Early phenotypic choices by neuronal precursors, revealed by clonal analysis of the chick embryo hindbrain

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SUMMARY

The mechanisms that generate diverse neuronal phenotypes within the central nervous system are thought to involve local cues or cell-cell interactions acting late in neurogenesis, perhaps as late as the last precursor cell division. We describe here a clonal analysis of neuronal development in the chick hindbrain, using an intracellular tracer to mark single precursor cells, that suggests the operation of an alternative strategy. The majority of clones, ranging from 1 to 46 cells, contained neurons of only one of several possible phenotypes. These single-phenotype clones were not positionally restricted within a rhombomere but were interspersed with other clones containing distinct phenotypes. The assignment of neuronal phenotype in this brain region may, therefore, be made in early precursors and remembered through several rounds of mitotic expansion and dispersal.

Key words: hindbrain, rhombomeres, fate restriction, clonal analysis, clones, chick embryo

INTRODUCTION

The mature central nervous system contains a remarkable variety of specialised cell phenotypes, derived from the epithelium of the embryonic neural tube. Most discussions of phenotype segregation propose mechanisms that involve exclusively local signals and cell-cell interactions (Yamada et al., 1991, 1993; Altshuler and Cepko, 1992; Watanabe and Raff, 1992; Harris and Messersmith, 1992); the early neuroepithelium is predicted to contain uncommitted multipotent precursor cells, with phenotypic determination occurring during late S or early G2 in the final cell division of each neuron (McConnell and Kaznowski, 1991). In some systems, such as the primary motor neurons of the zebrafish, it appears that some specific details of neuronal identity do not become fixed until axonogenesis has begun (Eisen, 1992). In a region of neuroepithelium containing a mixture of neuronal phenotypes, single precursor cells would thus be expected to generate daughter cells of more than one type. Consistent with this, clonal analyses in the retina (Wetts and Fraser, 1988; Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990), optic tectum (Galileo et al., 1990; Gray and Sanes, 1991) and spinal cord (Leber et al., 1990) have demonstrated the descent of diverse cell phenotypes from single precursors. Such studies have argued against the possibility of a role for cell lineage in the process of cell determination (but see discussion in Williams and Goldowitz, 1992).

In contrast, recent clonal analyses using the recombinant retroviral marking technique suggest that environmental factors alone may not be sufficient to account for the generation of glial and neuronal cell types in the rat cerebral cortex. While some studies argue that cortical neurons and glia may arise from the same precursors (Price and Thurlow, 1988; Williams et al., 1991; Walsh and Cepko, 1992) and that oligodendrocytes and astrocytes may share the same precursor in the subventricular zone (Levison and Goldman, 1993), others argue that astrocytes, oligodendrocytes and neurons may each arise from their own dedicated precursors (Luskin et al., 1988, 1993; Parnavelas et al., 1991; Grove et al., 1993). Moreover, Luskin et al. (1993) suggest that the two major neuronal classes of the cortex, pyramidal and non-pyramidal neurons, also arise from distinct precursors. These results raise the possibility that a lineage-dependent mechanism may control cell identity in some areas of the vertebrate CNS. However, one problem is that clonal analysis alone does not test the developmental potential of precursors and stereotyped or limited cell movements could result in determinate-looking cell lineages arising from multipotent precursors. A more critical test of the respective roles of environment and lineage is provided by an examination of both the dispersal and the phenotypes of the descendants of a single cell.

A number of considerations suggest that the chick embryo hindbrain provides an attractive system in which to pursue the role of lineage in neuronal determination. Recent studies have shown that this system is open to experimental manipulation (Guthrie and Lumsden, 1991); it is possible to mark individ-
ual precursor cells very early in development and follow their dispersal (Fraser et al., 1990); and early neuronal patterning of this region is based on a relatively simple set of neuronal phenotypes whose spatial organisation has been characterised (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993; Simon and Lumsden, 1993) and whose development is based on a segmental plan (Lumsden and Keynes, 1989). Despite widespread dispersal of clonally related cells within each segment (rhombomere), cell mixing between the rhombomeres is restricted (Fraser et al., 1990) and rhombomere transposition experiments have shown that the fates of individual rhombomeres are autonomous from an early stage (Guthrie et al., 1992). The early determination of regional identity in the hindbrain raises the possibility that cell identity may also be determined early and for some period of development may be independent of environmental influences.

