neuomal clones were found in E16 cultures grown in the presence of Xenopus bFGF.

Panel A shows the effects of bFGF on E16 cultures; effects of Xenopus bFGF are shown in panel A(i), while that of Human recombinant bFGF is shown in panel A(ii). On average, 14 ±7 clones per coverslip were found in cultures grown in the absence, and 24 ±12 clones in cultures grown in the presence of Xenopus bFGF (panel Ai). These numbers were 12 ±4 and 21 ±5, respectively, for data presented in panel A(ii). Panel B shows the effects of Xenopus bFGF on the numbers of different clone types found in E14 cultures. The average number of clones per coverslip found in these experiments were 19 ±7 in the absence, and 28 ±5 in the presence of bFGF.

Type and frequency of clones found in 7 day cultures of dissociated embryonic cortical cells grown on monolayers of cortical astrocytes in the presence or absence of bFGF.

A. E16 cultures

(i) Effects of 10 ng/ml Xenoups bFGF

Clone type	Control	+ Xen.bFGF	Percentage change
Neurones 1 Cell N.	606 (80%)	930 (69%)	+35%
2+ N.	48 (6%)	247 (18%)	+81%
Oligodendrocytes	42 (5.9%)	31 (2.5%)	- 27%
Astrocytes	1 (0.1%)	7 (0.5%)	+86%
Unidentified cells	59 (8%)	141 (10%)	+58%
Total	756	1356	

(ii) Effects of 10 ng/ml Human Recombinant bFGF

Clone type	Control	+Hu.Rec. bFGF	Percentage change
Neurones 1 Cell N.	203 (84%)	298 (68%)	+31%
2+ N.	12 (5%)	94 (22%)	+88%
Oligodendrocytes	17 (7%)	10 (2%)	- 41%
Astrocytes	0 (0)	0 (0)	0
Unidentified cells	11 (4%)	34 (8%)	+67%
Total	243	436	

B. E14 cultures

Effects of 10 ng/ml Xenopus bFGF

Clone type	Control	+ Xen.bFGF	Percentage change
Neurones 1 Cell N.	68 (60%)	59 (38%)	-12%
2+ N.	35 (31%)	74 (48%)	+53 %
Oligodendrocytes	7 (6.1%)	6 (3%)	-15 %
Astrocytes	1 (0.9%)	2 (1%)	+50%
Unidentified cells	4 (3%)	16 (10%)	+ 75%
Total	115	157	

Figure 4.1 Distribution of the size of neuronal clones in E16 cultures grown in the presence or absence of bFGF. As evident from this graph, over 90% of neuronal clones in cultures grown in the absence of bFGF, were single-cell clones and clones greater than this size rarely contained more than 2 cell. The 10-cell clone shown in the figure represents only two clones from a total of 756 clones. In contrast to the restricted size of neuronal clones in controls, neuronal clone sizes ranging from 2 to 20+ cells could be found when cultures were grown in the presence of bFGF.

Statistical analysis using the *two-sample Kolmogorov-Smirnov test* showed that the distribution of neuronal clone sizes grown in the presence of bFGF was significantly different from that grown in its absence. The values obtained from the *Kolmogorov-Smirnov test* for this analysis were as follows:

Comparison of distribution for	$F_m(x) - G_n(x)$	$p \leq 0.05$	$p \leq 0.01$
E16 cortical cells grown in the absence versus			
the presence of bFGF	0.14	0.07**	0.08**

where ** denotes significance at the shown level of confidence.

However, despite the increase in the frequency and size of neuronal clones due to the presence of bFGF, single-cell neuronal clones still made up just under 80% of all neuronal clones in bFGF-treated cultures, the significance of which is discussed in section 4.4.1.4.

Distribution of the size of neuronal clones in E16 cultures grown in the presence or absence of bFGF
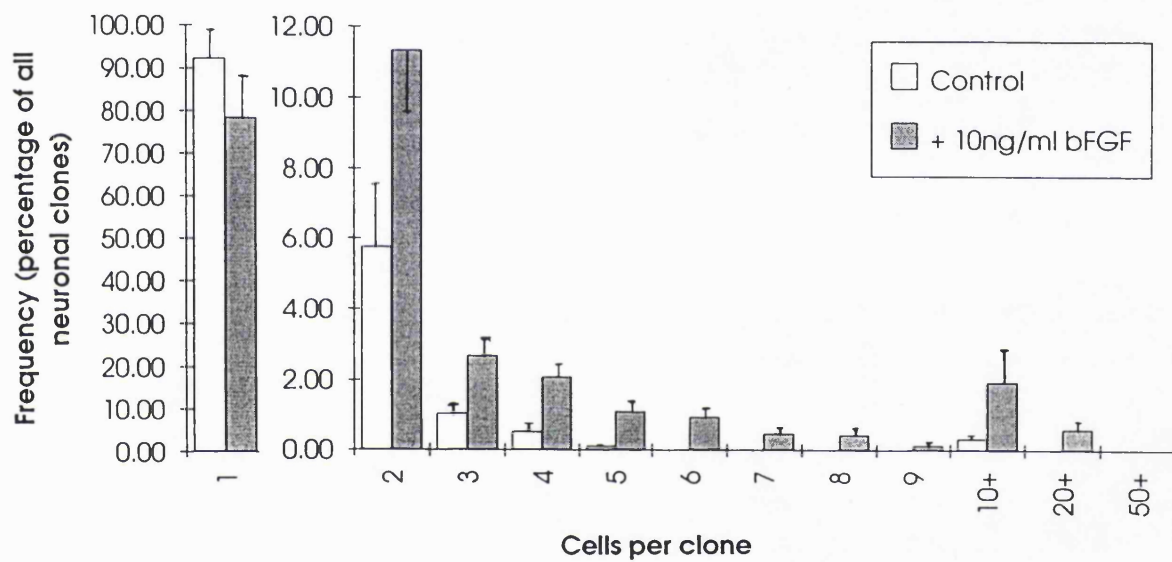
Figure 4.2 Examples of large neuronal clones found in 7-day cultures of E16 cortical cells grown in the presence of bFGF on monolayers of cortical astrocytes. Frame A shows a 6-cell neuronal clone as detected by anti-β gal antibody. All six cells are positive for TUJ1 as shown in frame B, thus confirming their neuronal identity. Frames C and E are examples of 4-cell and frame D of an 11-cell neuronal clone, as detected with anti-β gal antibody. Frame F, on the other hand, shows detection of another 11-cell neuronal clone with substrate X-gal.

Frames C to E came from coverslips that had been stained with MAP-2; the cells shown were negative for MAP-2. Nonetheless, a clear neuronal morphology can be seen in frames C to F; in each, β gal-positive neurones also demonstrate their characteristic bright spec (arrows).

Two important points should be noted from this figure. The first is the tight clustering of neurones (compare this with the large non-neuronal clones shown in chapter II), and Second, how members of a clone are remarkably similar in morphology. Scale bar in A to E= 10μm, and in F= 30μm.
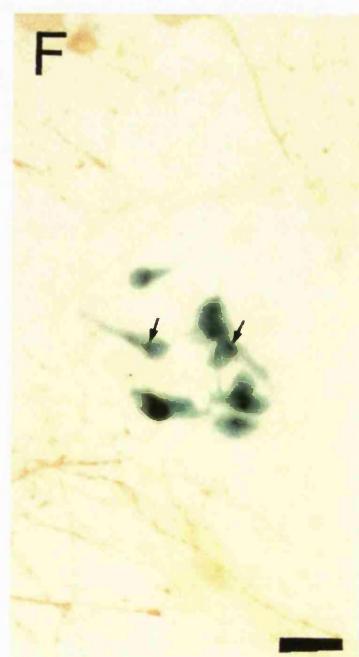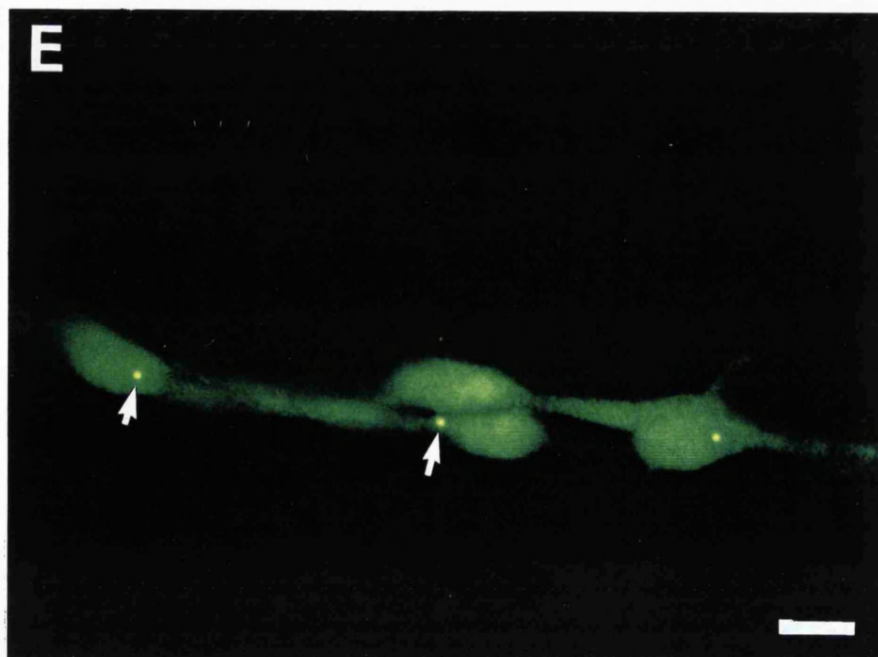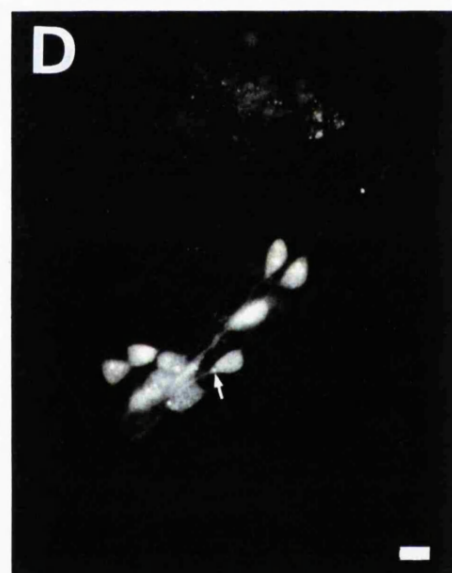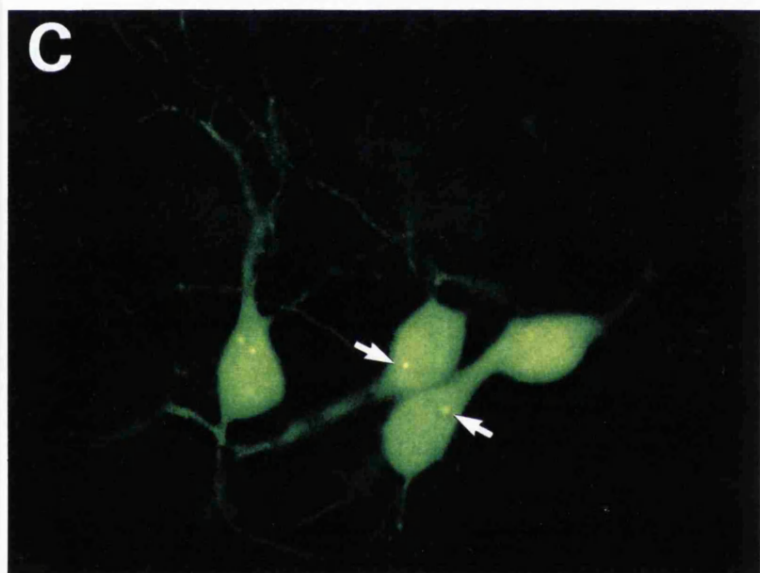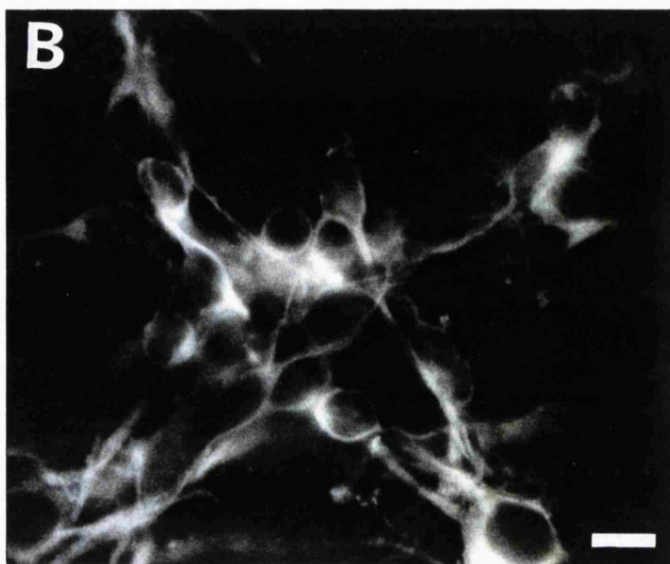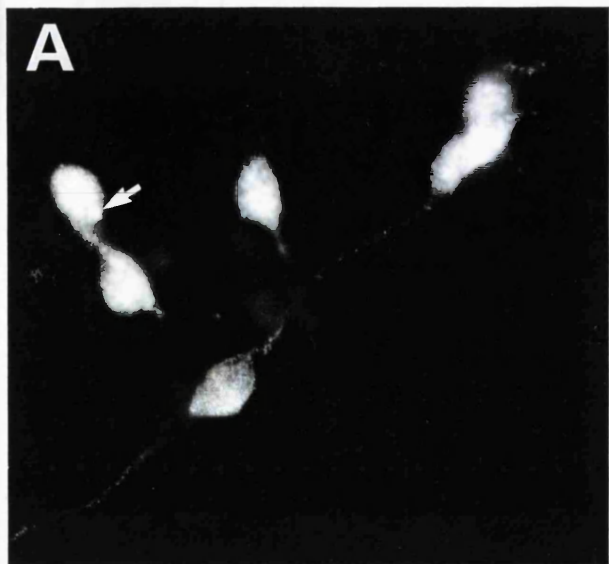
Figure 4.3 Distribution of the size of neuronal clones in E14 cultures grown in the presence or absence of bFGF. This figure shows that in contrast to E16 cultures, where presence of bFGF did not result in a significant change in the frequency of single-cell clones (figure 4.1), in E14 cultures (shown opposite) this frequency was almost halved. Concurrent with this reduction, bFGF increased the frequency of 2-cell and greater neuronal clones above a level found in its absence in E14 cultures. This, as discussed in section 4.3.1.3, suggested that bFGF may have acted as mitogen for some neuronal precursors.

In any event, statistical analysis using the *two-sample Kolmogorov-Smirnov test* showed that indeed the distribution of neuronal clone sizes in bFGF-treated cultures was significantly different from that of controls. The values obtained from the *Kolmogorov-Smirnov test* for this comparison were as follows:

Comparison of distribution for	$F_m(x) - G_n(x)$	$p \leq 0.05$	$p \leq 0.01$
E14 cortical cells grown in the absence versus the presence of bFGF	0.19	0.17**	0.2

Where ** denotes significance at the shown level of confidence.

