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Different clonal dispersion in the rostral and caudal mouse central nervous system

Luc Mathis* and Jean François Nicolas‡

Unité de Biologie moléculaire du Développement, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France
*Present address: Beckman Institute (139-74), California Institute of Technology, Pasadena, CA, 91125, USA
‡Author for correspondence (e-mail: jfnicola@pasteur.fr)

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SUMMARY

We have performed a systematic clonal analysis to describe the modes of growth, dispersion and production of cells during the development of the mouse neural system. We have used mice expressing a *LaacZ* reporter gene under the control of the neuron specific enolase promoter to randomly generate LacZ clones in the central nervous system (CNS). We present evidence for (1) a pool of CNS founder cells that is not regionalized, i.e. give descendants dispersed along the entire A-P axis, (2) an early separation between pools of precursors for the anterior and posterior CNS and (3) distinct modes of production of progenitors in these two domains. More specifically, cell growth and dispersion of the progenitors follow a relatively coherent pattern throughout the anterior CNS, a mode that leads to a progressive regionalization of cell fates. In contrast, cell growth of progenitors of the SC appears to involve self-renewing stem cells that progress caudally during regression of the mode. Therefore, at least part of the area surrounding the node is composed of precursors with self-renewing properties and the development of the trunk is dependent on pools of stem cells regressing from A to P. Taken together with our analysis of the cell growth changes associated with neuromere formation (Mathis, L., Sieur, J., Voiculescu, O., Charnay, P. and Nicolas, J. F. (1999) Development 126**, 4095-4106), our results suggest that major transitions in CNS development correspond to changes in cell behavior and may provide a link between morphogenesis and genetic patterning mechanisms (i.e. formation of the body plan).**

Key words: Cell lineage, Central nervous system, Clonal analysis, Brain, *laacZ*, Mouse embryo, Neural tube, Spinal cord, Stem cell

INTRODUCTION

Key patterning events for the regional organisation of the vertebrate central nervous system (CNS) occur during the longitudinal development of the neural plate and neural tube. In the mouse embryo, early signals for forebrain development are provided by the anterior visceral endoderm (Beddington and Robertson, 1999), and the first regionalised expression of genetic markers in the neuroepithelium (e.g. otx-2) is established at the early primitive streak stage (E6.5) (Rhinn et al., 1998; Varlet et al., 1997). Inductive signals emanating from the node (Bouwmeester et al., 1996; Ruiz i Altaba, 1994; Selleck and Stern, 1992; Stern et al., 1995; Storey et al., 1998) the pre-chordal plate (mesendoderm) (Pera and Kessel, 1997) and the paraxial mesoderm (Muhr et al., 1999; Woo and Fraser, 1997) are also involved in the early A-P patterning of the CNS. Morphological regionalization of the neural tube becomes evident with the subdivision of the longitudinal axis into four major domains: the prosencephalon, the mesencephalon, the rhombencephalon and the spinal cord (SC). The neuroepithelium of these subdivisions is then patterned into smaller gene expression domains, which define a segment-like neuromeric organization (Figdor and Stern, 1993; Fraser et al., 1990; Puelles and Rubenstein, 1993; Rubenstein et al., 1994).

Whereas accumulating evidence suggests a progressive genetic regionalization of the anterior and posterior neural plate (Lumsden, 1990; Lumsden and Krumlauf, 1996; Rubenstein and Beachy, 1998), little information is available concerning the cellular behavior underlying the morphogenesis of the CNS. In the early primitive streak-stage embryo (E6.5), neurectoderm precursors are found at the distal tip of the epiblast, anterior to the node, and the descendants of these cells then display a widespread axial distribution. They contribute to the anterior axial ectoderm as well as to the anterior lateral part of the primitive streak (Lawson et al., 1991). At the late primitive streak stage (E7.5), the precursors of the different CNS territories (SC, rhombencephalon, mesencephalon and prosencephalon) are roughly situated according to their future order (Tam, 1989).

Although very valuable, these fate maps give limited information on how the early regionalization of CNS precursors is established. There remain numerous questions regarding the mechanisms involved in CNS regionalization: are the different regions produced by the progressive subdivision of an initial field or are they sequentially added during a polarised growth? Is the formation of the cellular domains uniform along the longitudinal axis? What is the relative importance of growth patterns, the restriction of cell mingling

and clonal boundaries in these processes? Answering these questions is crucial to our understanding of CNS morphogenesis because it will help to determine whether specific genetic events are associated with these different modes of regionalization.

We have performed a systematic clonal analysis to describe cell behavior during the formation of the mouse neural tube (Mathis et al., 1997, 1999). In this method, a β-gal$^+$/β-gal$^-$ mosaicism in somatic cells is initiated by spontaneous homologous recombination between the duplicated sequences in laacZ, resulting in the restoration of a functional *lacZ* gene that is clonally transmitted to the progeny of the labeled precursor cell (Bonnerot and Nicolas, 1993; Eloy-Trinquet et al., 1999). The use of the neuron specific enolase (NSE) promoter (Forss-Petter et al., 1990; Mathis et al., 1997, 1999) allows to visualize descendants of these precursors in the CNS. In the present study, we analyse the modes of A-P growth and cell dispersion in the CNS.