We have marked single cells in the early chick hindbrain and have found that, during the ensuing 48 hours of development, the large majority of resulting clones contained neurons of only one phenotype. Clones containing neurons and precursor cells were not restricted to small spatial domains, and neurons of distinct phenotypes appear to have been generated in overlapping territories. These results suggest that neuronal precursors may be restricted by lineage to the generation of distinct phenotypes from an early stage in the development of the hindbrain and that environmental factors are not the sole determinants of neuronal identity in this part of the CNS.

MATERIALS AND METHODS

Hens eggs were windowed at 24-48 hours of incubation, embryos were made visible by sub- blastodermal injection of India ink (Pelican Fount), and the vitelline membrane reflected over a small area of the hindbrain. Thin-walled aluminosilicate microelectrodes (A-M systems; resistance 25-40 megohms) were tip filled with 100 mg/ml lysinated rhodamine dextran (LRD, Molecular Probes D-1817) or D-3308) or lysinated fluorescein dextran (LFD, Molecular Probes D-1820 or D-3306) and backfilled with either 1.2 M LiCl or 1 M KCl. The electrode was positioned over a selected area of the neural plate or neural tube under bright-field epi-illumination, advanced until a surface potential was recorded, and a cell impaled by briefly rinning the microelectrode with the negative capacitance control. The dye was injected with positive current pulses (250 msec, 4 nA at 2 Hz for up to 30 seconds) and the success of filling a single cell was confirmed by direct observation under low-intensity epifluorescence. After microinjection, a drop of Howard’s Ringer was put on the exposed surface of the embryo, the window closed with adhesive tape and the egg returned to a humidified incubator for a further 1-2 days. After incubation to stage 13-19 (Hamburger and Hamilton, 1951), embryos were fixed in 4% formaldehyde for 2 hours. Hindbrains were either removed and prepared as a ‘kipper’-mount in buffered glycerin (Lumsden and Keynes, 1989) or whole heads were sectioned transversely at 100 µm using a vibratome. Thick vibratome sections and flat, whole-mounted hindbrains, observed from their outer (pial) aspect, were examined under either green or blue excitation on a fluorescence microscope equipped with an image intensifying (SIT) camera or on a laser scanning confocal microscope (BioRad MRC 600) using the 488 nm and 568 nm lines of the krypton/argon laser. Positions and shapes of the labelled cells were recorded from SIT camera images at serial focal planes by tracing onto acetate sheets on the face of the monitor. Confocal fluorescence and bright-field images

Fig. 1. The principal classes of projection neuron in rhombomere 4 of the stage 18 chick hindbrain (data from Clarke and Lumsden, 1993). am, alar plate neurons with medially directed axons; bm, basal plate neurons with medially directed axons; brm, branchiomotor neurons; cam, neurons with ascending projections in the contralateral mlf; cdm, neurons with descending projections in the contralateral mlf; cva, contralaterally migrating vestibulocoustic sensory efferent neurons (arrow indicates direction of cell body migration); iai, neurons with ascending projections in the ipsilateral llt; iam, neurons with ascending projections in the ipsilateral mlf; idl, neurons with descending projections in the llt; idm, neurons with descending projections in the ipsilateral mlf; MLF, medial longitudinal fasciculus. Divisions of the neural tube into alar, basal and floor plates are shown, as is the exit point for the VII/VIIIth nerve.