Distribution of the size of neuronal clones in E14 cultures grown in the presence or absence of bFGF

Frequency (percentage of all neuronal clones)

Cells per clone

Control

+ 10ng/ml bFGF
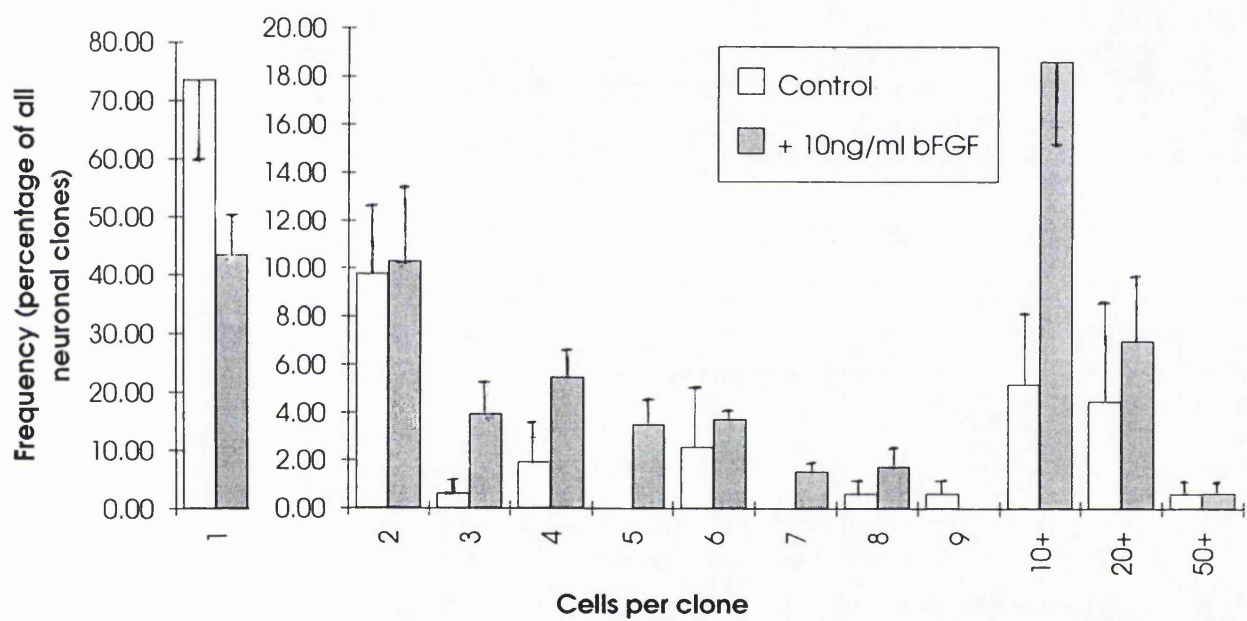
4.3.2 Titrating the effects of bFGF in E16 cultures
4.3.2.1 Addition of bFGF in different combinations

In the experiments described above, bFGF was routinely applied in three 10 ng/ml doses (see methods); one at the time of plating, designated day 1 of culture, and two subsequent doses on days 2 and 4. However, with this particular regimen at least two issues would have remained unresolved. First, could it have been that more neuronal clones were found in the presence of bFGF simply because bFGF induced more neuronal precursors to divide and hence incorporate the BAG virus? As cultures were infected with BAG on day 1, and as BAG has a half life of 4 hours, it would have been expected, were this phenomenon occuring for it to occur in the presence of the first bFGF dose. This possibility, however, was discounted when the first of the three-dose combination was omitted; in the absence of this first dose, one or two subsequent bFGF doses were still able to produce more neuronal clones (figures 4.4 and 4.5). This observation showed that rather than inducing more cells to incorporate BAG, bFGF was enhancing the survival of precursors that had already become labelled with BAG virus.

The second issue was whether particular combinations of bFGF could produce different effects, or, indeed which of the three doses were critical for bFGF's survival effect? To address this issue, a variety of combinations were applied. As already alluded to (figure 4.4), it was discovered that a single dose on days 1 or 2 was sufficient in enhancing the survival of neuronal precursors, although a combined dose on days 1 and 2, designated dose '1,2', was more effective. However, out of all the combinations tested, maximum survival was obtained with a 1,2,4 dose (figure 4.4).

Noteworthy, was that with all combinations, the survival of 2-cell and greater neuronal clones was always in proportion to the survival of single-cell neuronal clones, suggesting that the survival effect of bFGF on neuronal precursors was uniform under the different dose combinations. Worthy of mention also, was that identical results were obtained when Hu. Rec. bFGF was used in different combinations (figure 4.5).

4.3.2.2 Addition of different concentrations of bFGF

It was of interest to know whether or how different concentrations of bFGF would have affected the survival of neuronal precursors. bFGF was, therefore, tested at concentrations ranging from 2 to 50 ng/ml. Significantly more neuronal clones were detectable in E16 cultures even in the presence of 2 ng/ml bFGF (figure 4.6). Moreover, this increase affected the numbers of both single-cell and 2-cell and greater neuronal clones, suggesting a uniform survival effect even at these lower concentrations of bFGF. This survival was, however maximal at 10 ng/ml bFGF (figure 4.6); when bFGF was used at 50 ng/ml, there was no significant increment in the number of neuronal clones above that seen at 10 ng/ml. Similar numbers of 2-cell and greater neuronal clones were detected in both 10 and 50 ng/ml treated cultures (figure 4.6) but a comparison of the size distribution of neuronal

clones in the presence of 10 or 50 ng/ml indicated that bFGF's effects may have fallen at 50 ng/ml; in the presence of 50 ng/ml bFGF, the size range of 2-cell and greater neuronal clones was restricted to 2 to 4 cells, a range that was extended to 20 cells in the presence of 10 ng/ml bFGF (not shown). Interestingly, this phenomenon was also observed when the effects of 10 and 50 ng/ml Hu. Rec. bFGF were compared.

There could be two possible interpretations of this result: either 10, but not 50 ng/ml bFGF had an additional mitogenic effect on some neuronal precursors, or, a sub-population of neuronal precursors with higher proliferative potential did not respond to the survival effect of bFGF when used at 50 ng/ml.

4.3.3 Effects of bFGF in the presence of heparin

Studies of the effects of acidic FGF in vitro, have shown that these effects can be potentiated by the presence of heparin. To test whether or not this is true of bFGF's effects on neuronal precursors could be potentiated by heparin, cultured cortical precursors were grown in the presence or absence of 10 ng/ml bFGF, with or without heparin. 10 ng/ml, heparin had no effect on the increase in the number of single-cell or 2-cell and greater neuronal clones that was normally observed in the presence of 10 ng/ml bFGF (figure 4.7). At 100 ng/ml, however, heparin reduced the survival effect of bFGF on neuronal precursors, lowering the number of neuronal clones normally found in the presence of bFGF to control levels. Intriguingly, this reduction was mainly in the number of single-cell clones, with that of 2-cell and greater neuronal clones remaining largely unaffected (figure 4.7). More intriguingly was that although 100 ng/ml heparin did not affect the number of 2-cell and greater neuronal clones, it did influence their size range. In the combined presence of 100 ng/ml heparin and 10 ng/ml bFGF, these larger neuronal clones were composed solely of 2-cell clones, while in the presence of bFGF alone, clones as large as 6 or 9 cells were evident (not shown).

Although these findings represented the results of only one experiment, it was significant that similar effects were observed when bFGF was used in a different dose combination (1,2 as opposed to 1,2,4) in the presence of 100 ng/ml heparin (figure 4.7). Also noteworthy was that the health of cultured cells was not affected by the presence of 100 ng/ml heparin.

4.3.4 Testing the effects of other FGFs on E16 cortical precursors

In two experiments, effects of some other members of the FGF family were tested on E16 cortical precursors grown on cortical astrocytes. These were: acidic (FGF-1); int-2 (FGF-3); hst (FGF-4); FGF-5; and KGF (FGF-7).

As with bFGF, these FGFs had no effect on the type of clones obtained in E16 cultures. Moreover, some of these FGFs, such as FGF-5, did not affect the frequency or the size of clones above that seen in controls. Of those that did, however, the effects of int-2 (FGF-3)

Figure 4.4 and 4.5 Titration of bFGF's effects; addition of different dose combinations to cultures of E16 cortical precursor cells. As routine, bFGF was added to the embryonic cultures in three 10 ng/ml doses; one on the day of plating, designated for this purpose, as day 1of culture, and two subsequent doses on days 2 and 4 (i.e. 24 and 72 hours after plating). However, to test the effects of different dose combinations, in a series of parallel experiments, bFGF was added as indicated in the legend of the figure opposite. For example, '1 Dose 1' means a addition of a single dose of bFGF on the day of plating (day 1) and '2 Dose 1,2' means addition of 2 doses of bFGF, one on the day of plating and one 24 hours later (day 2). As evident from this comparison, maximum effect, i.e. increase in the number of neuronal clones, was obtained with a 1,2,4 dose. Moreover, this was true of both Xenopus (figure 4.4) and Human recombinant bFGF (figure 4.5).

In each graph, Total represents the total number of clones; N1, the number of single-cell neuronal clones, and N2+, 2-cell and greater neuronal clones.

Results presented in Figure 4.4A are from 4, while those in 4.4B are from 6 experiments. Similarly, results presented in Figure 4.5A are from 2 those of 4.5B are from 3 experiments.

Figure 4.4

A   Effects of Xen. bFGF on neuronal clones; comparison of different dose combinations I

+ 10 ng/ml Xenopus bFGF
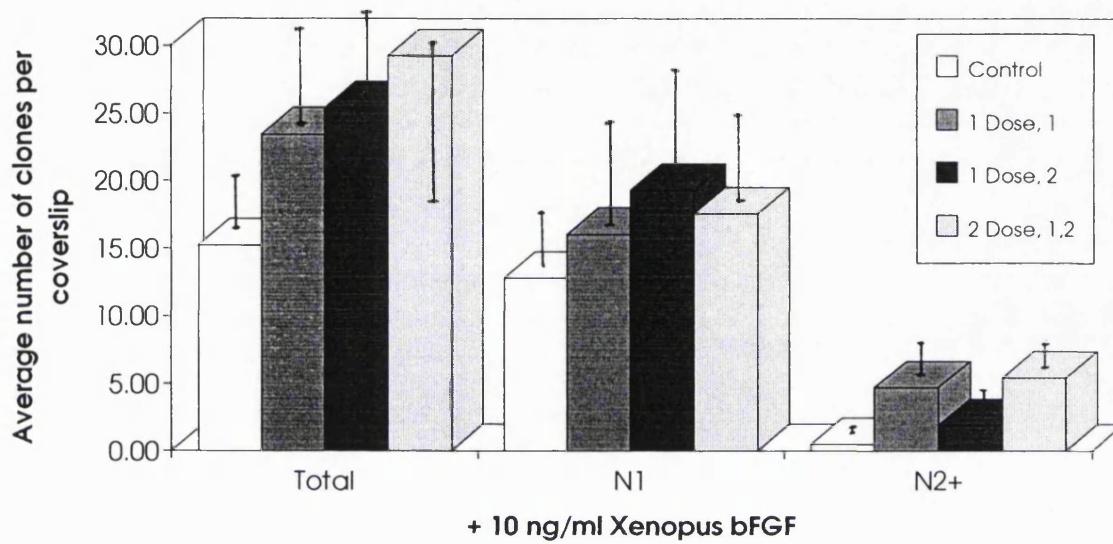
B   Effects of Xen. bFGF on neuronal clones; comparison of different dose combinations II

+10 ng/ml Xeopus bFGF
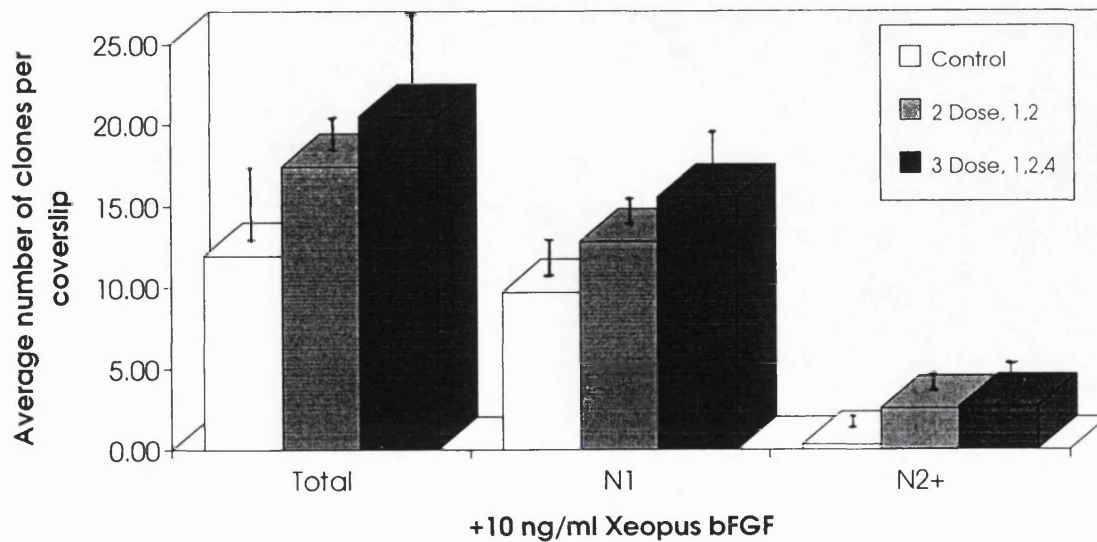
Figure 4.5

A Effects of Hu. Rec. bFGF on neuronal clones; comparison of different dose combinations I
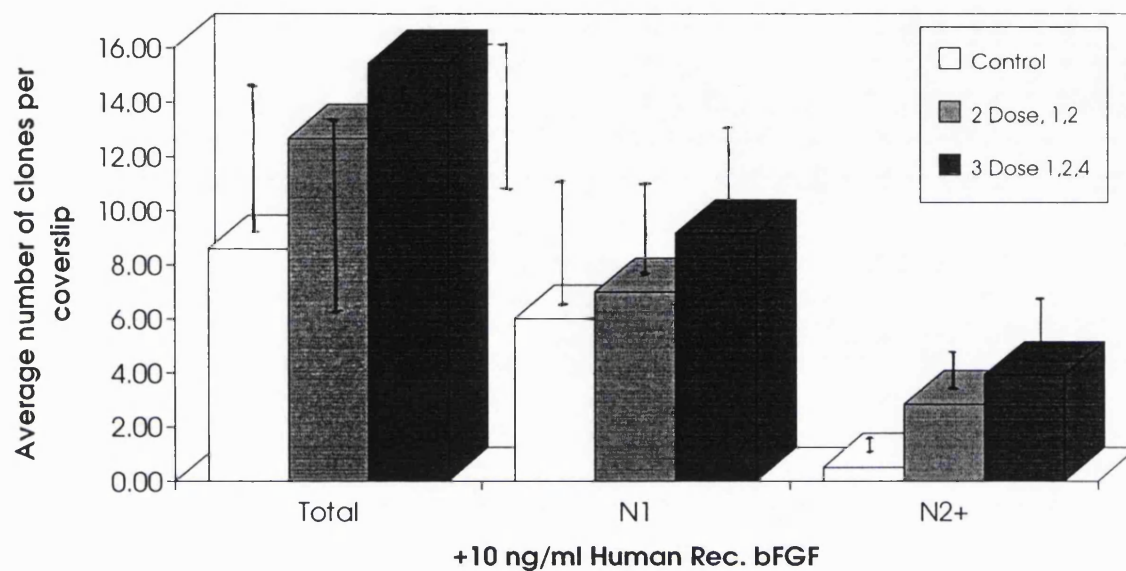
Legend: Control; 2 Dose, 1,2; 3 Dose 1,2,4

x-axis: Total, N1, N2+

+10 ng/ml Human Rec. bFGF

y-axis: Average number of clones per coverslip

B Effects of Hu. Rec. bFGF on neuronal clones; comparison of different dose combinations II

Legend: Control; 2 Dose, 2,4; 3 Dose, 1,2,4

x-axis: Total, N1, N2+

+ 10 ng/ml Human Rec. bFGF

y-axis: Average number of clones per coverslip
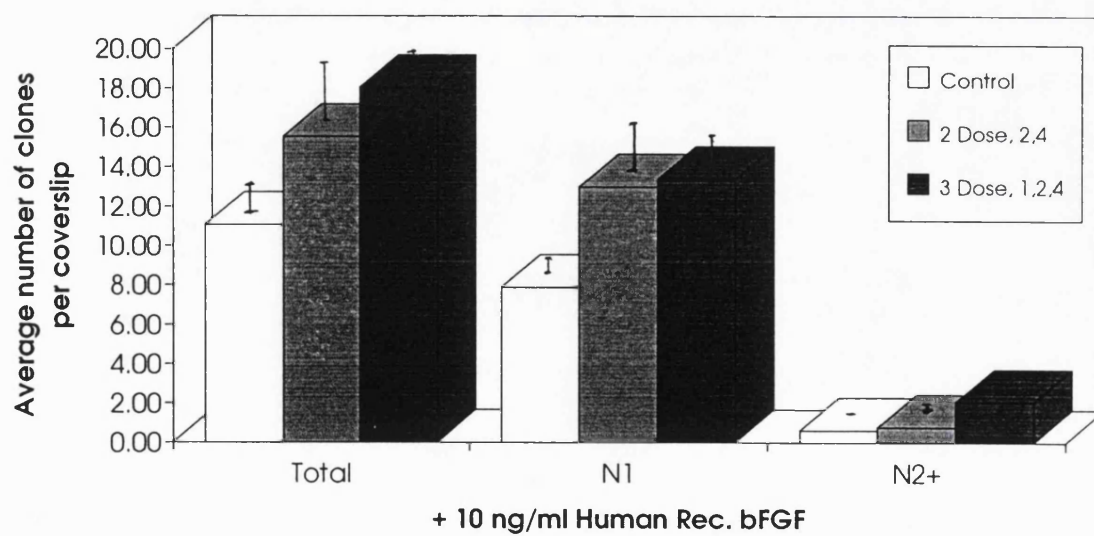
Figure 4.6 Titration of bFGF's effect; addition of different bFGF concentrations to cultures of E16 cortical precursor cells. Results presented throughout this chapter were obtained with either Xen. or Hu. Rec. bFGF was used at 10 ng/ml. This concentration was chosen because three dose response experiments (shown opposite) showed that maximum number of neuronal clones, both that of single-cell and 2-cell and greater neuronal clones, is obtained at this concentration. This was true both of Xenopus bFGF (graph A) and of Hu. Rec. bFGF (graph B). Results presented in graph A, are from 3, while those in graph B is from a single experiment.