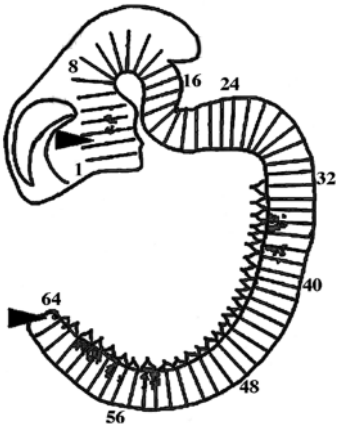

Fig. 1. Description of the clones. The position of β-gal$^+$ cells in the clones is described in relation to the 64 subdivisions of equal length in the neural tube. Clone 95-716 is taken as an example. The most anterior border is subunit 4, and the most posterior border is subunit 64 (arrowheads). The longitudinal extension, L=61.

MATERIALS AND METHODS

Production of β-gal$^+$ cells in the embryos

The embryos from crosses between homozygous *NSE-LaacZ* males (from the NSE-1 transgenic line, Mathis et al., 1999) and *C57BL/6×DBA/2* wild-type females were analysed at embryonic day E12.5. The morning of copulation is taken as E0.3. After 30 minutes of fixation in 4% PFA, the embryos were washed and incubated in X-gal staining solution for 2 days at 30°C, then for 1 week at 4°C. The embryos were then cleared for 48 hours in 70% glycerol in PBS (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.3) and bilaterally sectioned. The screen for embryos with cells was performed by observation of the embryos at 60× magnification (Mathis and Nicolas, 1998).

Description of the clones

For the description of the clonal distribution, the brain stem was longitudinally subdivided into 64 arbitrary units with an axial extension of 200 μm (Fig. 1). In the prosencephalon, these units were defined using the longitudinal organization defined in the prosomeric model (Rubenstein et al., 1994). These units do not strictly correspond to neuromeres. The prosencephalon extends from divisions 1 to 9, mesencephalon from 10 to 14, rhombencephalon from 15 to 24, spinal cord from 25 to 64; forelimbs correspond to subdivisions 34 to 39 and hindlimbs to 50 to 55 (Fig. 1). The position and numbers of β-gal$^+$ cells in the clones (Fig. 2) were recorded with a camera lucida drawing (Fig. 3). Each clone was characterized by three geometrical parameters, its anterior border (anteriormost subdivision to which they participate), its posterior border and its longitudinal extension, L. Because lineage analyses suggest a progressive limitation of global cell movements (Mathis et al., 1999; Tam et al., 1997; this study), L is a parameter that is probably related to the date of birth of the clones. The participation of labeled cells to the subdivisions was recorded from the drawings of 163 clones. Independent descriptions gave rise to maximal differences of 1 or 2 subdivisions in the determination of the location of labeled cells. The numerical descriptions of the clones were treated using EXcell.

Nomenclature

In this article, we define the ancestor cells of a structure (the CNS, the brain, the D-V clusters, etc.) as any cell that will contribute at least some descendants to this structure. Founder cells of a structure are defined as the first cells of a lineage whose descendants will preferentially populate that structure. Therefore, founder cells exhibit clonal growth, whereas ancestors to founder cells are characterized by non-clonal growth within the structure (Eloy-Trinquet et al., 1999 and references therein). We define coherent growth as a mode of growth where cells remain close after division; coherent intermingling is a mode of growth where cells intersperse but only with their closest neighbours. Finally, growth is called dispersive when daughter cells are widely separated after division; this can occur due to absence of coherence or through a self-renewing stem cell system progressing in a polarized way (Nicolas et al., 1996). For a given domain, restricted clones are those that give descendants only in that domain. Non-restricted clones give descendants into other domains. Polarized clones (towards caudal for instance): group of clones with posterior borders in the caudalmost domain and anterior borders at variable axial levels.

RESULTS

Production and description of β-gal$^+$ clones in the E12.5 mouse CNS

In NSE-1 E12.5 embryos, the *laacz* transgene is strongly expressed along the entire CNS and, at this stage, expression includes the ventricular zones; in the spinal cord, expression is mainly in the ventral tube (Mathis et al., 1999). We harvested 163 CNS exhibiting β-gal$^+$ cells from a total of 3000 E12.5 embryos (Fig. 3). Embryos that exhibited labeling restricted to the telencephalon, including the lateral and medial ganglionic eminences, were excluded from this analysis. The low frequency of labeling in the CNS verifies that most of the β-gal$^+$ cells derived from a single recombination event, since the probability of more than one recombination event in the same embryo is about 2.5×10^{-3} .The position of β-gal$^+$ cells along the CNS was scored according to their participation in each of 64 transverse subdivisions (Fig. 1). We have analyzed the clonal pattern based on two different representations: by their position along the A-P axis in relation to their longitudinal extension (Fig. 4), which gives a general idea of the genealogical history of the structures, and by the relationship between their anterior and posterior clonal borders (Fig. 5), which allows for direct grouping of clones according to their

dispersive properties. Figs 4 and 5 show that each axial subdivision is populated many times by clones possessing the highest longitudinal extension (L>32), as well as by smaller clones. Therefore, all axial levels can be analyzed with our collection of clones.