Fig. 2. Marking single hindbrain cells with fluorescent dextran results in single phenotype clones. All images have been displayed in pseudo colour. (A) A single neuroepithelial cell in situ immediately after iontophoresic injection of lysinated rhodamine dextran (LRD) at stage 10. The three bright spots represent the basal and apical endfeet and the interposed cell body. (B) A ten cell clone at stage 13, 24 hours after marking at stage 9– (6 somites); confocal z-series projection from a transverse 100 µm vibratome section at the level of r4. Nearly all the cells display the columnar morphology characteristic of neuroepithelial cells. Superimposed fluorescence/phase contrast image. (C) A 12 cell clone at stage 14, 48 hours after marking at stage 8 (4 somites); confocal z-series projection from a transverse 100 µm vibratome section. Cells can be seen in various stages of the cell cycle; the large round cell at the ventricular (upper) surface is in M phase, those at the pial (lower) surface are probably differentiating neurons, the remainder are in S and G phases. (D-H) Clones viewed from the pial aspect in whole-mount hindbrain preparations. (D) Clone of am (alar) cells in r3 at stage 19, 58 hours after marking at stage 8+ (5 somites); all of the visible axons, some with visible growth cones, have extended medially and crossed the basal/floor plate border (dashed line). (E) Clone of brm cells (branchial motor neurons) in r5 at stage 17, 48 hours after marking at stage 8+ (5 somites); all of the visible growth cones course laterally, cross the r4/r5 boundary and reach the exit point of the VII/VIIIth nerve (dashed circle). (F) Very large clone of brm cells in r8 at stage 20, 80 hours after marking at the head process stage (stage 5); all axons have a lateral trajectory. (G) A clone of brm cells in r4 at stage 18, 48 hours after marking at stage 11– (12 somites); all visible axons have a laterally directed trajectory towards the VII/VIIIth nerve exit in the same rhombomere. (H) A clone of iam cells (ipsilateral ascending mlf) neurons in r5 at stage 18, 53 hours after marking at stage 8+ (5 somites); all visible axons (arrowheads) enter the ipsilateral mlf. (I) The spatial overlap of diverse phenotypes, revealed by double labelling of motor neurons (green, SC1/DM2 motor neuron-specific antibody) and both cdm and idm cells (red, retrograde trace with LRD from the right side mlf). 75 µm transverse vibratome section through r4 at stage 16, dashed lines highlight the ventricular and pial surfaces of the section (see also Clarke and Lumsden, 1993). Scale bar, 50 µm for A and B; 75 µm for F and I; 100 µm for C, E and G; 125 µm for D and H.
were obtained for each of several focal planes and the positions and shapes of labelled cells were analyzed from individual z-series images.

RESULTS

At the end of the third day of embryonic development (Hamburger and Hamilton stage 20) the chick hindbrain contains a simple set of neuronal phenotypes (Fig. 1) which can be characterised by the axonal pathway chosen by their axons (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993). The main longitudinal axon tract at this time is the medial longitudinal fasciculus (mlf) which lies adjacent to the floor plate and which contains both ascending and descending axons from ipsilateral and contralateral neurons. Those neurons with descending axons probably form the reticulo-lospinal neurons of the more mature brain (Glover and Petursdottir, 1991). More lateral and separated from the mlf by an axon sparse region is the lateral longitudinal tract (llt) which at these stages contains ascending and descending axons almost exclusively from laterally placed ipsilateral neurons. Some neurons at this stage have only short medially directed axons and growth cones. We call these alar circumferential cells (am cells) or basal circumferential cells (bm cells) according to their position. Finally the embryonic hindbrain contains a medial column of motor neurons whose axons run either laterally to exit the brain in the branchial and visceral cranial nerves or exit the brain medially in the somatic cranial nerves.