Titrating the effects of bFGF on cultured E16 cortical precursor cells

A. Xenopus bFGF

Legend:
- Total
- 1 Cell Neuronal
- 2+ Neuronal

Y-axis: Average number of clonse per coverslip

X-axis (Xenopus bFGF): Control, 2 ng/ml, 10 ng/ml, 50 ng/ml

B. Human Recombinant bFGF

Legend:
- Total
- 1 Cell Neuronal
- 2+ Neuronal

Y-axis: Average number of clones per coverslip

X-axis (Hum. Rec. bFGF): Control, 2 ng/ml, 10 ng/ml, 50 ng/ml
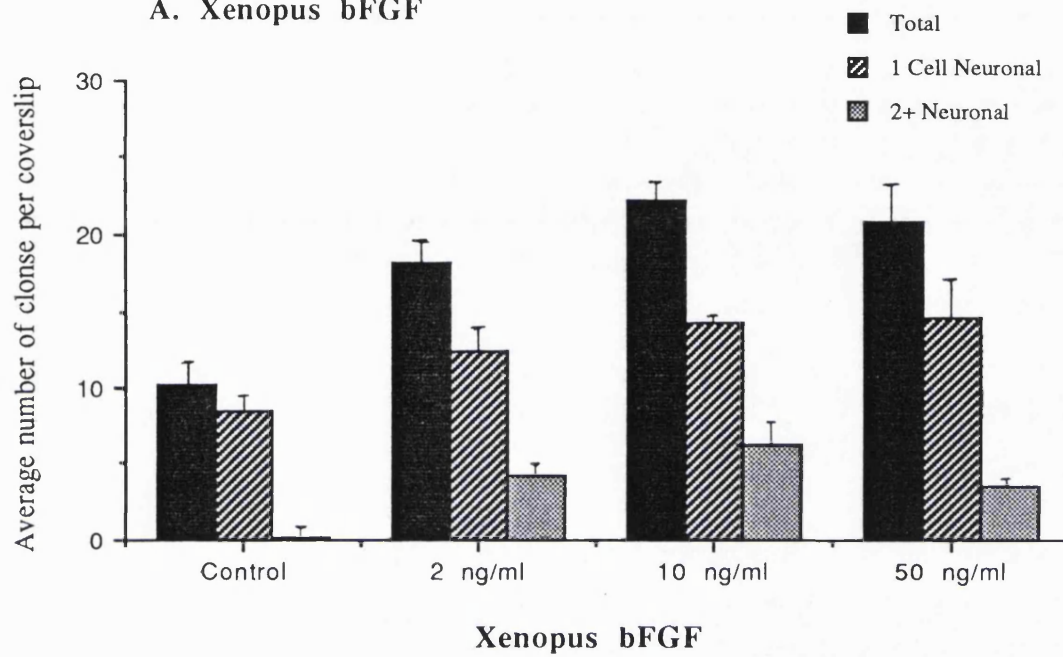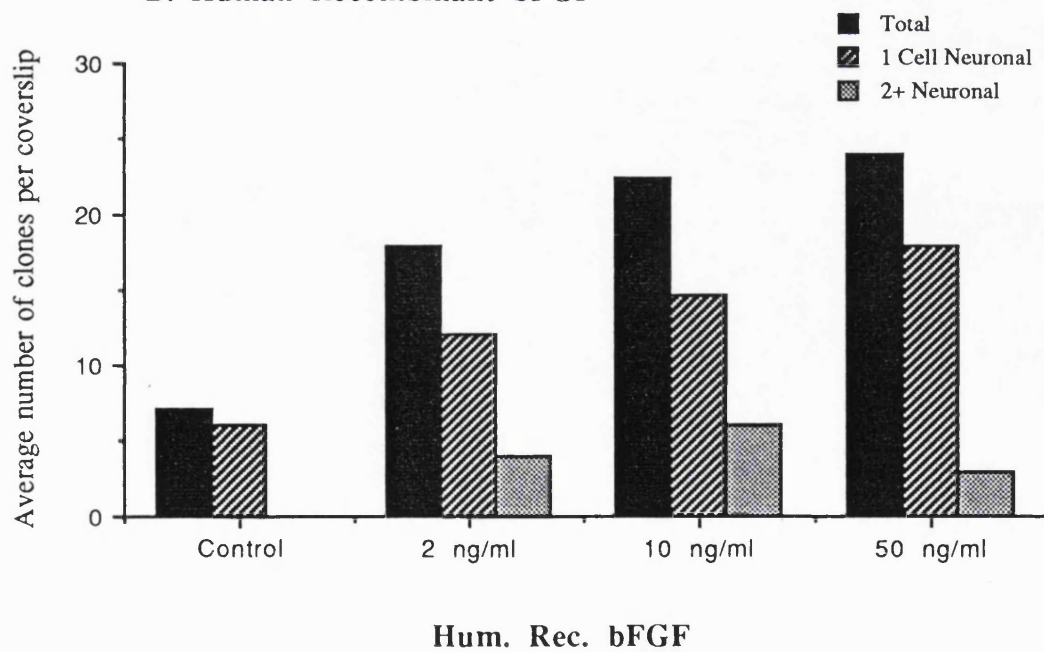
Figure 4.7 Analysis of the effects of bFGF on cultured E16 cortical precursor cells in the presence or absence of heparin. To test whether bFGF's effects (described in the results section) could be potentiated by heparin, effects of 10 and 100 ng/ml heparin were tested in E16 cultures grown in the presence of either 2 doses (1,2) or 3 doses (1,2,4) of 10 ng/ml Human Rec. bFGF. As evident from the graph opposite, which represents the results of only one experiment, presence of 100, but not 10 ng/ml heparin infact inhibited the survival effect of bFGF on neuronal precursors, bringing the average number of neuronal clones detected per coverslip in the presence of bFGF (F and C), close to control levels (H and E). This reduction, however, was mainly in the number of single-cell clones (striped columns).

Analysis of the effects of bFGF on cultured E16 cortical precursor cells in the presence or absence of heparin

A = Control
B = A+ 100 ng/ml heparin
C = bFGF 1,2
D = C+ 10 ng/ml heparin
E = C+ 100 ng/ml heparin
F = bFGF 1,2,4
G = F+ 10 ng/ml heparin
H = F+ 100 ng/ml heparin

■ Total
▨ 1 Cell Neuronal
▩ 2+ Neuronal

Average number of clones per coverslip

Conditions
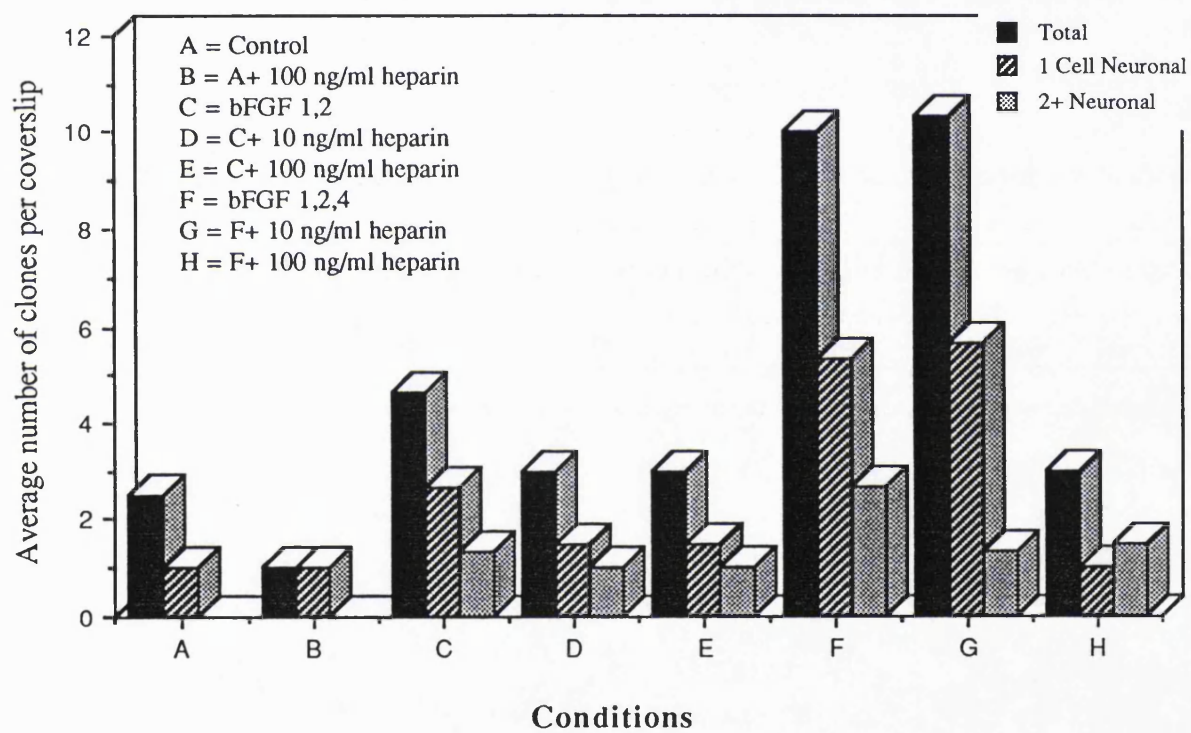
Figure 4.8 Comparing the effects of different FGFs on cultured E16 cortical precursor cells. In three experiments, effects of KGF (FGF-7), hst (FGF-4), aFGF (FGF-1), F5 (FGF-5) and Int-2 (FGF-3), obtained from in vitro translates of reticulolysates of cells transfected with a particular FGF cDNA), were tested alongside Hu. Rec. bFGF (FGF-2). The results of these experiments (shown opposite) showed that only hst and Int-2 were capable of increasing the average number of neuronal clones detected per coverslip in a magnitude similar to that of bFGF. Two sets of controls were run in these experiments; one containing only Sato's medium and another (denoted 'control med.' in the figure) containing medium or buffer in which the reticulolysates were collected.

Comparing the effects of different FGFs on cultured E16 cortical precursor cells
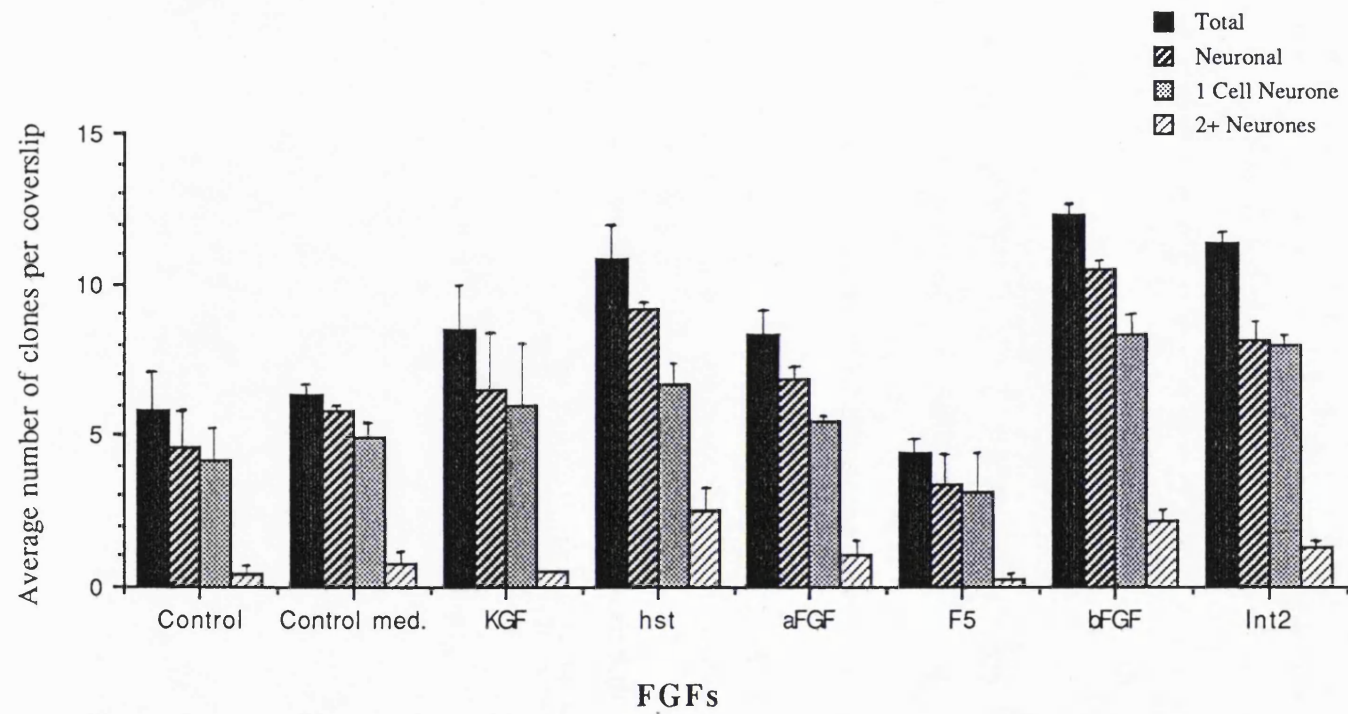
and hst (FGF-4) were remarkably similar to that of bFGF; in the presence of either of these factors, significantly more and larger neuronal clones were detectable (figure 4.8).

4.3.5 Analysis of bFGF's effects in the absence of cortical astrocyte monolayer

It was conceivable that bFGF's effects on embryonic cortical cells may have been mediated by cortical astrocytes, cells on which the precursors were routinely cultured. To investigate this possibility, parallel experiments were conducted without astrocytes. In preliminary experiments, embryonic cortical cells were grown on a monolayer of air-dried cortical astrocytes (referred to as 'astrocyte matrix') in the presence or absence of bFGF. In most such experiments, however, all embryonic cortical cells died within 48 hours of plating on matrix alone. Although cultures grown in the presence of bFGF did survive for slightly longer, death of control cultures meant that a quantitative clonal comparison could not be made between the two treatments. In order to test whether a different matrix/ substrate may enhance the survival of cultures, identical experiments were carried out by plating embryonic cortical cells on laminin (50 µg/ml) or poly-D-lysine coated coverslips or air-dried monolayers of NIH-3T3 cells (3T3 cell matrix). The degree of survival obtained on these substrates, however, was similar to that obtained on astrocyte matrix. Moreover, astrocyte conditioned medium consistently failed to enhance the survival of embryonic cells grown in the absence of cortical astrocytes.

In another approach to boost the survival of cultures in the absence of astrocytes, embryonic cells were plated at a slightly higher density: 3.5×10^5 cells per coverslip compared to 2.0-2.5×10^5 cells in the initial experiments. Only in 6 out of 20 such experiments, however, did the cultures survive the seven day period in the absence of astrocytes. Nonetheless, the few cultures that did survive made it possible to analyse and compare the effects of bFGF on cortical precursor cells grown in the presence or absence of cortical astrocytes. The results of this analysis are described below.

4.3.5.1 Analysis of clones found in E16 cultures grown in the absence of cortical astrocytes

Several important observations were made when clones derived from E16 precursors that were grown in the absence of cortical astrocytes were analysed. For the sake of analysis, these observations will be divided into three different groups: observations made both in the presence and absence of bFGF; those made exclusively in the presence of bFGF; and those that were exclusive to the absence of cortical astrocytes.