Two major phases in the formation of the CNS

Several clones exhibited β-gal$^+$ cells distributed along the entire A-P axis, from the prosencephalon and/or mesencephalon to the caudal part of the spinal cord (Fig. 2A-C and Fig. 3). These very long clones are clearly identified as a group in Fig. 5 (clones in the L>42 region). There are 11 such clones whose longitudinal extension is between 64 and 45. The very long clones show that a pool of CNS precursor cells gives rise to descendants dispersed along the entire longitudinal axis. This observation reveals a period of development during which CNS precursor cells undergo widespread longitudinal clonal dispersion.

The very long clones were either bilateral (6/11) or strictly unilateral (5/11) (Figs 2A-C, 3A, 4; clones in purple or in black, respectively). The observation of very long unilateral clones (Figs 2A, 3C) suggests that bilaterality could be mainly due to precursor position in the neural plate and that despite the widespread A-P cell dispersion, some constraints act on cell movements and dispersion.

Another striking observation is that labeled cells in the CNS are distributed into small separate clusters (Figs 2, 3). These clusters are oriented D-V in the spinal cord, the rhombencephalon and the mesencephalon (Fig. 3), and their orientation is more random in the prosencephalon. The smallest clones had the characteristics of these clusters, in particular concerning their D-V orientation and cell numbers, suggesting that the clusters define subclones within the large clones (Mathis et al., 1999). To further test this point, we analyzed the A-P dimension of the D-V clusters from the different domains of the CNS (Pros-, Mes-, Rhombencephalon and SC) by measuring their participation to the 64 subdivisions of the neural tube. Among these different domains, D-V clusters participated in similar numbers of subdivisions within clones containing a single labeled cluster or several separate clusters that were either distributed into only one of these domains (restricted clones) or dispersed into other domains (non-restricted clones) (Fig. 6). This observation strongly suggests that D-V clusters define subclones within the larger clones.

If D-V clusters are clonal sub-units, then it is possible to define the founder cells of these units as the first cell in a lineage whose descendants will all populate a D-V cluster. The ancestors of the D-V clusters are the precursors that give rise to more than one D-V cluster (see Materials and Methods for nomenclature). We have tried to identify the class of clones corresponding to the ancestors of the D-V clusters. In the spinal cord, rhombencephalon and mesencephalon, more than 95% of the D-V clusters were distributed in fewer than two consecutive subdivisions; and in the prosencephalon, 83% of the D-V clusters populated fewer than two consecutive subdivisions (Fig. 6). The higher A-P dispersion of clusters in the prosencephalon is due to the variability in their orientation (Fig. 3D-G; see also Mathis et al., 1999), whereas our subdivisions have been chosen with a D-V orientation. Therefore, most of the clones with L≤2 give rise to a single D-V cluster, whereas the majority of clones with L>2 give rise to at least two D-V clusters. These data support the notion that the clones with L>2