In order to assess neuronal lineage relationships we marked single hindbrain precursor cells in ovo by microinjection with reliable and unambiguous fluorescent lineage tracers (lysinated fluorescent dextrans, Gimlich and Braun, 1985; Fig. 2A). Most parent cells were marked between stages 6 (0 somites) and 12 (17 somites), but a few were marked as early as the head process stage (stage 5, early in the development of the head notochord). Descendent labelled cells were analysed first by direct observation under fluorescence excitation and subsequently by confocal and intensified video microscopy. Analyses were performed between 24 (Fig. 2B-2C) and 48 hours (Fig. 2D-2H) after marking, when embryos had reached stage 13-20 (Hamburger and Hamilton, 1951). The time interval between marking and analysis represented between 3 and 6 rounds of cell division. The fluorescent dye dispersed throughout the cytoplasm of descendent cells, usually rendering accurate morphological detail of individual neurons and their processes. Neurons were recognised by their fluorescent axons and growth cones. The position of the clone with respect to morphological landmarks, such as the floor plate and rhombomere boundaries, the number of labelled cells, and the number and trajectories of labelled axons could all be visualised by direct observation. The analysis presented here comes from 132 clones in which labelled axons could be unambiguously identified.

Neuronal composition of clones

Neurons were phenotyped according to the axonal pathway chosen by their axons as established by Clarke and Lumsden (1993). Using these criteria we found that the majority of clones (93/132) contained neurons of only a single phenotype. Clones containing only branchiomotor neurons (brm cells, 34/93; Figs 1, 2D-F, 3A) or only alar circumferential neurons (am cells, 33/93, Figs 1, 2G, 3B) were most commonly observed (Table 1). Alar circumferential neurons are relatively young cells whose axons are growing circumferentially towards the ventral midline, they have avoided growing into the llt but have not yet reached the mlf. The next most frequent were clones each containing a single variety of neuron whose axons all ran in one direction within the mlf (iam, idm, cam or cdm cells, 20/93, Figs 1, 2H, 3D-F). Two clones contained only neurons identified as contralaterally migrating sensory efferent neurons of the vestibulocoustic nerve (cva cells, Fig. 3H), and one clone contained only neurons with axons running in a single direction in the lateral longitudinal tract.

A further 22/132 clones contained two or more closely related phenotypes. We describe these phenotypes as closely related because all their axons run in the same major longitudinal tract (either the mlf or the llt) but the direction of growth within these tracts is mixed. As described by Clarke and Lumsden (1993), neurons with axons in the mlf divide into four possible groups according to whether their axons grow in the ipsilateral or contralateral mlf and whether they ascend or descend in these tracts. In 20 of these clones all of the axons have grown in the mlf but in these cases the direction of growth is not uniform. In 16/20 clones only 2 of the 4 possible mlf phenotypes were found, while 3/20 contained 3 and 1/20 contained all 4 of the possible mlf phenotypes; 10/20 contained only ipsilateral axons, 3/20 contained only contralateral axons, and 6/20 contained both ipsilateral and contralateral axons. There was no obvious trend with stage of marking. Two additional clones contained both ascending and descending lateral longitudinal tract neurons (ial and idl cells; Fig. 3F). These 22 clones demonstrate the presence of precursors capable of generating two or more closely related phenotypes.

In only 14/132 clones did the axon trajectories suggest the presence of two markedly distinct phenotypes. Each of these

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**Fig. 3.** Survey of the variety of single and dual phenotype clones. Tracings from the video monitor of clones containing neurons with fluorescent axons, superimposed on a diagram of a stage 19 hindbrain. Examples are shown of each class whose direction of axonal growth suggested either a single neuronal phenotype or dual phenotypes. (A) Branchial motor (brm) neuron-only clones in the basal plate of single rhombomeres. (B) Alar-medial (am) clones in r2 and a clone containing contralateral ascending medial neurons (cam) cells in r5. (C) Clones containing both ascending and descending ipsilateral mlf neurons (iam and idm) cells. (D) Two further iam + idm clones and a iam-only clone in r5. (E) Clone containing both ipsilateral and contralateral ascending mlf (iam and cam) neurons in r5. (F) An idm clone in r1, a clone of cdm cells in r2, a clone of neurons with axons descending in the ipsilateral lateral longitudinal tract (idl) cells in r4, and a clone containing both ascending and descending llt neurons (idal cells). (G) Mixed phenotype clones containing both brm cells and idm cells. The clone in right-side r4 contains 8 brm neurons and 8 idm neurons. (H) Another mixed motor neuron and mlf neuron clone in r5 and a floor plate clone whose neurons project to the VII/VIIIth nerve exit point in r4 (cva cells - vestibulocoustic sensory efferent neurons). (I) Clones with unascribable phenotypes in r4 and the floor plate, together with an am clone in r2 showing an unusual error of axon navigation. a, alar plate; b, basal plate; f, floor plate.
clones contained a mixture of axons running in two distinctly different phenotypic pathways. All of these clones were located in the basal plate and all contained a mixture of branchiomotor neurons and mlf neurons (Figs 3G-H, 5, Table 1). The remaining 5/132 clones contained neurons with unascrivable or aberrant phenotypes (Fig. 3I).