4.3.5. 2 Observations made in the presence and absence of bFGF
4.3.5.2.1 Clone type restrictions and proportions

The types of clones found in cultures that were grown in the absence of astrocytes, either in the presence or absence of bFGF, were found to be identical to those seen in E16

cultures grown on cortical astrocytes. i.e. four types of clones, each containing a single cell type were noted. These were clones of neurones, astrocytes, oligodendrocytes, or cells that could not be identified (Table 4.2). Of these four clone types, neuronal clones were found to be the most frequent clone type, both in the presence and absence of bFGF. For example, in cultures that were grown on astrocyte matrix, neuronal clones made up 83% of all clones in the absence, and 81% of all clones in the presence of bFGF.

4.3.5.3 Effects exclusive to the presence of bFGF

4.3.5.3.1 Direct survival effect on precursor cells

The poor survival of cultures grown in the absence of cortical astrocytes was reflected in the number of clones obtained in such cultures. In parallel experiments, for example, on average only 8.6 clones per coverslip were found when E16 cells were cultured on PDL alone, compared to 27.6 clones per coverslip when grown on cortical astrocytes (Table 4.2). Similarly, in separate sets of experiments, an average of 17.2 clones per coverslip were found in cultures that were grown on astrocyte matrix, compared to 28.6 clones in cultures grown on cortical astrocytes. In the presence of bFGF, however, dramatically more clones could be found in cultures that were grown in the absence of astrocytes. In cultures grown on PDL, for example, the average number of clones detected per coverslip was increased from 8.6 to 32.5 by the addition of bFGF. A similar increase in clone numbers was also observed in cultures that were grown on astrocyte matrix in the presence of bFGF; from 17.2 clones per coverslip to 30.6. Importantly though, it was always the number of neuronal clones that showed the greatest increase in the presence of bFGF (Table 4.2). These observations suggested, therefore, that even in the absence of cortical astrocytes, bFGF could act as a survival factor for E16 cortical neuronal precursors. To investigate whether bFGF may have also acted as a mitogen for neuronal precursors, the size distribution of neuronal clones found in the presence and absence of bFGF was analysed (figures 4.9 and 4.10). However, this analysis revealed an important effect on the size of neuronal clones, not due to the presence of bFGF *per se*, but due to the absence of cortical astrocytes. This effect is considered below.

4.3.5.4 Effects exclusive to the absence of cortical astrocytes

Two striking effects were noted when E16 cultures were grown in the absence of astrocytes. First, more and larger clones of unidentified cells were recorded in cultures that were grown in the absence of astrocytes, either on astrocyte matrix or PDL-coated coverslips (Table 4.2). Cells in some of these clones were similar in appearance to those found in cultures grown on astrocytes; these cells had an astrocytic morphology, and stained with anti-GFAP. A proportion of unidentified clones, however, were clearly different from those found in the presence of cortical astrocytes. Cells in these clones,

which occasionally contained 50 or more cells, appeared as undifferentiated precursor cells (figure 4.11)

The second effect was on the frequency and size of 2-cell and greater neuronal clones. Despite the poor survival of cultures in the absence of astrocytes, significantly more 2-cell and greater neuronal clones were found in these cultures than those grown on astrocytes. For example, in cultures that were grown on astrocyte matrix, 20% of all neuronal clones contained 2 or more cells, compared to only 5% in cultures grown on astrocytes (Table 4.2). Moreover, when the size distribution of neuronal clones under the two culture conditions was analysed, it was discovered that even though the majority of neuronal clones in the presence and absence of astrocytes contained only a single-cell, a wide range of larger neuronal clones were observed when astrocytes were absent (figure 4.9). For example, neuronal clones as large as 20 or 40 cells (figure 4.12 and 4.13) were recorded in cultures that were grown on astrocyte matrix, clone sizes that were never found in cultures grown on astrocytes.

The crucial point about these observations, however, was that they could not be related to a particular culture substrate, such as astrocyte matrix, because, larger neuronal clones could also be found in cultures grown on PDL or 3T3 cell matrix (figure 4.13). It thus seemed as though the detection of more and larger unidentified as well as neuronal clones in these cultures was a direct consequence of the absence of cortical astrocytes.

Against this background of large neuronal clones found in cultures grown in the absence of astrocytes, therefore, it was impossible to distinguish whether in addition to its survival effect, bFGF had a mitogenic effect on E16 derived neuronal precursors (figures 4.9 and 4.10).

4.3.5.5 Analysis of clones found in E14 cultures grown in the absence of cortical astrocytes

In several experiments, bFGF's effects on E14 cortical precursors that were grown in the absence of cortical astrocytes were also tested. In only one experiment, however, did cells survive the seven day culture period. More unfortunately, sister cultures of E14 cortical precursors grown on astrocytes in this experiment were lost in processing, making it impossible to analyse any effects due to the absence of astrocytes. Nonetheless, comparison of cultures grown on astrocyte matrix alone with those grown in the presence of bFGF showed that bFGF had a small survival effect on E14 neuronal precursors; on average 26 neuronal clones were found in the absence, compared to 29 neuronal clones in the presence of bFGF. When the size distribution of neuronal clones in this experiment was analysed, however, it was noted that presence of bFGF had caused a shift in the frequency of neuronal clones sizes away from single and 2 cell clones toward 10 and 20 cell clones (figure 4.14). As discussed in section 4.3.1.3, there could be two explanations: this observation could represent a mitogenic effect on a subpopulation of precursor cells by

bFGF, or could be explained purely as a differential survival response by a population of neuronal precursors with higher proliferative potential. Whatever the effect, the fact that it took place in the absence of cortical astrocytes, showed that as well as having a direct influence on E16 precursors, bFGF also had a direct effect on E14 cortical precursors.

Table 4.2 Comparison of the type and frequency of clones obtained in 7-day cultures of E16 cortical cells grown on different substrates in the presence or absence of bFGF. Several important points can be noted from this table. First, culturing E16 cortical cells on different substrates does not alter the type or the overall relative proportions of different clone types. Second, survival of cultures is dramatically reduced when cortical precursors are grown in the absence of cortical astrocytes, either on astrocyte matrix, PDL or NIH-3T3 cell matrix. The poor survival of cultures grown on these matrices is reflected in a comparison of the average number of clones obtained in these cultures (third column in the table) compared to that obtained in the presence of cortical astrocytes (second column). Third, in the absence of cortical astrocytes, bFGF is capable of enhancing the survival of E16 neuronal precursor cells, as shown by the increase in the average number of clones obtained in its presence (fourth column) compared to its absence (third column).

Data presented in the opposite figure are from a total of six experiments, two for each substrate.

A comparison of type and frequency of clones obtained in 7 day cultures of E16 cortical cells grown on different substrates, in the presence or absence of bFGF.

(a) Cultures grown on astrocytes or astrocytes matrix

Clone type	On cortical astrocytes	On astrocyte matrix	On astrocyte matrix + 10 ng/ml bFGF
Neurones			
1 Cell N.	26 (91%)	10.8 ±2.3 (63%)	19.6 ±8.3 (64%)
2+ N.	1.3 (5%)	3.4 ±1.7 (20%)	5 ± 0.7 (16%)
Oligodendrocytes	0.3 (1%)	0.4 ±0.1 (2%)	0.3 ±0.1 (1%)
Astrocytes	0 (0)	0.2 ±0.2 (1%)	0.3 ±0.3 (1%)
Unidentified cells	2.3 (3%)	2.4 ±1.0 (14%)	5.4 ±4.7 (18%)
Total	28.6	17.2	30.6

(b) Cultures grown on astrocytes or PDL

Clone type	On cortical astrocytes	On PDL	On PDL + 10 ng/ml bFGF
Neurones			
1 Cell N.	24.6 ±0.4 (87%)	5.8 ±1.2 (67%)	24 ± 1.7 (74%)
2+ N.	2.6 ±0.9 (9%)	0.7 ±0.3 (8%)	5.3 ±0.7 (16%)
Oligodendrocytes	0.2 ±0.2 (1%)	0.3 ±0.3 (4%)	0.3 (1%)
Astrocytes	0 (0)	0.3 (4%)	0.2 ±0.2 (1%)
Unidentified cells	0.8 ±0.2 (3%)	1.5 ±0.2 (17%)	2.7 ±0.7 (8%)
Total	28.2	8.6	32.5

(c) Cultures grown on astrocytes or NIH-3T3 cell matrix

Clone type	On cortical astrocytes	On 3T3 cell matrix	On 3T3 cell matrix + 10 ng/ml bFGF
Neurones			
1 Cell N.	8.2 ±0.2 (79%)	2.5 ±0.5 (45%)	nd
2+ N.	0.5 ±0.5 (4%)	2.3 ±1.3 (41%)	nd
Oligodendrocytes	0.6 ±0.4 (6%)	0 (0)	nd
Astrocytes	0 (0)	0 (0)	nd
Unidentified cells	1.1 ±0.8 (11%)	0.8 ±0.2 (14%)	nd
Total	10	5.6	nd

Figures 4.9 and 4.10 Distribution of the size of neuronal clones in E16 cultures grown on either on cortical astrocytes or on other substrates in the presence or absence of bFGF.

Collectively, these two figures highlight the difference between the frequency and size range of 2-cell and greater neuronal clones in cultures grown in the presence compared to those grown in the absence of cortical astrocytes; as a whole such clones significantly are more frequent in the absence of cortical astrocytes. This significance was tested using the *two-sample Kolmogorov-Smirnov test*; the values obtained for difference comparisons were as follows.

For figure 4.9,

Comparison of distribution for	$F_m(x) - G_n(x)$	$p \leq 0.05$	$p \leq 0.01$
(i) Cells cultured on astrocytes vs on astrocyte matrix	0.16	0.16**	0.23
(ii) Cells cultured on astrocytes vs on matrix + bFGF	0.15	0.11**	0.13**
(iii) Cells cultured on matrix vs on matrix + bFGF	0.08	0.19**	0.22**

and, for figure 4.10,

Comparison of distributions for	$F_m(x) - G_n(x)$	$p \leq 0.05$	$p \leq 0.01$
(i) Cells cultured on astrocytes vs on PDL	0.06	0.22	0.26
(ii) Cells culture on astrocytes vs on PDL + bFGF	0.10	0.11	0.13
(ii) Cells cultured on PDL vs PDL + bFGF	0.08	0.24	0.28
(iv) Cells cultured on astrocytes vs NIH-3T3 cell matrix	0.37	0.29**	0.35**

where ** denotes significance at the level shown.

Thus the distribution of the size of neuronal clones in culture grown on astrocytes were significantly different from those grown on astrocyte matrix or NIH-3T3 cells but not on PDL. It should be noted, however, that although 2-cell and greater neuronal clones were not significantly more frequent in cultures grown on PDL as compared to astrocytes, the size of such clone show a clear difference, i.e. although the data presented is from 2 experiments, clones as large as 7 cells could be found in PDL cultures.

A second important point noted from figs. 4.9 and 4.10 however, is that the majority of neuronal clones found in E16 cultures contained only a single cell whether grown in the presence or absence of cortical astrocytes. The significance of this observation is discussed in section 4.4.1.4.

Figure 4.9

Distribution of the size of the neuronal clones in E16 cultures grown on astrocytes, or astrocyte matrix +/- bFGF
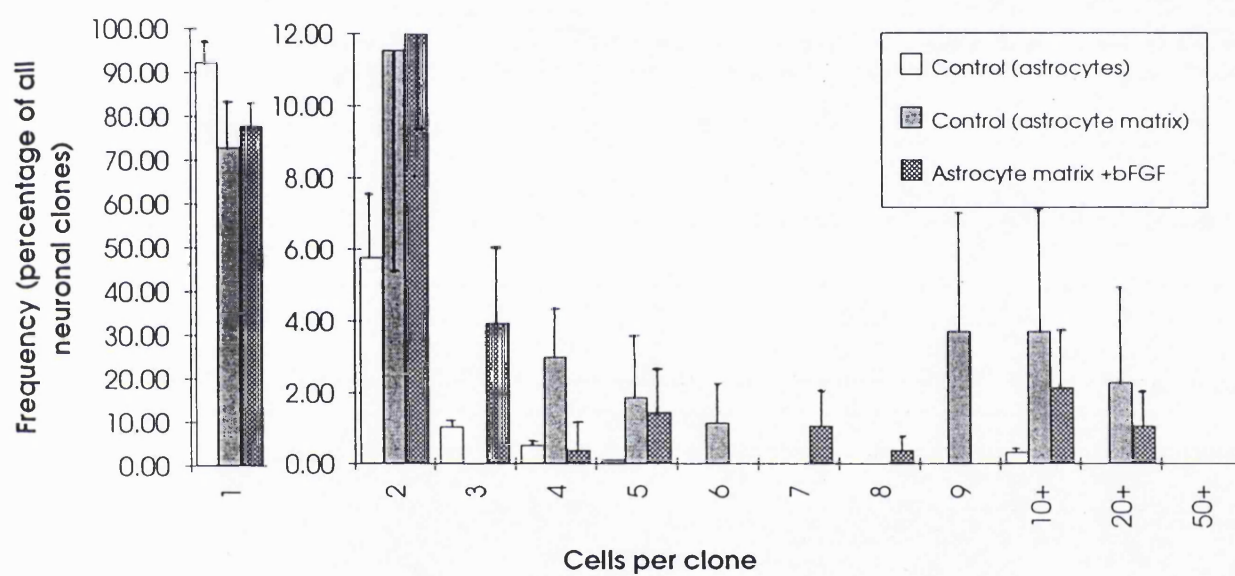
Figure 4.10

A Distribution of the size of neuronal clones in E16 cultures grown on astrocytes, or PDL-coated coverslips +/- bFGF
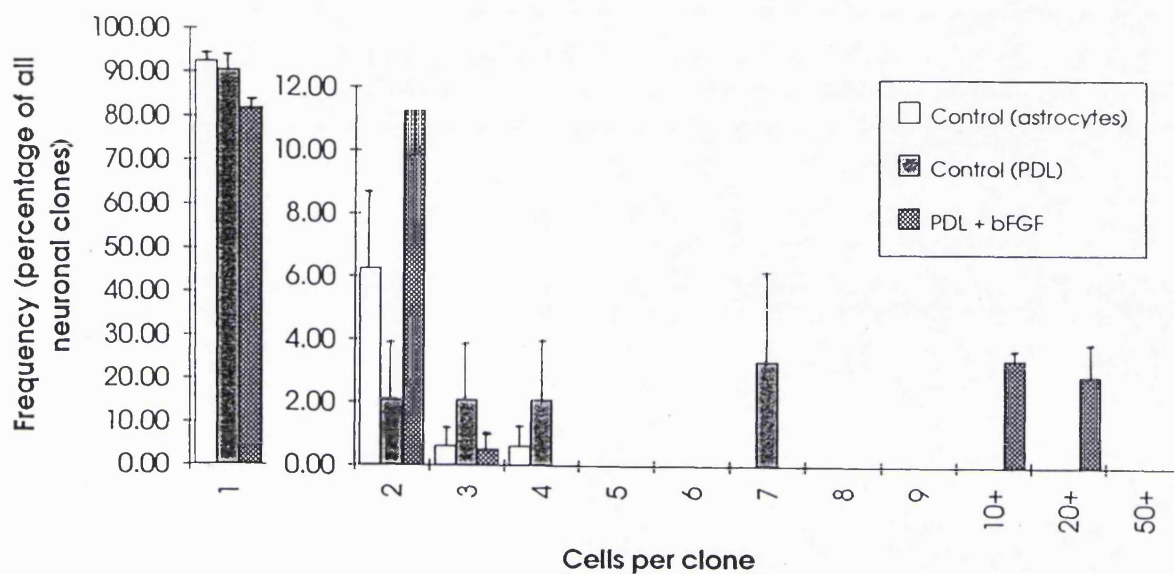
Legend:
- □ Control (astrocytes)
- ▨ Control (PDL)
- ▨ PDL + bFGF

Y-axis: Frequency (percentage of all neuronal clones)
X-axis: Cells per clone

B Distribution of the size of neuronal clones in E16 cultures grown on astrocytes, or NIH-3T3 cell matrix