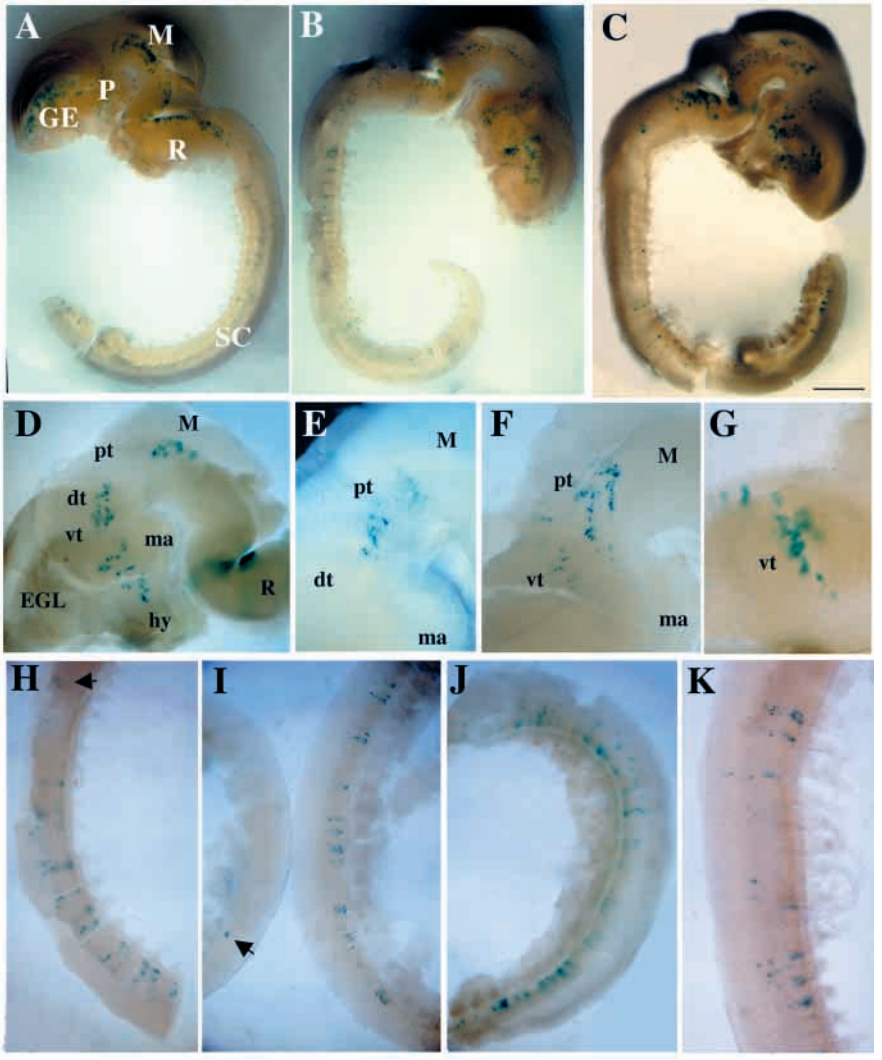

Fig. 2. Clonal cell arrangement in very long and long lacZ clones. (A-C) Examples of clones dispersed along the entire CNS. (A,B) Clone 94-906, very long bilateral clone. (C) Clone 95-534, very long unilateral clone. Note the organisation in separate clusters of β-gal$^+$ cells. (D-G) Examples of clones in the brain. (D) Clone 95-918.2. (E) Clone 94-926.1. (F) Clone 95-516.1. (G) Clone 95-717. (H-K) Examples of long clones dispersed along the SC. (H) Clone 94-850. β-gal$^+$ cells located in the anterior SC are indicated by an arrow. (I) Clone 94-901.2, bilateral clone (contralateral cells indicated by the arrow). (J) Clone 95-882.2. (K) Clone 94-928. SC, spinal cord; R, rhombencephalon; M, mesencephalon; P, prosencephalon; GE, ganglionic eminences; pt, pretectum; dt, dorsal thalamus; vt, ventral thalamus; ma, mamillary area; Hy, hypothalamus. Bar, 800 μm (A-C).