**Patterns of division**

Given the 48-hour survival period after cell marking there would have been maximally six rounds of mitosis (assuming a cell cycle time of 8 hours; Guthrie et al., 1991). With a pure stem cell pattern of generation this is time to generate only 7 cells (6 post-mitotic neurons and 1 mitotically active stem cell).
With a multiplicative or symmetrical mode of division a maximum of 64 cells can be generated. In fact clone size varied widely from 1 to 46 cells (Fig. 4), the average being 16.5 cells, including 7.3 neurons, per clone. This suggests that modes of division are complex at these stages of development, but most of the injected progenitors must have gone through a few rounds of multiplicative division before either generating neurons or switching to a stem cell pattern of division. In addition to recognizable neurons, the majority of the clones also contained cells that lacked labelled processes. In the brightest clones most of these cells could be identified as columnar cells that span the neuroepithelium from ventricular to pial surface and are thus probably mitotically active precursor cells. Other axon-less cells could be young glia, young neurons prior to axon outgrowth, or neurons whose processes contained insufficient dye to be detectable. At the stages analysed, no known markers of glial cells are yet expressed.

Single phenotype clones were obtained from precursors injected as early as the head process stage of development (stage 5, Fig. 5). Neural tube closure in the hindbrain region is complete by about stage 9+ (8 somites) and 45/78 (58%) of precursors injected before this time have generated neurons of only a single type and 21/78 (27%) have generated neurons of only closely related phenotypes.

Motor neuron clones (n = 34) and mlf neuron clones (n = 40) were obtained with similar frequency and these generated almost equal numbers of motor neurons (n = 330, 52.5%) and mlf neurons (n = 299, 47.5%) over this period of development. This suggests that clones are not biased towards single phenotype composition by the relative abundance of one neuronal type over another. To assess quantitatively the non-random nature of the phenotypes found in association here, a simple multinomial test was applied. Using actual frequencies of identifiable types within our aggregate data, we calculated the odds of the clones containing the observed sets of cell phenotypes. Just as it would be possible to calculate the probability of a word of 16 letters containing 8 of the same letters, we determined that the odds of obtaining the associations of phenotype observed here by chance were exceedingly small (Table 2). In these calculations, axonless cells within each clone were taken as being of unknown phenotype.

**Spatial distribution of clonal derivatives**

By using retrograde axonal tracing to mark mlf neurons in combination with antibodies that specifically label motor neurons we have previously demonstrated that these two neuronal types are interspersed with each other within the basal plate (Clarke and Lumsden, 1993; Fig. 2I). In support of this, branchiomotor-only clones and mlf neuron-only clones show considerable spatial overlap when superimposed (Fig. 6 and compare position of motor neuron clones in Fig. 3A with mlf neuron clones in Fig. 3C-E). It is not clear how this overlap arises, however. It may be that early precursors dedicated to the production of distinct neuronal types are indeed interspersed at an early stage (Fig. 7A), or undedicated, multipotent precursors may initially occupy distinct spatial domains but this separation is then lost by subsequent intermixing of
neuronal lineage in the chick hindbrain

either late, and now dedicated precursors or the neurons, or both (Fig. 7B).