Legend:
- □ Astrocytes
- ▨ NIH-3T3 cell matrix

Y-axis: Frequency (percentage of all neuronal clones)
X-axis: Cells per clone
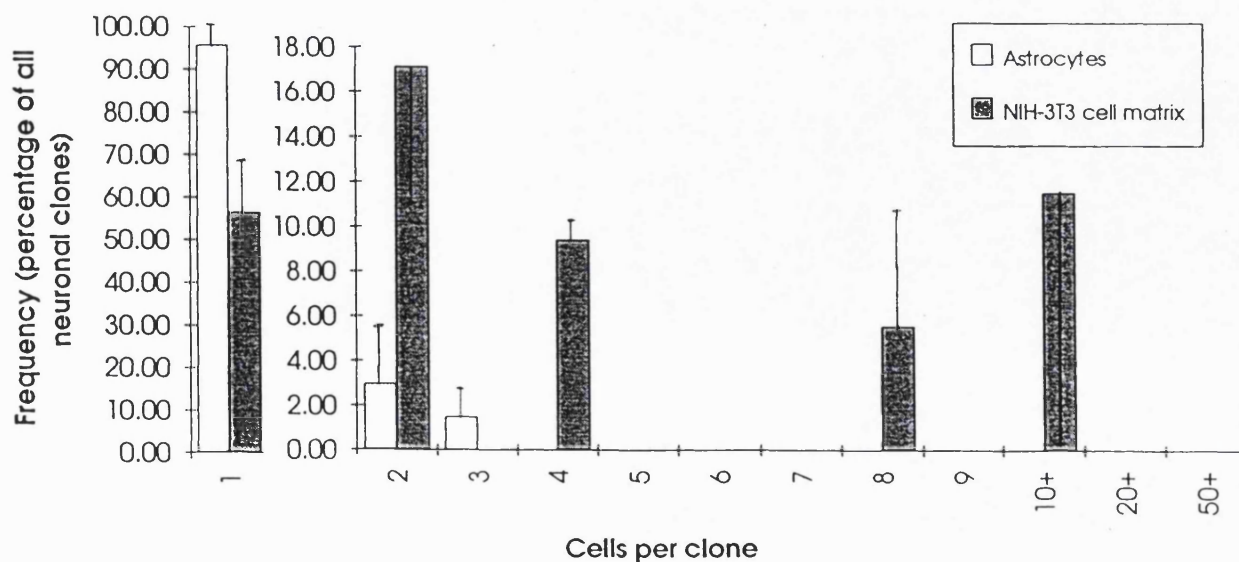
Figure 4.11 Examples of large unidentified clones seen in 7 day-cultures of E16 cortical cells grown on PDL-coated coverslips in the absence of bFGF. This figure shows that although such clone types could not be identified, cells in some of these clones (frames A to C) have an undifferentiated precursor like appearance, while others (frames D and E) appear differentiated. Cells in D appear to have an astrocytic morphology and those in E resemble immature oligodendrocyte precursors. Note how in contrast to D and E, cells in frames A to C are tightly clustered, suggesting that they may be neuronal. However, in the absence of immunoreactivity with MAP-2, from where these clones came from, they were not counted as neuronal.

Note also that the clone in A contains almost 100 cells, suggesting that cells within this clone must have divided geometrically (see section 2.4.3 of chapter II for this reasoning). Scale bar frames= 20μm.
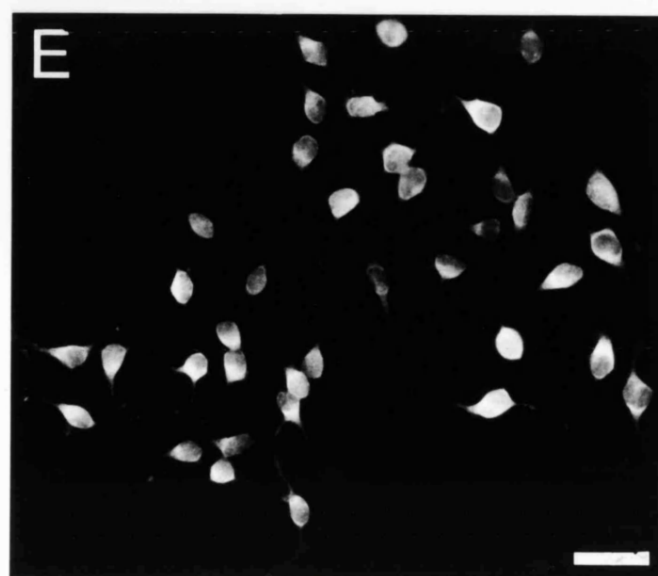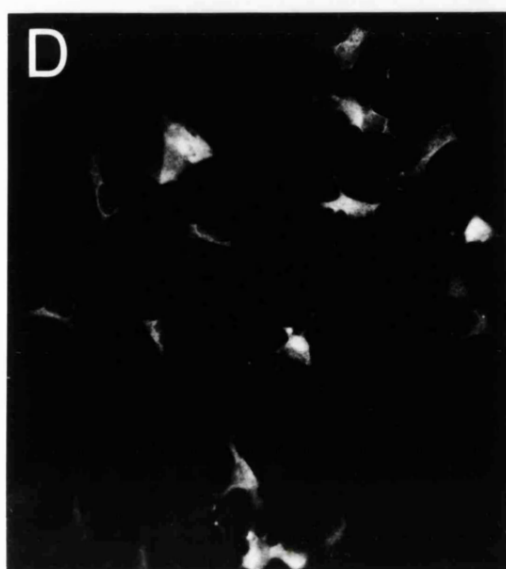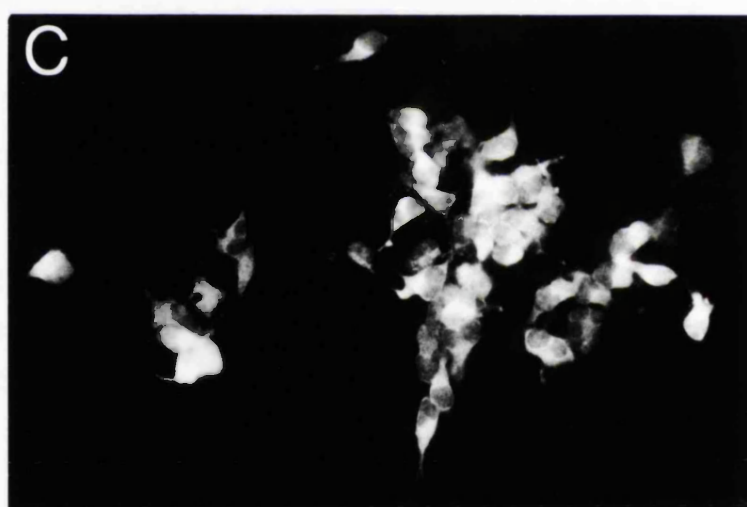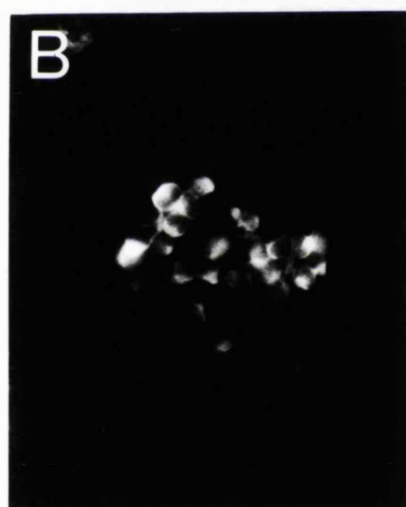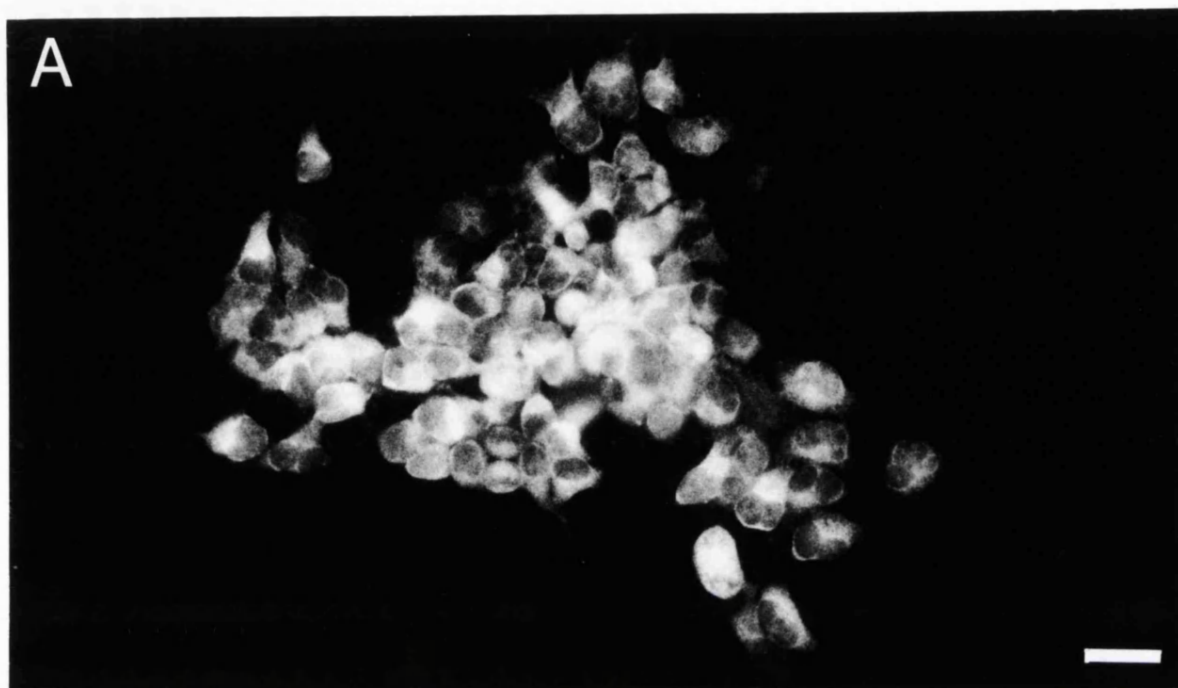
Figures 4.12 and 4.13   Examples of large neuronal clones found in 7-day cultures of E16 cortical cells grown on in the absence of cortical astrocytes.

Figure 4.12 Frame A shows a low magnification of an 18-cell neuronal clone as detected with anti-β gal antibody staining. Frame B shows the same clone at higher power. When stained with anti-TUJ1, most members of this clone were found to be positive, the clearest of which are indicated by arrows. No member of this clone was positive for O4 antibody, the third primary antibody with which this coverslip was stained. Scale bars in A and C= 10μm.

Figure 4.13 Frame A of this figure shows a 22-cell clones (estimated) of β gal positive neurones found in cultures grown on astrocyte matrix. As far as the resolution of staining allowed, all members of this clone were found also to be TUJ1-positive (frame B), although this is not so obvious in the photomicrograph due to quality of the negative and photographic reproduction. Nonetheless, clear positive TUJ1 immunoreactivity by three members of this clone are indicated with small arrows. Frame C and D show a cluster of neurone-like cells in cultures grown on NIH-3T3 cell matrix. Frame C shows the phase contrast appearance of this cluster, typical of neuronal aggregates found in cultures of embryonic cortical cells. Frame D (anti-β gal staining) shows the clonal relationship between members of this cluster; note how some cells in this cluster posses the characteristic neuronal spec. Scale bar in frames A to D= 20μm.
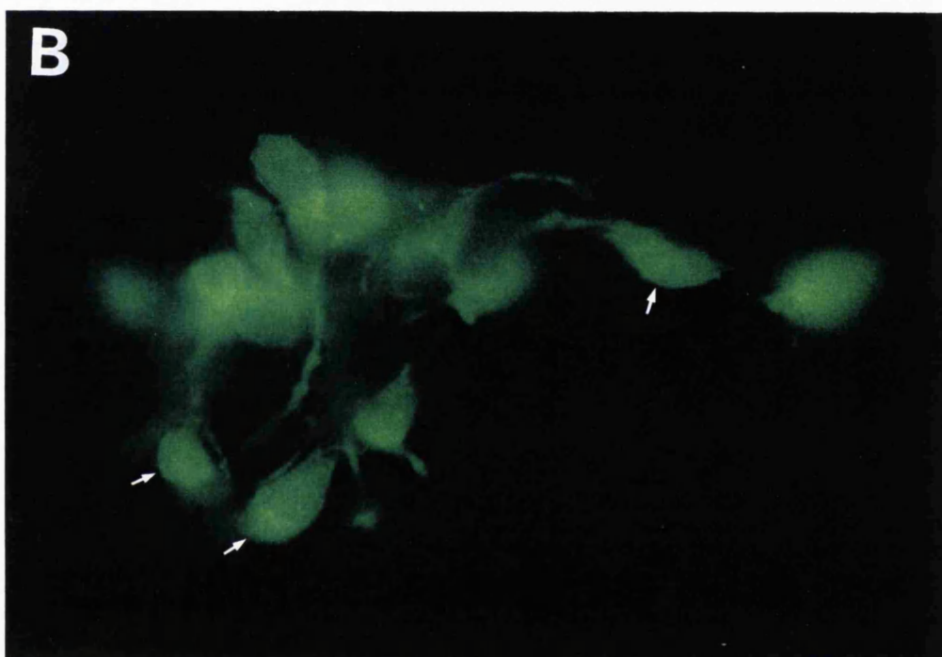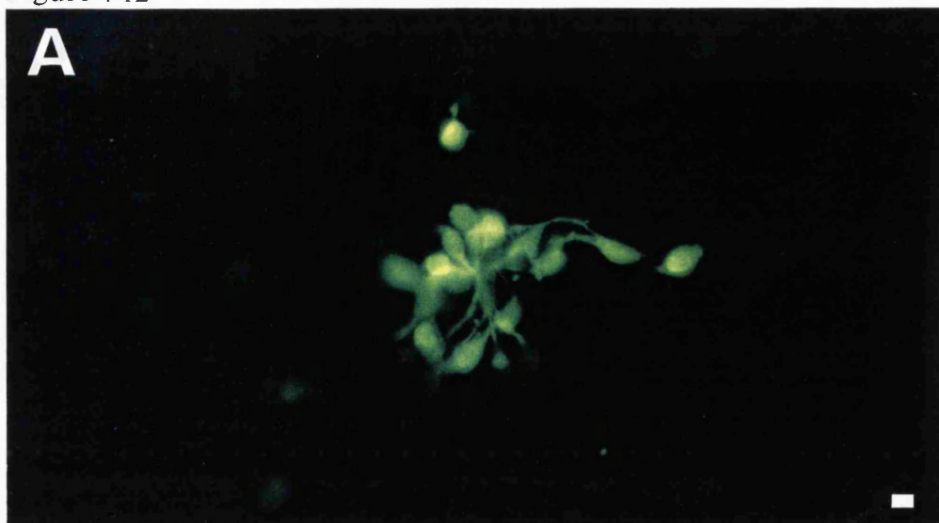
Figure 4.12