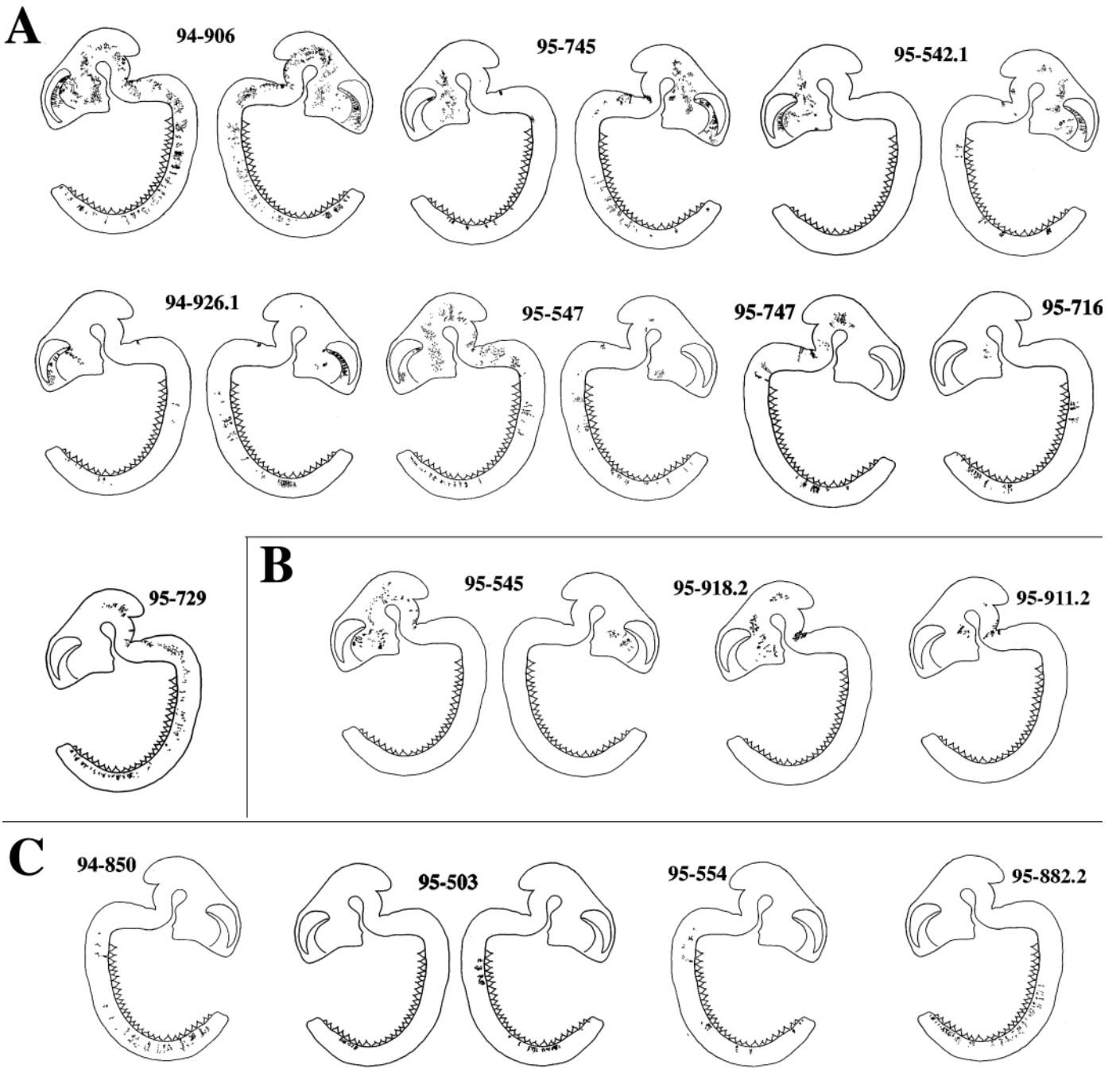

Fig. 3. Camera lucida drawing of the distribution of β-gal$^+$ cells in representative very long and long clones distributed along the entire CNS (A), the brain (B) and spinal cord (C). The number on top corresponds to the identification of the clones.

correspond to the labeling of ancestors of D-V cluster founders, because these clones derive from the labeling of precursors that give rise to more than one D-V cluster.

The cellular arrangement of the clones into separate D-V clusters identifies two major phases in the formation of the CNS: an initial phase of longitudinal dispersion of ancestors to the D-V cluster founders, followed by local growth of the descendants of the founders to produce the D-V clusters. We have estimated the number of D-V clusters in the 11 clones that populate the entire A-P axis. The mean value of 36 D-V clusters (s.e.m. ±6) (Table 1) suggests that about 5-6 cell generations separate founder cells of the CNS from the D-V cluster founders.

The remainder of our analysis focuses on the modes of growth and dispersion and production of the descendants of the CNS founder cells, which correspond to the ancestors of the founders for the D-V clusters during this period of 5-6 cell generations.

Different clonal patterns in the anterior and posterior neural system

Examination of the data presented in Fig. 5 suggests that a

Table 1. Number of DV clusters in very long clones

Number of DV clusters per clone	<16	16-31	32-63	64-127
Number of clones	0	7 (16, 17, 19, 21, 28, 29, 31)	3 (33, 50, 57)	1 (92)
Number of cell generations (n)	n<4	4≤n<5	5≤n<6	6≤n<7

We determined the number of DV clusters in the 11 very long clones distributed along the entire CNS. Clones are arranged in classes of exponentially increasing numbers of DV clusters to match a linear increase of cell generations.

The numbers in parentheses are the individual values of the number of DV clusters per clone. The mean value is 36±6 (±s.e.m.), which indicates that 5≤n<6.