**DISCUSSION**

In some parts of the vertebrate CNS the determination of neuronal phenotype appears to be independent of lineage (Galileo et al., 1990; Gray and Sanes, 1991; Leber et al., 1990; Wetts and Fraser, 1988; Holt et al., 1988). In related studies, grafting and ablation experiments (Yamada et al., 1991; Goulding et al., 1993) on the early spinal cord have demonstrated a critical role for both cell-cell interactions (Placzek et al., 1993) and diffusible factors (Yamada et al., 1993; Basler et al., 1993) in establishing the spatial organisation of neuronal pattern. In this paper we present evidence that during the early development of the chick hindbrain most neuronal precursors produce neurons of only a single phenotype, and that clones containing a particular neuronal type can be spatially mixed with clones containing a different neuronal type. This result suggests that local environmental signals alone may be insufficient to account for the determination of neuronal phenotype in the hindbrain and a lineage-dependent mechanism may be engaged for at least part of the early development of this region (Fig. 7).

Our analysis has been restricted to the first 48 hours of neuronal development in order to examine the manner of generation of cell phenotypes identified and mapped up to stage 20 (Clarke and Lumsden, 1993). This early stage of neurogenesis is marked by tangential clone growth and the production of an essentially two-dimensional sheet of neurons in the outer (pial) region of a mitotically active neuroepithelium. Later neurogenesis involves a transition to radial clone growth (Hemond and Glover, 1993) as the mantle layer undergoes rapid thickening. Although well suited to the study of early clones, our method of lineage-tracing by direct dye injection would not allow clones to be followed into later stages, on account of dye dilution by further cell division and increase in neuronal volume. At stage 17-20, the large majority of clones also

**Table 2. Statistics: a simple polynomial test of proportions has been applied for the first 100 clones we obtained (see text)**

<table>
<thead>
<tr>
<th>Population ratio in the basal plate:</th>
<th>271 brm (0.53)</th>
<th>143 idm (0.28)</th>
<th>97 cdm (0.19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>for 4/4 idm cells in 1 clone</td>
<td>0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>for 8/8 brm cells in 5 clones</td>
<td>2x10^-24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>for 28/28 cdm cells in 1 clone</td>
<td>6x10^-21</td>
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(2) Considering all clones containing both neurons and non-neuronal (axon-less) cells, what is the probability that \( n \) of these cells would be neurons of only a single type?

- for brm clones: \( 2.9 \times 10^{-70} \)
- for idm clones: \( 5.8 \times 10^{-34} \)
- for cdm clones: \( 8.0 \times 10^{-39} \)

**Fig. 6.** Spatial overlap of distinct single-phenotype clones. Tracings from the video monitor of clones in the same position in the hindbrains of animals marked and recovered at identical times. Floor plate (borders shown dashed) and rhombomere boundaries have been superimposed. A shows an mlf neuron clone (black) interspersed in r2 with a brm clone (unfilled), some of whose cells (light grey) lie in the floor plate; both clonal progenitors were marked at stage 9- (6 somites) and the animals killed at stage 18. B shows a cdm clone (black) interspersed with a brm clone (neurons, unfilled; floor plate cells, light grey) in r4; both clonal progenitors were marked at stage 8- (5 somites) and the animals killed at stage 18. fp, floor plate; bp, basal plate.
This intermixing arises. It may be that differently fated precursors may subsequently be lost at the time of analysis by later intermixing of descendant, now determined precursors, neurons or both (Fig. 7B). A critical evaluation of these alternatives requires a more precise analysis of the distribution and movements of both precursors and recently born neurons throughout the 48-hour period of clonal expansion examined in this study.