Figure 4.13
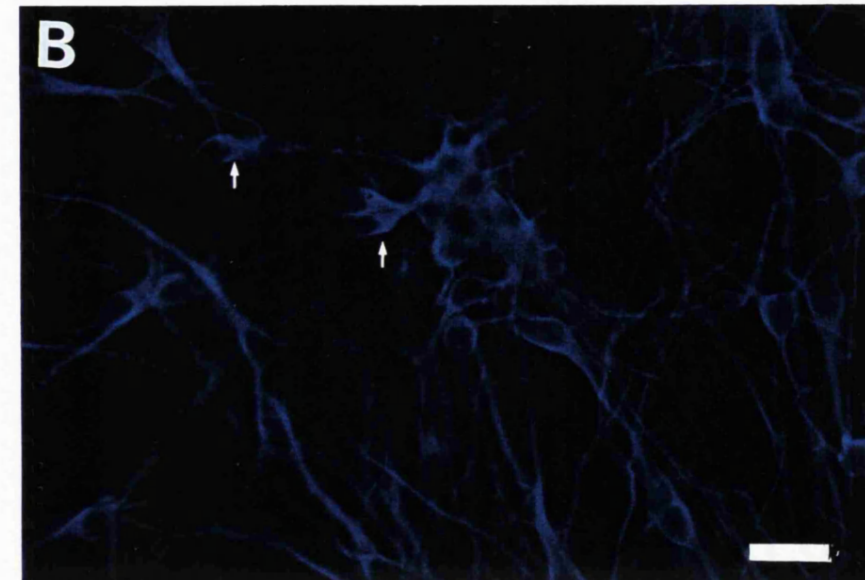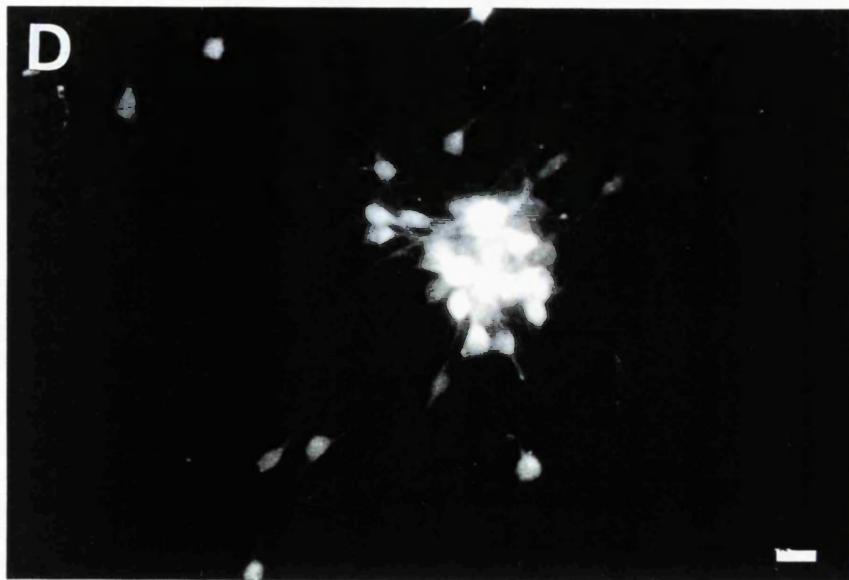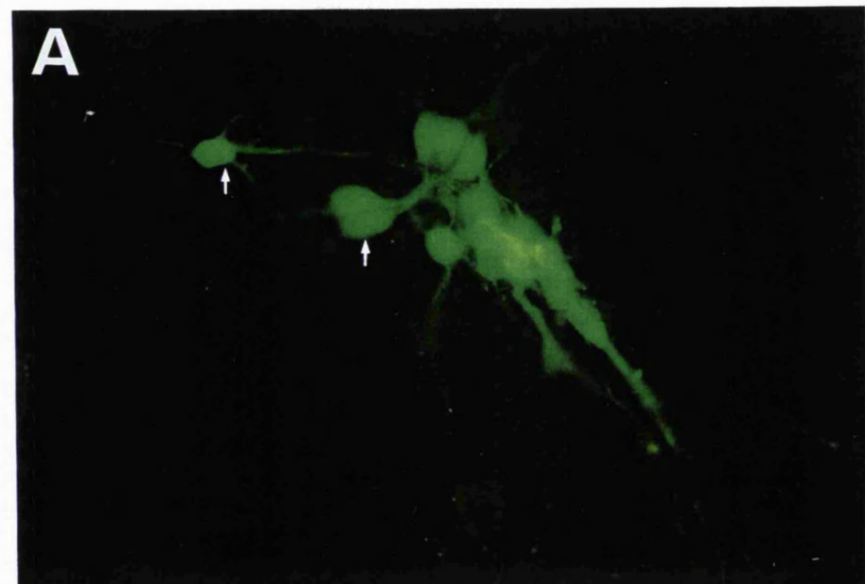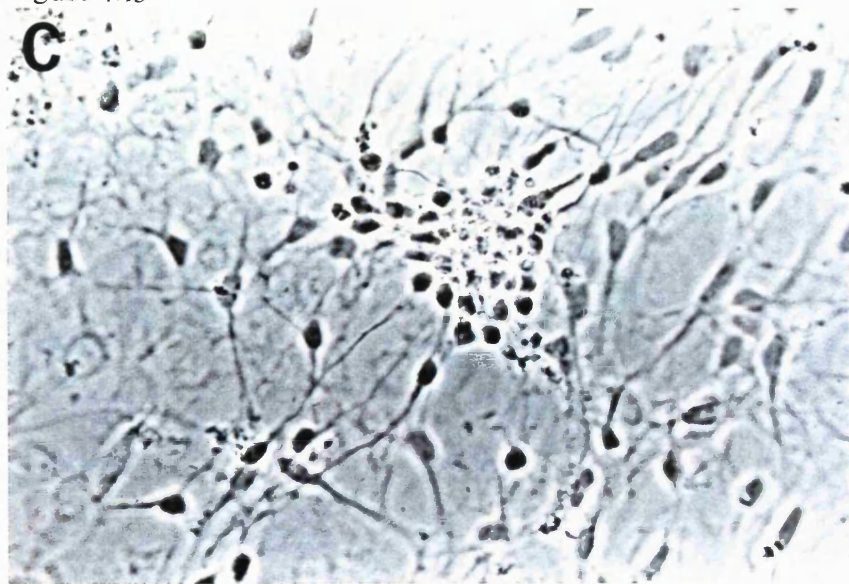
Figure 4.14 Distribution of the size of neuronal clones in E14 cultures grown on astrocyte matrix in the presence or absence of bFGF. Although this figure represent data from only one experiments, it shows that as in E16 cultures, 2-cell and greater neuronal clones are more frequent when E14 cortical precursors are cultured in the absence of cortical astrocytes. Because E14 cortical precursors normally have a higher proliferative potential than their E16 counterparts (results of Chapter II), the increment in the frequency and size of neuronal clones due to absence of cortical astrocytes is not be as obvious as that seen in E16 cultures (figure 4.9).

Distribution of the size of neuronal clones in E14 cultures grown on astrocyte matrix in the presence or absence of bFGF

Cells per clone

Frequency (percentage of all neuronal clones)

□ Astrocyte matrix
▧ Astrocyte matrix + bFGF
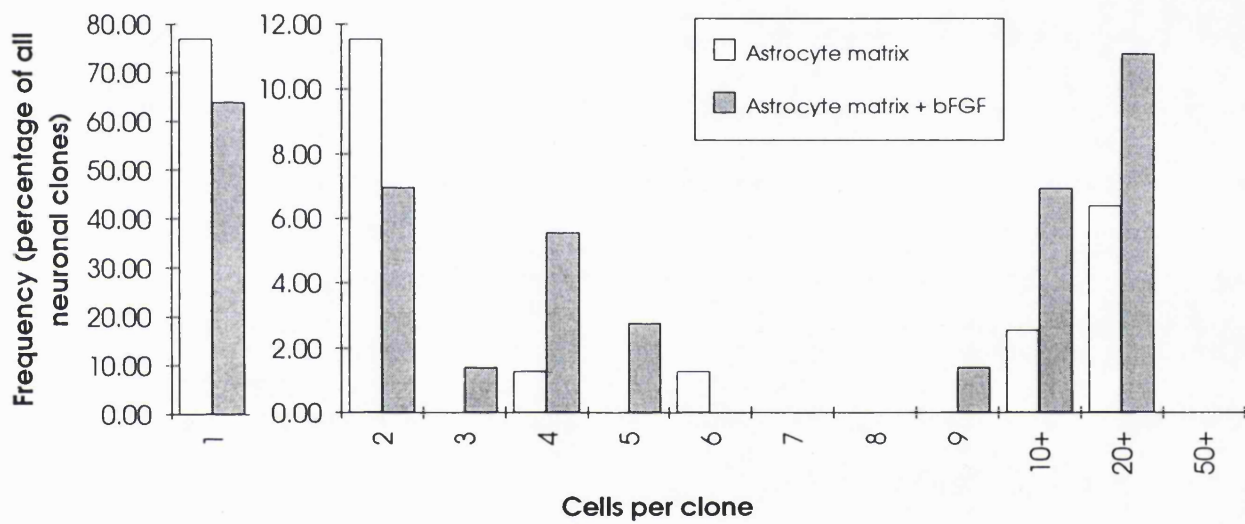
4.4   Discussion

By analysing and comparing the fate of embryonic cortical precursors grown under a variety of different conditions, this study has been able to investigate whether a particular culture condition can or can not influence the development of distinct cortical precursors cells in vitro. The prime finding of this work is that under all conditions tested, including the presence of bFGF, embryonic cortical precursors generate only one particular cell type, reinforcing the conclusion reached in chapter II that by embryonic day 16 (E16), if not E14, the overwhelming majority of cortical precursors have become specified in their phenotypic potential. In view of this phenotypic restriction, this study was then able to ask whether or how distinct types of precursor cells were affected by a particular culture condition, such as the presence of a growth factor. Accordingly, this study has identified several factors that can influence the survival and proliferation of cortical neuronal precursors, in vitro. It was shown that cortical astrocytes and bFGF can independently enhance the survival of cultured cortical neuronal precursors, although maximal survival was achieved when both factors were present. Moreover, it appeared as though cortical astrocytes inhibit the proliferation of neuronal precursors. At least two sets of questions are begged by these observations. First, how were these effects brought about; for example, did cortical astrocytes and bFGF enhance the survival of neuronal precursors by a common mechanism or by different mechanisms, and second, what may be the in vivo relevance of these findings?

4.4.1 Factors influencing the survival of cortical neuronal precursors in vitro

In these experiments cortical precursors were separated from their neighbours by dissociation of embryonic cortex and plated as isolated cells. In the absence of any initial contact with neighbouring embryonic cells, therefore, several factors could have been crucial to the survival of cortical precursor cells; among these are adhesion of precursors to a particular substrate and/ or exposure to cell survival factors. To analyse these possibilities, it is necessary to consider the survival of precursors under three experimental conditions: in cultures grown (i) in the presence of bFGF alone, (ii) in the presence of cortical astrocytes alone, and (iii) in the presence of cortical astrocytes and bFGF.

4.4.2   Survival of cortical precursors in the presence of bFGF alone

The observation that in the absence of cortical astrocytes, bFGF could enhance the survival of neuronal precursors, is a strong argument for a direct effect by bFGF. This direct effect is mostly likely to have been mediated by binding of bFGF to FGF receptors on neuronal precursors. Although this study has not shown that neuronal precursors carry FGF receptors, there is ample evidence that cortical precursors cells of ages similar to that used in these cultures possess such receptors in vivo and in vitro. FGF receptor transcripts, for example, have been detected in the cells of rat cortical ventricular zone

between E12 and E17 (Powell et al., 1991; Wanaka et al., 1991). Moreover, E18 cortical cells have been shown to express bFGF receptors (Walicke et al., 1989). However, involvement of bFGF receptors could perhaps be demonstrated by blocking such receptors with anti-FGFR-1 antibodies.

In any event, binding of bFGF to FGF receptors, a type of receptor tyrosine kinase, could have activated a cascade of second messengers (see section 1.7.2.1.2) leading to altered patterns of gene expression and ultimately cell survival. There is evidence, however, that receptor-bound bFGF can be directly internalised (Carmen et al., 1990; Ferguson et al., 1990; Walicke and Baird, 1991). Walicke and Baird (1991), who have studied this phenomenon in E18 rat hippocampal neurones, report that following its internalisation, bFGF is metabolised into several fragments, some of which translocate to the nucleus and become associated with cellular chromatin. Thus, bFGF could also directly influence gene expression at chromatin level.

Although bFGF is most likely to have had a direct effect on neuronal precursors, the possibility that this effect may have been an indirect one must also be considered. In this senario, bFGF's effect may have depended on other cells or factors present in these cultures. One possible candidate is the astrocytes derived from embryonic cortical precursors themselves. Almost invariably more such astrocytes could be found when cultures were grown on substrates other than cortical astrocytes, suggesting in itself that the proliferation of embryonic astrocytic precursors was normally inhibited by astrocytes of the monolayer. In this respect, it is interesting to note that O-2A progenitors grown on monolayers of cortical astrocytes in the absence of serum, also fail to produce type-2 astrocytes (Williams et al., 1985). Returning to bFGF's effects, two groups have claimed that in cultures of embryonic hypothalamus or mesencephalon, embryonic astrocytes mediate bFGF's neurotrophic effects (Petroski et al., 1991; Engele and Bohn, 1991). On the other hand, there is evidence that bFGF can have a direct survival effect on neurones (Walcike and Baird, 1988; Unsicker, et al., 1992). Results of this study also argue for an effect independent of embryonic astrocytes. In these experiments bFGF was applied only within the first 72 hours of culture, a period during which non-neuronal cells had not developed in these cultures. However, until bFGF's effect are tested on a purified population of neuronal precursors, it would be difficult to categorically rule out involvement of other cell types or their precursors in bFGF's survival effect on neuronal precursors.

It is also possible that bFGF's survival effect on neuronal precursors depended on the presence of factors in the medium, such as thyroxine, tri-iodothyronine and insulin. Cortical cells in this study were routinely cultured in modified Sato's medium which contains 2 mg/ml thyroxine, 1.5 mg/ml tri-iodothryronine and 5 μg/ml insulin (Bottenstein and Satos (1979); ffrench-Constant and Raff (1986). In this respect, Pons and Torres-Aleman (1992) have shown that bFGF treatment can upregulate insulin-like growth factor I

(IGF-I) receptor expression in cultured E15 rat hypothalamic neurones. Moreover, Drago et al. (1991b) have demonstrated that bFGF's 'mitogenic' effect on E10 mouse telencephalic and mesencephalic neuroepithelial cells depends on the presence of IGF-I, and that in response to bFGF treatment, these neuroepithelial cells upregulate IGF-I expression. Neither study, however, has shown that bFGF's effects on *neuronal* precursors is either mediated by, or depends on, the presence of insulin/ IGF-I. Drago et al. (1991b), for example, did not show which type of precursor had responded to the mitogenic effect of bFGF. This is important because bFGF is known to act as a mitogen for glial cells as well as neuronal precursors. Indeed, because of their mitogenic effects on astrocytes, acidic and basic FGF were originally also known as astroglial growth factors (AGF)(Brockes et al., 1980). Thus, it is not certain that in these experiments insulin mediated bFGF's survival effect on neuronal precursors.

4.4. 2 Survival of neuronal precursors grown on cortical astrocytes alone

Numerous studies have shown that neonatal astrocytes are able to promote the survival as well as neurite outgrowth from neurones in cultures of embryonic rat and mouse CNS. Examples of these include: survival of neurones derived from embryonic hippocampus (Banker, 1980), cerebellum (Alliot et al., 1988), spinal cord (), cortex (Ibata et al., 1991; Walsh et al., 1992), and that of olfactory receptor neurones (Pixley, 1992). By analysing the fate of single precursor cells grown in the presence of cortical astrocytes, this study has shown that primary astrocytes can also enhance the survival of cortical neuronal precursors in vitro; cultures of embryonic cortex either did not survive or contained 60% fewer neuronal clones when grown in the absence (on PDL) as compared to the presence of astrocytes. The ensuing question, however, is how did cortical astrocytes exert their survival effect? In theory, this could have been mediated either by direct cell-cell contact, or by production of neurotrophic factors, or both.

As already mentioned, in these experiments cortical embryonic cells were routinely plated as dissociated single cell suspensions, a condition that would have disrupted any communication between embryonic cells. This communication, however, is known to be important to the survival of embryonic cells; in the absence of cortical astrocytes, cultures survived better when embryonic cells were plated at higher cell densities (see results). In the absence of this communication, therefore, physical contact with cortical astrocytes may have served as an important survival factor. In this respect, Schmalenbach and Müller (1993) and Matthiessen et al. (1989) have identified a high MW complex present on cortical astrocyte membrane that can promote the survival of E18 rat hippocampal cells in vitro. These authors have found that this complex contains negatively charged, highly sulphated β-laminin chains as well as sulphated proteoglycans. Moreover, it has been shown that cultured astrocytes produce and release high levels of both laminin (Chiu et al., 1991) and fibronectin (Price and Hynes, 1985; Matthiessen et al., 1989). These or similar

extracellular matrix molecules may then have enhanced the survival of neuronal precursors by one of two possible mechanisms. First, cell surface molecules present on cortical astrocytes may have helped anchor precursors, thus improving the plating efficiency of cultures. However, there is evidence that beside mere anchorage, adhesion to substrate can also trigger a cascade of second messengers within the adhering cells. An example of this type of signal transduction is the neurite promoting activity of Neural-cell adhesion molecule (N-CAM) that is thought to be mediated by a change in intracellular calcium levels (see review of Doherty and Walsh, 1992). Another example, outlined by Gumbiner (1993), is interactions of cell-surface integrins with cytoskeletal and second messenger proteins, such as talin, vinculin and paxilin, at what are known as the focal adhesion plaques.