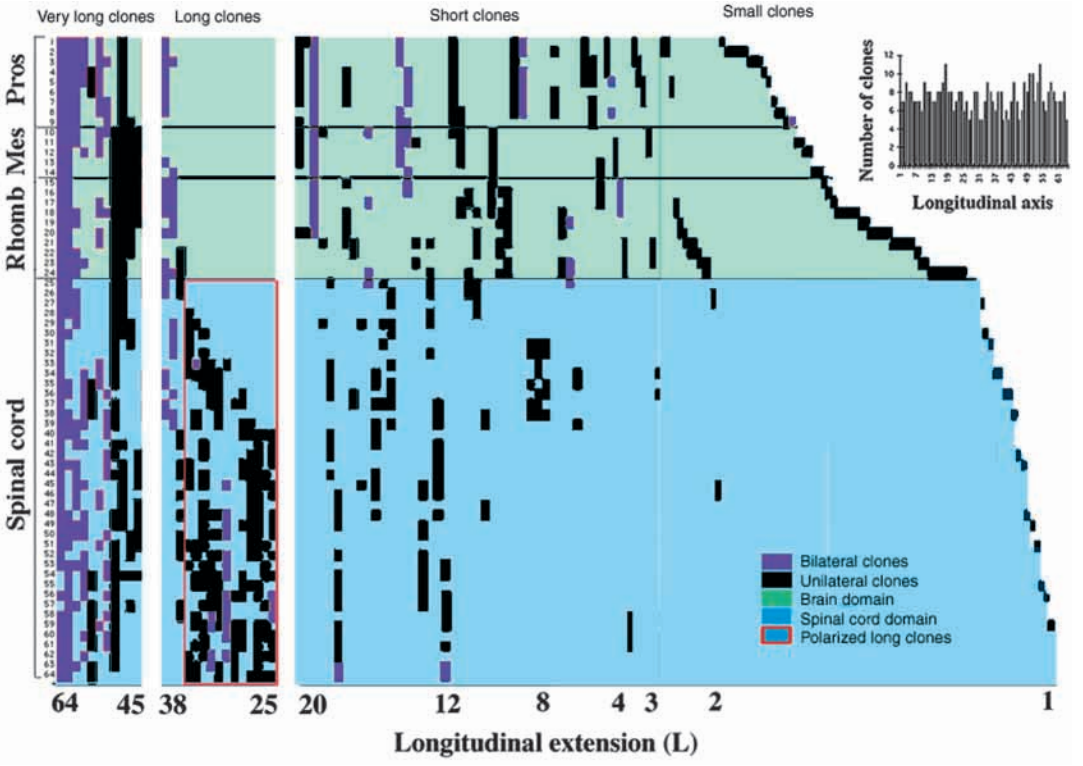

Fig. 4. Representation of the pattern of the 163 clones by their longitudinal extension. Clones with the same length are arranged by their anterior position along the longitudinal axis. Each clone (x axis: a single vertical column represents a clone even if bilateral) is represented by its participation to the 64 subdivisions (y axis). Inset: number of times (y axis) the different subdivisions (x axis) are populated by clones of L>32.

group of clones (represented in red) participates systematically in the most caudal part of the SC. Furthermore, these clones (delimited by the red rectangle in Fig. 4) are restricted to the SC and exhibit A-P polarity, i.e. the position of their anterior border correlates with their longitudinal extension (Fig. 4). To assess the statistical reality of this group of clones, we calculated that in a uniform distribution of long clones along the A-P axis, only 27% of them are expected to be restricted to the SC, whereas the observed value is 80% (n=12/15). The expected value was estimated by the ratio of the surface, defined by 21<L<42 and an anterior border above 25 (Fig. 5 hatched blue triangle), to the surface defined by 21<L<42 (Fig. 5 whole hatched domain).

To extend this observation, we compared the frequencies of longitudinal extension of clones that give rise to the descendants in the brain and/or SC (Fig. 7). To take into account the difference in the size of the two domains, we compared the observed values (Fig. 7A) to the values expected if the clonal distributions were random, calculated as above by the ratio of the surfaces of the different domains in Fig. 5 (Fig. 7B). The ratio between these values clearly shows that the anterior (brain) and the posterior (SC) neural system are populated in different manners by clones of different L values (Fig. 7C). The anterior is populated mainly by very long clones (L>42) and by short clones (2<L<21). In marked difference, the posterior is frequently populated by long clones. Therefore, we conclude that the mode of growth and/or dispersion are different in the anterior and posterior neural system.

Different modes of growth in the anterior and posterior neural system

We next analyzed the basis for this difference. To facilitate the numerical analysis of growth and dispersion, it is helpful to present the patterns of clones and the distribution of longitudinal extensions expected with the LaacZ/LacZ labeling system, in which each precursor cell has the same probability of being labeled. The main possible models are represented and detailed in Fig. 8. This analysis predicts that (1) different shapes of cell distribution for exponentially increasing longitudinal extensions are obtained in an ordered mode of growth (Fig. 8C) or through a dispersive (Fig. 8B) or stem cell mode (Fig. 8D) and (2) the shape of the distribution of longitudinal extensions is independent of both the rate of intercalation and the number of cell generations.

Application to cell dispersion in the anterior neural system

The brain is populated by clones of all different L values (Fig.