The factors that initially allocate the precursors into distinct sets are likely to include interactive signals arising from the dorsal (roof plate) and ventral (floor plate and notochord) midline (Yamada et al., 1991, 1993; Basler et al., 1993). Once established, however, we suggest that precursors may become refractory to environmental signals for several rounds of division; thus precursor specification is stabilised despite migration and intermixing. This contrasts with the development of other regions of the nervous system, where a single parent cell produces a variety of neuronal phenotypes and the fates of cells must be decided, presumably by cell-cell and environmental interactions, later in development. The early hindbrain may employ an alternative strategy as a consequence of other features of its developmental programme, namely the early stabilisation of cell position by rhombomere boundaries (Fraser et al., 1990) and the early determination of regional fate along the anteroposterior axis (Guthrie et al., 1992). The independence of rhombomere fate from its local environment and the mechanisms that impose this regional autonomy could also render individual neuronal precursors refractory to environmental influences.

Clones containing distinct or related phenotypes
A minority of clones contained two quite distinct neuronal phenotypes (i.e. branchiomotor neurons and mlf neurons, n=14/132; Table 1) and a further group, of mlf neuron-only clones that we have described as containing closely related phenotypes, since they contained a mixture of ascending and descending and/or ipsilateral and contralateral projections (n=20/132). The former group of clones are evidence that the developmental state of a few precursors remains labile despite the apparent fate-restriction of many of their neighbours, and the second group suggests that the determination process may proceed in two steps; first, the decision is made to project into the mlf rather than become, say, a motor neuron; later, for the mlf neuron, the more specific choice of direction is made. Since 50% of the total 40 mlf neuron-only clones contained axons growing in different directions, this aspect of phenotype may be determined by factors other than lineage.

Early cell fate determination would be reminiscent of the cell lineages characteristic of invertebrate development (Doe et al., 1985; Taghert and Goodman, 1984; Witten and Truman, 1991; Doe and Technau, 1993), and strengthens the possibility that the hindbrain region retains ancestral developmental strategies. One explanation for this is that early fate determination and amplification by multiplicative division may provide a reliably rapid means of generating the diversity and necessary numbers of cells to establish the early functioning circuitry of the region, establishing the basic scaffold of neuronal organisation upon which later complexity can be built. In contrast to the predominantly circumferential growth of early neuronal clones, later clone growth is radial; following

Fig. 7. Two possible lineage tree diagrams showing the development of distinct yet interspersed single phenotype clones. Circumferential (dorsoventral) position within the neural tube is represented by left-to-right position across the page. In A, precursor cells are specified early; their progeny disperse and intermix with the progeny of other, differently specified precursors. In B, early precursors are unspecified and mixing is restricted until a final late stage following commitment, when the young neurons disperse widely and mix with those of a distinct positional origin. Not all cells are necessarily postmitotic neurons at this stage. In this model, early wide-spread dispersal would result in dual-phenotype clones.

contain non-neuronal cells. At least some of these cells have the morphology of neuroepithelial cells and may be neuronal precursors as ventricular cells marked with retroviruses at stage 17 do produce neurons (Hemond and Glover, 1993). Thus, the presence of non-neurons in our clones allows for the possibility that an early uniform composition (at stage 17-20) could later acquire heterogeneity.

The interspersion of different single phenotype clones raises the interesting possibility that their respective precursors had been specified at least four cell divisions earlier. In general, the existence of single phenotype clones cannot be taken as evidence for irreversible commitment, the test for which requires moving the cell to an alternate environment. Although moving single cells by surgical means within the chick neuroepithelium has not yet been successful, the interspersion of differently fated clones reported here may represent a natural transposition experiment. However, we do not yet know how this intermixing arises. It may be that differently fated precursors are intermixed from an early stage in development (Fig. 7A). Alternatively, an early spatial segregation of differently fated precursors may subsequently be lost at the time of analysis by later intermixing of descendant, now determined precursors, neurons or both (Fig. 7B). A critical evaluation of these alternatives requires a more precise analysis of the distribution and movements of both precursors and recently born neurons throughout the 48-hour period of clonal expansion examined in this study.

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this transition, the hindbrain may utilize a more conventional strategy of neurogenesis with cell type-determination depending on radial position (Hemond and Glover, 1993).

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