The second mechanism would be by direct interaction of ECM molecules with distinct receptors on neuronal precursors, which, like growth factor receptors can also activate a cascade of second messengers (see review of Juliano and Haskil, 1993). An example of such molecules is laminin; Drago et al. (1991a), for example, have shown that E10 mouse neuroepithelial cells, derived from the developing telencephalon and mesencephalon, possess laminin binding sites and that laminin can induce their proliferation in vitro. Also, Reh et al. (1987) have demonstrated that binding to laminin alone can directly induce transdifferentiation of retinal pigment epithelial cells into cells of the neural retina. It is worth noting, however, that in the present study PDL/ laminin (50 µg/ml) coated coverslips could not support the survival of neuronal precursors.

It is equally likely that the survival effects of cortical astrocytes may have been mediated by diffusable factors. In this respect, several groups have shown that astrocyte conditioned medium can act a survival factor for embryonic CNS cells (Banker 1980; Ibata et al., 1991; Alliot et al., 1992; Gray and Patel, 1991). Also, conditioned-medium from an astrocyte cell line can rescue E10 mouse neuroepithelial cells from death in vitro (Kilpatrick et al., 1993). Moreover, astrocytes can exert their survival effect on embryonic cells in cultures in which the two cell types are separated by a transwell or a coverslip (Walsh et al., 1992; Ang et al., 1992). Nonetheless, Schmalenbach and Muller (1993) have demonstrated that optimal survival of CNS neurones may require simultaneous presence of both diffusable and non-diffusable astrocyte-derived factors. Interestingly, Alliot et al. (1992) who tested the effects of three morphologically-different types of cerebellar astrocytes, have found that conditioned medium collected from only two of these astrocytic types could enhance the survival of embryonic cerebellar precursors; survival induced by the third type required direct physical contact between the astrocytes and the embryonic precursors. Noteworthy here also is that homotypic interactions may be required for a survival effect by astrocytes; Yuzaki et al. (1993) have shown that cerebellar, but not cortical or hippocampal astrocytes, enhance the survival of cerebellar Purkinjie neurones in culture.

Against this background, this study failed to find any survival effect by astrocyte conditioned-medium on cultured cortical neuronal precursors. It may be, however, that the conditioned medium collected from bulk cultures of astrocytes that were free of embryonic cells did not contain the adequate concentration of diffusable neurotrophic factors, or, that such factors are only produced as part of communication between neuronal precursors and cortical astrocytes.

In any event, if the survival of neuronal precursors on cortical astrocytes is mediated by diffusable factors, what is the nature of such factors? Cultured cortical astrocytes are known to produce a wide array of neuropeptides, neurotransmitters, cytokines and growth factors (listed in a review by McMillan et al., 1994), several of which are known to support the survival of CNS neurones in vitro or in vivo. These include CNTF (Ip et al., 1991; Oppenheim et al., 1991), bFGF (see chapter I), NGF and NGF-related peptides, BDNF (Oppenheim et al., 1992; Yan et al., 1992), NT-3 (Arenas and Persson, 1994) and NT-4/5 (Friedman et al., 1993). Thus, one or more of these factors may have mediated the survival effect of astrocytes on neuronal precursors. However, CNTF and bFGF are the less likely candidates, because, both these proteins lack a secretory signal sequence and would normally not have been available to precursors cells. Rudge et al. (1992) have shown that although cortical astrocytes produce considerable quantity of CNTF mRNA, some 65 fold higher than their in vivo levels, no CNTF can be detected in their conditioned-medium or at their cell surface. It is possible that with astrocyte cell lysis these factors could escape into the culture medium. In this study, however, there was no apparent cell death in the astrocyte monolayer, rather, cortical astrocytes remained as an intact population of quiescent, contact-inhibited cells both in the presence and absence of embryonic cortical cells. One observation that supports the quiescent nature of these astrocytes is that despite being exposed to BAG during the infection of embryonic cells, none of the cells of the monolayer were ever labelled.

NGF is also an unlikely candidate, because, in contrast to peripheral neurones, very few central neurones are known to respond to NGF (Banker, 1980; Lindsay et al., 1987); cholinergic neurones of the basal forebrain are among a handful of such NGF-responsive CNS neurones (Hefti et al., 1989), even though a recent study has questioned the significance of this responsiveness (Crowley et al., 1994). Crowley et al. (1994) have shown that the cholinergic neurones of the basal forebrain can develop normally in mutant mice lacking NGF. Moreover, Cattaneo and McKay (1990) find that NGF alone is unable to support the survival of E14 mesencephalic neural precursors. The case for involvement of other NGF-related peptides, however, is stronger. First, in contrast to the low levels of NGF (trk-A) receptor and NGFR-mRNA expression (Lu et al., 1989), receptors for BDNF (trk-B) and NT-3 (trk-C) are expressed at high levels in the developing telencephalon, being restricted to the cells of the ventricular zone and cortical plate (Klein et al., 1990a, 1990b; Lamballe et al., 1994). Second, neurotrophins such as NT-3 but not

NGF are also found in high levels in the developing telencephalon (Maisonpierre, et al., 1990). However, more direct evidence is in hand. Ghosh et al. (1994) have recently demonstrated that BDNF can enhance the survival of cultured E17 and E18 cortical neurones in vitro and that in response to BDNF, NT-3 and NT-4/5 but not NGF treatment, these cells upregulate the immediate early gene, *c-fos*. Surprisingly though, Rudge et al. (1992) have found that BDNF was not among a range of factors produced by cortical astrocytes in vitro. Thus, a novel astrocyte-derived factor may be enhancing the survival of cortical neuronal precursors, a possibility that deserves an indepth investigation.

4.4. 3. Survival of neuronal precursor in the presence of astrocytes and bFGF

Results of this study showed that although cortical astrocytes enhanced the survival of neuronal precursors, this survival could be enhanced further in the presence of 10 ng/ml bFGF; 40-50 % more neuronal clones could be detected in cultures grown the presence of cortical astrocytes and bFGF, than in the presence of cortical astrocytes alone (Table 4.1). This difference could be explained in at least three possible ways, two of which would suggest that cortical astrocytes had a role in bFGF's survival effect on neuronal precursors. First, bFGF could have upregulated the production of neurotrophic factors by cortical astrocytes (discussed above). Yoshida and Gage (1991 and 1992), for example, have shown that aFGF can markedly increase the synthesis and secretion of NGF in cultured cortical astrocytes. Although NGF, as discussed, is an unlikely survival factor for cortical precursors, increased production of similar neurotrophic factors could have led to survival of more neuronal precursors. On the other hand, bFGF may have upregulated NGF receptor expression in neuronal precursors, making such precursors responsive to NGF, a phenomenon that has been observed in bipotential sympathoadrenal precursors when they become specified along the neuronal lineage (Birren and Anderson, 1990, see chapter I).

The second way in which astrocytes could be involved is not by any particular response to bFGF, but by their 'normal' production of extracellular molecules, such as heparin and sulphated proteoglycans (Schmalenbach and Müller (1993); Matthiessen et al., (1989)). bFGF is known to have a high binding affinity for these components of the extracellular matrix and this binding, which is known to protect bFGF from degradation (Saksela et al., 1988; Sommer and Rifkin, 1989), can be important in at least two respects. First, by binding to bFGF, sulphated heparin or proteoglycans may act as a reservoir of bFGF thus prolonging its availability to neuronal precursors. This would be significant since free bFGF has a half life of only 7 hours   in culture (Walicke ?). Second, cell-surface bound heparin and sulphated heparans are known to facilitate the presentation and binding of bFGF to its high affinity receptors (Bernard, et al., 1991; Rapraeger, et al., 1991; Yayon et al., 1991, and review of Klagsburn and Baird, 1991) which mediate bFGF's biological activity. bFGF's survival effects in the presence of astrocytes, could, therefore, have been potentiated by the presence of extracellular matrix molecules. This may also

explain why survival of neuronal precursors in bFGF-treated cultures was poorer when grown in absence of cortical astrocytes; i.e. PDL-coated coverslips and astrocyte matrix may have been either deficient or have contained the inappropriate extracellular matrix molecules.

In this study, addition of 100 but not 10 ng/ml heparin to cultures grown on cortical astrocytes in the presence of bFGF, markedly reduced bFGF's survival effect on neuronal precursors. In this respect, several other workers have discovered that 100 ng/ml heparin or excess heparan sulphates prevents binding of bFGF to its low and high affinity receptors, thereby impairing its biological activity (Neufled and Gospodarowicz, 1985; Hondermarck et al., 1992; Moscatelli, 1992; Roghani and Moscatelli, 1992). One possible explanation is that since in this study heparin and bFGF were mixed in suspension prior to their application, free heparin could have adsorbed bFGF or have masked its receptor binding sites thus preventing its presentation to bFGF receptors.

There is a third possible explanation of why more neuronal precursors survive in the presence of bFGF and cortical astrocytes than cortical astrocytes alone. It may be that a subpopulation of neuronal precursors do not respond to neurotrophic effects of cortical astrocytes, whatever their nature; bFGF then would have had a direct survival effect on such precursors. This direct effect was demonstrated in cultures grown in the absence of astrocytes. Alternatively, survival of the same subpopulation may have required the presence of both bFGF and cortical-astrocyte derived neurotrophins. Although these possibilities cannot be resolved, results of this study show that cultures of E16 cortex do contain subpopulations of neuronal precursors. The evidence for this comes from an analysis of the size distribution of neuronal clones in E16 and E14 cultures grown under different cultures conditions. This evidence is discussed below as part of an analysis of factors that may control the division of cultured cortical neuronal precursors.

4.4. 4 Factors that control division of neuronal precursors
4.4. 4.1 Inhibition of neuronal precursor proliferation by cortical astrocytes

This study showed that E16 cultures grown in the absence of cortical astrocytes, contain significantly larger neuronal clones than those grown on astrocytes, although under both culture conditions the majority of neuronal clones were single-cell clones (Table 4.2; figures 4.9 and 4.10). The dramatic difference in the size of neuronal clones found in these cultures could not be explained by differential survival of precursors, because, the survival of precursors was always poorer in the absence of cortical astrocytes. Consequently, two other possible explanations exist. First, neuronal precursors with a high proliferative potential could have been actively destroyed in cultures grown on cortical astrocytes. Giulian et al. (1993), for example, have shown that microglial cells that contaminate primary cultures of cortical astrocytes produce a 500 Da neurotoxic factor. This explanation, however, is highly unlikely since prior to plating of embryonic cortical cells,

microglia and other contaminating cells were routinely removed from the astrocyte monolayers such that 95% of cells in these monolayers were found to be positive for GFAP. The second explanation is that cortical astrocytes actively inhibit the proliferation of neuronal precursors as previously suggested by Yoshida et al. (1986). These workers studied ^{3}H-thymindine incorporation by cultured E15 cortical precursors and reported that 5% of neurones derived from cultures grown on astrocytes were labelled with ^{3}H-thymidine, compared to 15% when grown on PDL. They suggested that this effect may have been mediated by an astrocyte cell-surface molecule. In a later study, however, they suggest that the inhibition is mediated by soluble factors (Ibata et al., 1991).

Two other studies have analysed the role of astrocytes in regulating neuronal precursor cell division in vitro. Gao et al. (1991) showed that cerebellar astrocytes inhibit DNA synthesis by post-natal cerebellar granule cell precursors in vitro, an effect mediated by astrocyte membranes. By contrast, Temple and Davis (1994) show that astrocytes promote division of single E14 cortical precursors, an effect also attributed to astrocyte cell membranes. Despite their clonal analysis, however, Temple and Davis (1994) did not determine proliferation of which precursor type was promoted. Moreover, Temple and Davis (1994) analysed the effects of astrocytes, but not their membranes, in the presence of serum which could have complicated the findings.

These conclusions notwithstanding, results of this study suggest that proliferation of only a subpopulation of neuronal precursors was inhibited by cortical astrocytes, the evidence for which is as follows.

4.4.5 Evidence that E16 and E14 cultures contain subpopulations of neuronal precursors

Both in the presence and absence of cortical astrocyte, the majority of neuronal clones were found to contain only a single cell.

The high frequency of single-cell clones, suggests that single-cell clones represent the terminal round of division of arithmetically-dividing precursors, . If cortical astrocytes had inhibited the proliferation of all neuronal precursors, one would expect that in their absence, neuronal precursors would undergo at least one, if not more rounds of division. If this were to happen, then one would expect a shift away from the frequency of single-cell clones toward 2-cell and greater neuronal clones in cultures grown in the absence of astrocytes. No significant change in the frequency of single-cell clones, however, was observed in the absence of cortical astrocytes; single-cell clones made up as much as 70-80% of all neuronal clones in such cultures (figures 4.9 and 4.10), suggesting that a considerable number of neuronal precursors continued to undergo their terminal

round of arithmetic division. It may be that this terminal division is an inherent property of such precursors, in which case, E16 neuronal precursors can be divided into two subpopulations: a predominant population that have a limited proliferative potential, giving rise to single-cell clones, and a small population that have a higher proliferative potential, a potential that was inhibited by cortical astrocytes.

There is further evidence that E16 cultures contain subpopulations of neuronal precursors. When grown on cortical astrocytes alone, E16 derived neuronal clones rarely contained more than 2 cells, with the majority being single-cell clones. In the presence of bFGF, however, as well as a high frequency of single-cell clones, neuronal clones as large as 10 to 20 cells were evident. Detection of a high frequency of single-cell clones in both these cultures again is evidence for a predominant population of neuronal precursors that may be undergoing their terminal round of division, a population whose survival but not division was enhanced by bFGF. Presence of larger neuronal clones in bFGF-treated cultures grown on cortical astrocytes, on the other hand, could be explained in two different ways. bFGF may have either acted as a mitogen for a subpopulation of neuronal precursors, or through an unknown mechanism, may have overridden the inhibition imposed by cortical astrocytes on precursors of higher proliferative capacity. Alternatively, bFGF could have acted as a survival factor for a population of precursors with a higher proliferative capacity that did not normally survive in the presence of cortical astrocytes. Either way, these observations show that E16 cultures contain subpopulations of neuronal precursors that differ in their mitogenic response to bFGF or inherent proliferative capacity. As far as proliferative potential is concerned, E14 cultures were also noted to contain subpopulations of neuronal precursors (results and discussion of chapter II); precursors could be found in these cultures that gave rise to single-cell clones, and precursors that had divided geometrically, producing clones of 50 or more neurones in vitro. It may be that the 10-20 cell clones found in E16 cultures are derived from precursor belonging to earlier embryonic ages that would have persisted as late as E16 in the cortical VZ.

Evidence for existence of subpopulations of neuronal precursors, however, has also been provided by several other workers. The work of Luskin et al. (1993), for example, suggests that VZ of E16 rat cortex may contain separate precursors for the two major neuronal types found in mature cerebral cortex, namely the pyramidal and non-pyramidal neurones. These authors report that E15 or E16 BAG-labelled cortical neuronal precursors subsequently give rise to cell clusters that are composed of either pyramidal or non-pyramidal neurones. Similarly, Mione et al.'s study suggests that subpopulations of neuronal precursors may have been specified in their choice of neurotransmitter phenotype while within the VZ; these authors report that E14, E15, E16 or E19 BAG-labelled neuronal precursors, all give rise to cell-clusters that are composed entirely of either GABAergic or glutaminergic neurones. The results of Mione et al. (1994) also suggests

that GABAergic and glutaminergic clones are generated by precursors of different proliferative potential; glutaminergic clones generated by E14, E15 and E16 precursors were on average twice as large as GABAergic clones derived from precursors of comparable ages. More recently, however, Gotz et al. (manuscript submitted) have investigated whether cortical neuronal precursors are specified in their choice of neurotransmitter. In contrast to Mione et al.'s findings, these authors find that almost a third of cultured BAG-labelled E16 cortical precursors can produce clones composed of both GABAergic and glutaminergic neurones, suggesting that at the point of their isolation from E16 cortex, neuronal precursors were not specified in their neurotransmitter phenotype. However, Gotz et al. also find that 60% of neuronal clones are composed entirely of either GABAergic or glutaminergic neurones. Interestingly, in this study the ratio of pure GABAergic to glutaminergic clones was of the order of 1:4, a proportion remarkably similar to the abundance of GABAergic (non-pyramidal) and glutaminergic (pyramidal) neurones in vivo.

4.4.6 Possible relevance of the present findings to the development of cortical neuronal precursors in vivo

Studies of cortical development in vivo show that in contrast to glial cell production, production of cortical neurones is limited both spatially and temporally. In the rat, for example, cortical neuronal precursors divide only within the VZ, and cortical neurogenesis itself is limited to 7-8 days of CNS development; i.e.between E13 and E21. The question is therefore what factors limit cortical neurogenesis in vivo? In essence, neurogenesis may be regulated by the rate and mode of proliferation and/ or survival of neuronal precursors.