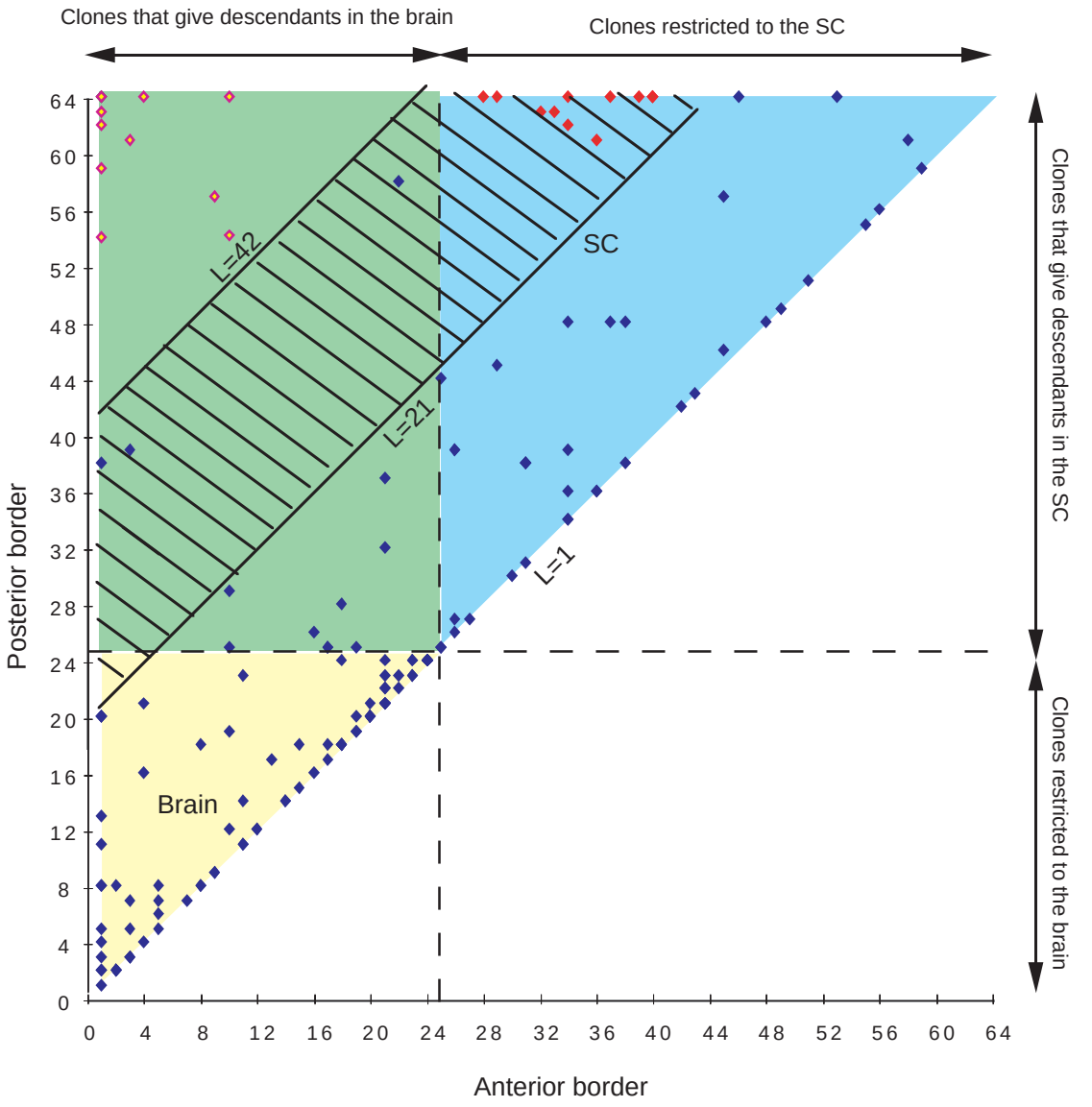

Fig. 5. Dispersive properties of the clones. Representation of the 163 clones by the value of their anterior (*x* axis) and posterior (*y* axis) borders. Note that when more than one clone have the same anterior and posterior borders they appear as a single dot. Clones with the same length (L) are arranged along the *y*=*x* axis. The lines L=21 and L=42 delimit very long clones (L≥42, open purple dots), long clones (21<L<42) and short clones (2<L<21). Note that most of 21<L<42 clones are restricted to the SC (clones in red). The horizontal (same posterior border) and vertical (same anterior border) lines define the limits of axial domains: clones with *P*<a posterior border value below 25 are restricted to the brain (in yellow) and clones with an anterior border above 25 are restricted to the SC (in blue). Clones that participate to both brain and SC are in green.

4, green domain), and we observed a decrease of the frequency of clones with exponentially increasing longitudinal extensions (Fig. 9A). These observations are inconsistent with cellular modes involving either dispersive cell intermingling (Fig. 8B) or self-renewing stem cells (Fig. 8D). The observed distribution of L values (Fig. 9A) is only expected in an ordered intermingling mode, in which cells can rearrange only with their closest neighbors (Fig. 8C). This mode of precursor dispersion was found in the caudal (rhombencephalon) and rostral (mesencephalon and prosencephalon) sub-domains of the brain (Fig. 9B-C). Higher frequencies of small clones (L<2) were observed in the r2-r7 area than in more rostral levels (Fig. 4). This is possibly related to a higher coherence of growth among descendants of the D-V cluster founders in this segmented domain of the CNS. Together, these data suggest a mode of relatively coherent intermingling of brain precursors.

Application to cell dispersion in the posterior neural system

The SC was frequently populated by long clones, whereas shorter clones were much less frequent (Figs 4, red rectangle, and 5, clones in red). This lack of short clones (Fig. 7C) and the high frequency of long clones compared to shorter clones (Fig. 9D) are expected in only two modes: a self-renewing stem cell mode and a dispersive intermingling mode (Fig. 8B,D). The long clones (L>21) restricted to the SC all participate in the posterior SC, contribute with decreasing frequency to more anterior levels of the SC (Figs 4, red rectangle, and 5, clones