In this respect, the present study has identified several factors that can influence the survival and proliferation of cortical neuronal precursors in vitro. The ensuing question, however, is whether such factors play any role in cortical neurogenesis in vivo.

4.4.6.1 A possible role for glial cells or their precursors

This study shows that type-1 astrocytes derived from neonatal rat cortex inhibit the proliferation of neuronal precursors in vitro, an effect possibly brought about by cell-cell contact. Although type-1 astrocytes are absent from E16 rat cortex, presence of radial glial cells, which are thought to give rise to astrocytes (Schmechel and Rakic, 1979; Voight, 1989; Gray and Sanes, 1992), tempt one to speculate that such cells may also inhibit neuronal precursor cell division in vivo. Contact with radial glial cell-process may induce neuronal precursor to become post-mitotic, thus allowing post-mitotic neurones to use the processes of these glial cells to migrate to their relevant cortical lamina.

Detection of GFAP-positive mitotic cells, regarded by some as astrocyte precursors (Levitt et al., 1981 and 1983) within the cortical VZ, may also add weight to an inhibitory role for glial precursors. Levitt et al. (1981 and 1983) report that at E47, 28% of all VZ

cells in the monkey visual cortex are GFAP positive, a proportion that rises to 80% at the peak of neurogenesis in this structure (around E80). Although GFAP positive cells have not been found in the rat cortical VZ during the peak of cortical neurogenesis at E16, there is evidence that during such period, the VZ does contain astrocytic precursors (Grove et al., 1993). In any event, GFAP positive cells are first detected in the rat cortex at E18 (Maric et al., unpublished observations). Thus glial cell precursors may also, through cell-cell contact, inhibit proliferation of neuronal precursors. Support for this hypothesis is provided by studies of neurogenesis in the rat cerebellum. In contrast to cerebral cortex, a subpopulation of cerebellar neurones, called granule cells, are generated during early post-natal life from a germinal zone located at the periphery (pial side) of the developing cerebellum. Although astrocytes are prominent in the postnatal cerebellum, anatomical studies show that such cells are separated from the neuronal precursors of the germinal zone by a layer of differentiated neurones. In this respect, Gao et al. (1991) have shown that contact between the cerebellar astrocytes and cerebellar neuronal precursors can inhibit their proliferation in vitro.

These observations, therefore, support the need for investigating the exact nature of the astrocyte-derived molecules that inhibit the proliferation of neuronal precursors in vitro. After this, the hypothesis that glial cells or their precursors inhibit neuronal precursor proliferation in vivo can be tested. If the inhibitory molecules are found to be cell-surface associated, in the first instance one may ask whether radial glia or GFAP-positive VZ cells carry them. Alternatively, it may be possible to test how the rate of neurogenesis is affected by high expression of such molecules in vivo; if encoded by a single gene, over-experssion of such molecules by VZ cells could be induced either by transgenic technology or by retroviral mediated gene-transfer.

4.4.6.2 Possible involvement of bFGF

Results of this study also showed that bFGF can influence the survival and possibly the proliferation of neuronal precursors in vitro. Moreover, bFGF treatment revealed the existence of subpopulations of neuronal precursors in cultures of E16 cortical cells; subpopulations that differed either in their proliferative potential or their responsiveness to a possible mitogenic effect by bFGF (see earlier discussion). Thus, bFGF may also play a role in regulating neuronal precursor cell division and/or survival in vivo.

However, the extrapolation of the in vitro effects of bFGF to the in vivo situation are complicated by the fact that bFGF is not a secretory molecule; i.e. it is not clear how neuronal precursors may acquire bFGF in vivo. In this respect, several possibilities exist. First, it may be that bFGF is synthesised by neuronal precursors themselves in vivo, and that this factor acts in an autocrine fashion; for example, bFGF may induce its effects by translocation from the cytoplasm to the nucleus. Such translocation of bFGF has been observed in presumptive mesodermal cells of Xenopus co-inciding with the time of

mesoderm induction (Shiruba et al., 1991), a process in which FGF plays a crucial role (see chapter I). Moreover, bFGF is known to exist as different molecular weight species, some of which bear a nuclear localisation sequence and are thus found exclusively in the nucleus (Quarto et al., 1991). However, although differentiating cortical neurones are known to synthesize bFGF (Weise et al), it is not certain that neuronal *precursors* can do so; studies of FGF expression in vivo merely point out that some VZ cells express FGF, acidic FGF in particular (Fu et al., 1991).

Alternatively, bFGF may act in a paracrine fashion becoming sequestered by neuronal precursors from cells that produce bFGF. In the absence of a secretory signal sequence, bFGF may become released by a novel non-Golgi dependant pathway, or after cell lysis. There is, however, a third possibility. It may be that the observed effects of bFGF in vitro, are not mediated by bFGF *per se* in vivo, but by FGF-related factors that are secreted and can interact with bFGF receptor (FGFR-1). The precedence for this argument comes from work on mesoderm induction and development of the vertebrate limb, in which FGF-4 (hst) was shown to mimic the in vitro effects of bFGF in these developmental processes in vivo (Paterno et al, 1989; Isaacs et al., 1992; Niswander et al., 1993). Moreover, preliminary findings reported in the present study shows that two other FGFs, namely int-2 (FGF-3) and hst (FGF-4), can also promote the survival of E16 precursors in vitro, in a magnitude similar to that of bFGF (figure 4.8, section 4.3.4). In this respect, int-2 is known to be expressed by cells of the developing mouse cortex in vivo (Mason et al., 1994), although the in vivo developmental expression of hst is, at present, unknown.

Naturally, the effects of such FGFs need to be investigated in vivo. Several approaches could be taken. As with studies of NGF function (Crowely et al., 1994), knockout of bFGF or bFGF-receptor genes, for example, could be attempted. However, this is not likely to be successful because unlike NGF, bFGF is involved in diverse developmental processes ranging from angiogenesis to bone and muscle development. Thus, elimination or mutation of bFGF gene may be lethal to the embryo at very early stages of development. Moreover, absence of bFGF may be compensated by other members of the FGF family, some of which can normally bind more than one type of FGF receptor; aFGF (FGF-1) and FGF-4 are capable of binding all four known types of FGF receptors albeit with different affinities.

However, it may be possible to overexpress bFGF in single neuronal precursors in vivo by retrovirus mediated-gene transfer; i.e. bFGF as well as a histochemical marker gene could be introduced in to such cells using what are called dual-expression vectors. In this regard, a retroviral vectors carrying *Lac-Z* as well as NGF gene has already been constructed (E. Grove personal communication). Thus, if bFGF was to act as an autorcine factor, its overexpression in single neuronal precursors may enhance their survival and/ or proliferation, an effect that could be monitored by following the fate of such virally-labelled cells using the product of the histochemical marker gene *Lac-Z*.

Alternatively, it may be possible to deliver a form of bFGF that possesses a secretory signal sequence (Blam et al., 1988). In this case, single virally-labelled cells could act as foci of bFGF expression and thus the effect of bFGF on neighbours cells of a precursor cell could be studied, either in vitro or in vivo.

An investigation of the effects of bFGF on the development of neuronal precursors in vitro: a summary of findings and conclusions: A Summary of findings

Effects of bFGF on cortical neuronal precursors were studied by comparing the fate of single E16 or E14-retrovirally labelled cortical precursor cells grown for a week amongst a population of unlabelled precursors, on a monolayer of cortical astrocytes in the presence or absence of bFGF. Such a comparison revealed that bFGF does not override the phenotypic specification of cortical precursor cells (reported in chapter II); i.e. clones generated by retrovirally-labelled precursors were composed solely of a single cell type both in presence and absence of bFGF (Table 4.1). bFGF also did not alter the proportion of different clone types found in 7-day cultures of E16 or E14 cortical cells; the majority of clones in such cultures were found to be neuronal, both in the presence and absence of bFGF (Table 4.1). bFGF did, however, enhance the survival of cortical precursor cells, specifically that of neuronal precursors; almost 50% more clones could be found in bFGF-treated cultures of E16 cortical cells than in controls, the majority of which were neuronal (Table 4.1). Furthermore, bFGF could act as a survival factor for neuronal precursor in cultures of E16 rat cortex grown on substrates other than a monolayer of cortical astrocytes.

Further to these observations, it was of particular interest to know whether in addition to its survival effect, bFGF had acted as a mitogen for cortical neuronal precursors. For this purpose the size distribution of neuronal clones were analysed (figure 4.1). This analysis showed that both in the presence and absence of bFGF, the majority of neuronal clones remained as single-cell clones. Results and discussions presented in chapters II and III suggested that these clones are the likely products of precursors undergoing terminal division, where, only one daughter of such precursors inherits the viral marker genes and thus becomes detectable as a single cell clone. In this regard, bFGF did not influence this terminal division and therefore was unlikely to have acted as a mitogen for neuronal precursors. However, another set of observations suggested that bFGF may have acted as a mitogen for a *subpopulation* of E16 neuronal precursors. In the presence of bFGF, a small proportion of E16 neuronal precursors generated clones ranging in size from 2 to 20 cells (figure 4.2). In the absence of bFGF, by contrast, such 2-cell and greater neuronal clones rarely contained more than 2 neurones (figure 4.1). This observation could not be taken as absolute evidence for a mitogenic effect, however, since detection of larger neuronal clones in the presence of bFGF could reflect survival of a subpopulation of E16 neuronal precursors with a higher proliferative potential.

In a series of experiments, effects of bFGF were titrated by using different concentrations of bFGF ranging from 2 to 50 ng/ml; or by applying bFGF in different dose combinations; or by applying bFGF in the presence of heparin. Results of these analyses showed that survival of embryonic cortical precursors was maximal when bFGF was applied at 10 ng/ml in three doses: one on at the time of plating, designated day 1 of culture, and two subsequent doses on days 2 and 4 (figures 4.4, 4.5 and 4.6). This analysis also showed that 100 but not 10 ng/ml heparin could markedly reduce bFGF's survival effect on cortical neuronal precursors (figure 4.7), suggesting that heparin had adsorbed and masked bFGF's binding sites thereby blocking its biological activity.

Worthy of mention here is that identical results were obtained with two different sources of bFGF: Xenopus and Human recombinant bFGF (Table 4.1). Furthermore, in another set of experiments, effects of some other members of the FGF family were tested alongside that of bFGF. These were: KGF (FGF-7); hst (FGF-4); aFGF (FGF-1); FGF-5; and int-2 (FGF-3). Of these factors, however, only int-2 and hst were found to have a survival effect on neuronal precursors of a magnitude similar to that of bFGF (figure 4.8).

Conclusions

The above results show that cultured neuronal precursors derived from embryonic day 16 rat cortex can respond to bFGF as well as two other members of the FGF family most related to bFGF, suggesting that rat cortical neuronal precursors carry bFGF receptors in vitro. This finding complements reports of bFGF-receptor expression by embryonic cortical precursors express bFGF-receptors in vivo (Powell et al., 1991) and suggests that bFGF may play a role in the regulation of rat cortical neurogenesis in vivo; i.e. bFGF may also govern the survival of neuronal precursors in vivo.

By analysing the developmental potential of cortical precursors in the presence or absence of bFGF, this study has also provided evidence for existence of subpopulations of neuronal precursors at E16 in the rat cortex; i.e. subpopulations that differ inherently in their proliferative potential (if detection of 10-20 cell clone in the presence of bFGF was to be interpreted as purely a survival effect), or in their mitogenic response to bFGF (if bFGF were to have acted as a mitogen specifically on these precursors).

Influence of cortical astrocytes on the development of embryonic rat cortical precursors in vitro

In the experiments described in chapters II and IV, E16 or E14 cortical precursors were routinely cultured on monolayers of astrocytes derived from neonatal cerebral cortex. In this respect, it was conceivable that cortical astrocytes may somehow have influenced the development of embryonic cortical precursors in vitro. In order to investigate such a possibility, the fate of retrovirally-labelled cortical precursors were analysed in the presence or absence of cortical astrocytes. This analysis showed that cortical astrocytes enhance the survival of embryonic neuronal precursors; cultures grown in the absence of cortical astrocytes, either on PDL-coated coverslips, or astrocytes matrix or NIH-3T3 cell matrix, either died or contained 50-70% fewer clones (Table 4.2). However, clones generated by cortical precursors grown in the absence of cortical astrocytes were still found to be composed of a single cell type, supporting the hypothesis that E16 or E14 cortical precursors are specified in their phenotypic choice. Moreover, the majority of clones found in such cultures were neuronal, supporting the conclusion reached in chapter II that neuronal precursors constitute the majority of precursors present in the E16 or E14 rat cortex in vivo.

However, beside their survival effect, cortical astrocytes were also found to influence the proliferation of a subpopulation of cortical neuronal precursors in vitro. Neuronal clones consistently showed a broader size range whenever E16 cortical precursors were cultured in the absence compared to the presence of cortical astrocytes (figures 4.9 and 4.10); some of such clones contained as many as 10 or 20 neurones (figure 4.12). Because the survival of cortical cultures was always poorer in the absence of cortical astrocytes, this observations suggested that cortical astrocytes normally inhibit the proliferation of E16-derived neuronal precursors. However, this inhibition affected only a subpopulation of neuronal precursors since the majority (70-90%) of neuronal precursors continued to give rise to single-cell clones even in the absence of cortical astrocytes (figures 4.9 and 4.10). This observation was important because it suggested that (a) the majority of E16 neuronal precursors appear to undergo terminal division, no matter which substrate they are plated on, and (b) E16 cortex appears to contain subpopulations of neuronal precursors that differ in their proliferative potential.

Against this background, it remains to be determined what factors limit the proliferation of cortical neuronal precursors in vivo, since neuronal clones containing as many as 10 or even 20 cells have never been reported by cortical cell-lineage studies in vivo; i.e. it remains to be determined wether astrocytes or astrocyte precursors have any role in limiting the proliferation of cortical neuronal precursors in vivo.

Conclusions

Conclusions

Collectively, the findings of this study extend our knowledge of type, proportion and behaviour of different neural precursors present in the embryonic cortex of the rat during the peak of cortical neurogenesis at E16. Cortical cell lineage studies in vivo had suggested that the rat cortical VZ is a mosaic of different precursor types; evidence had been presented for existence of separate precursors for neurones, astrocytes and oligodendrocytes. Moreover, pyramidal and non-pyramidal neurones were shown to arise from separate precursors (Parnavelas et al., 1991; Luskin et al., 1993). These observations suggested that by E16 cortical precursors are specified in their phenotypic fate and that this restriction may take place in the VZ itself. This conclusion was tentative, however, since cell lineage studies analysed the presumptive fate and not the developmental potential of cortical precursor cells. In this regard, the present study has analysed the developmental potential of E16 rat cortical precursors to discover that majority of such precursors are specified in their phenotypic potential; clones generated by single-retrovirally labelled cells were found to contain only one cell type. In addition, this study has shown that as much as 80-90% of E16 cortical precursors are neuronal precursors and that E16 cortex may contain subpopulation of neuronal precursors that differ in their proliferative potential. Also, this study has highlighted a clear difference between the proliferative potentials of E16 and E14 cortical neuronal precursors; neuronal clones were found to be larger in size in E14 than in E16 cultures. Furthermore, it was shown that E14 cortex contains neuronal precursors that divide geometrically in vitro.

The study of modes of retroviral integration, present in chapter III, added a third dimension. These results, which showed that retroviral integration is into post-replication host DNA, provided further evidence that most neuronal precursors of the cerebral cortex divide asymmetrically, thus also providing a sound explanation for the occurrence of single-cell neuronal clones reported by all cortical cell lineage studies in vivo and in vitro.

Further to these, this study was also able to identify factors that may play a crucial role in controlling cortical neurogenesis in vivo. basic fibroblast growth factor was identified as a factor that enhances the survival and possibly the proliferation of cortical neuronal precursors. This observation complemented the overwhelming evidence for the presence of bFGF and its receptors in the developing cerebral cortex of the rat, thus suggesting that bFGF may have similar influence on the development of neuronal precursors in vivo. In addition, this study provided evidence that an unknown factor derived from cortical astrocytes inhibits the proliferation of E16 cortical neuronal precursors.

In this regard, further studies are required to identify the molecular mechanisms by which bFGF or cortical astrocytes influence the development of neuronal precursors; this identification would help determine whether such factors play similar roles in vivo.

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