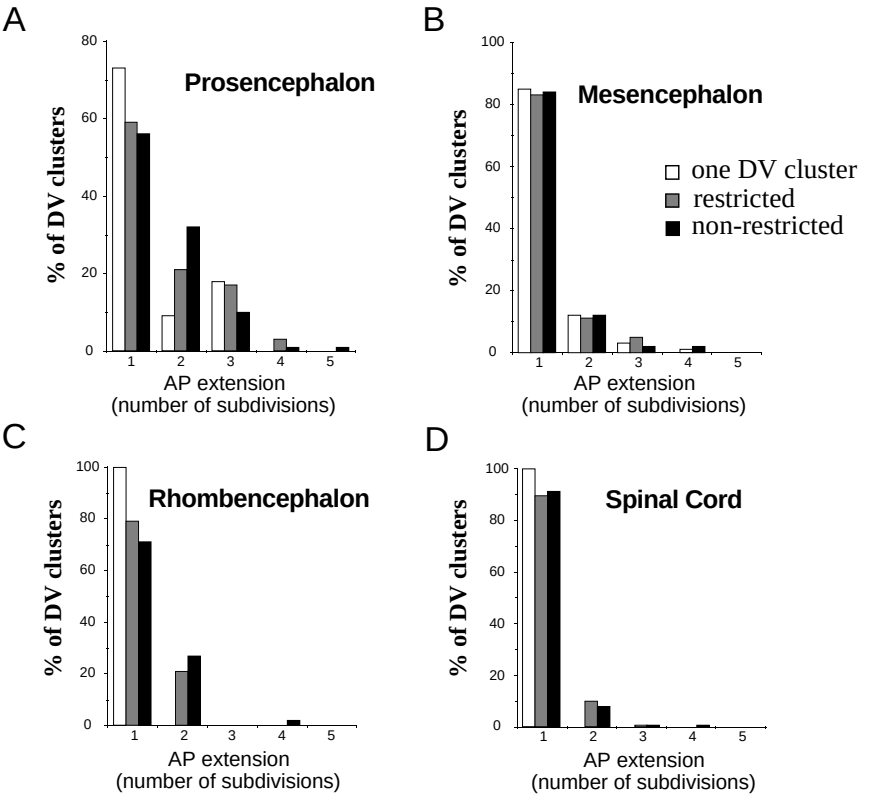

Fig. 6. Percentage of the number of D-V clusters (*y* axis) participating in consecutive subdivisions (*x* axis) in the different A-P domains of the CNS. The calculations were made for clones with a single D-V cluster and for clones restricted and non-restricted to these different A-P domains of the CNS.

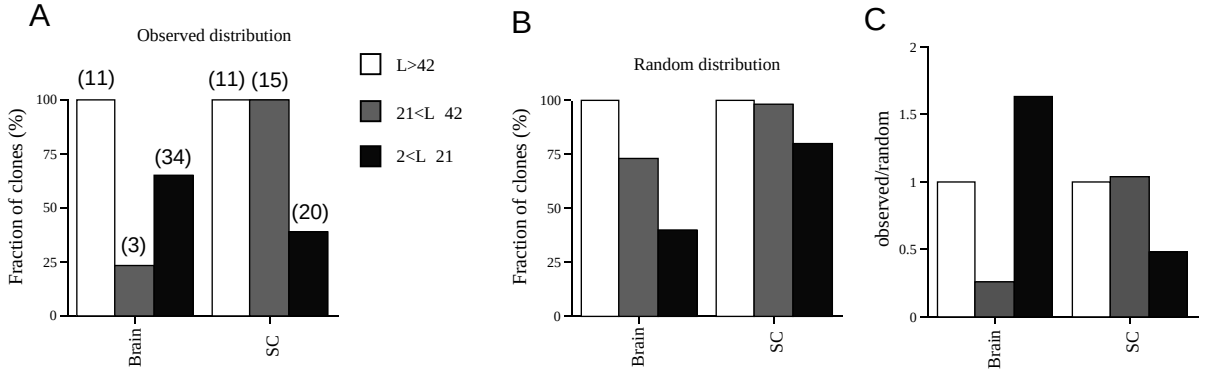

Fig. 7. Distribution of the very long, long and short clones in the SC and brain. (A-B) Fraction (%) of the number of clones of each category that give rise to descendants in the brain or SC. (A) Observed distributions. The individual numbers of clones are given in parentheses. The total number of clones in each class are: 11 very long clones (L≥42), 15 long clones (21<L<42) and 52 short clones (2<L<21). (B) Theoretical values, if the clones in each class were distributed at random along the A-P axis (calculated from the ratio of surfaces in Fig. 5). (C) Ratio of A to B.

in red), and present a direct relationship between their length and their anterior border (Fig. 9F), suggesting a polarity of cell dispersion in the SC. In a self-renewing cell mode, cell dispersion is polarized and, in addition, the clonal complexity of long clones (the number of times a given axial domain is populated by clones) is expected to increase along with the progression of the self-renewing cell pool (Fig. 8D). We observed that the clonal complexity of the 12 long clones (L>21) restricted to the SC increases significantly along the SC from A to P (Fig. 9E). These observations strongly suggest that the A-P dispersion of SC precursors is due to a rostral-caudal progression of a permanent pool of self renewing stem cells.

In conclusion, we deduce that two different modes of epithelial growth are at work along the CNS. Cell dispersion is ordered (meaning that clonal coherence is high) in the anterior CNS, whereas cell dispersion appears to be due to the A-to-P progression of a pool of self-renewing stem cells in the posterior CNS.

A unique pool of cells at the origin of the CNS?

We have examined the possibility that the pools of precursors restricted to the anterior and posterior CNS derive from a pool of precursors that give rise to descendants dispersed throughout the CNS. The random nature of the labeling event

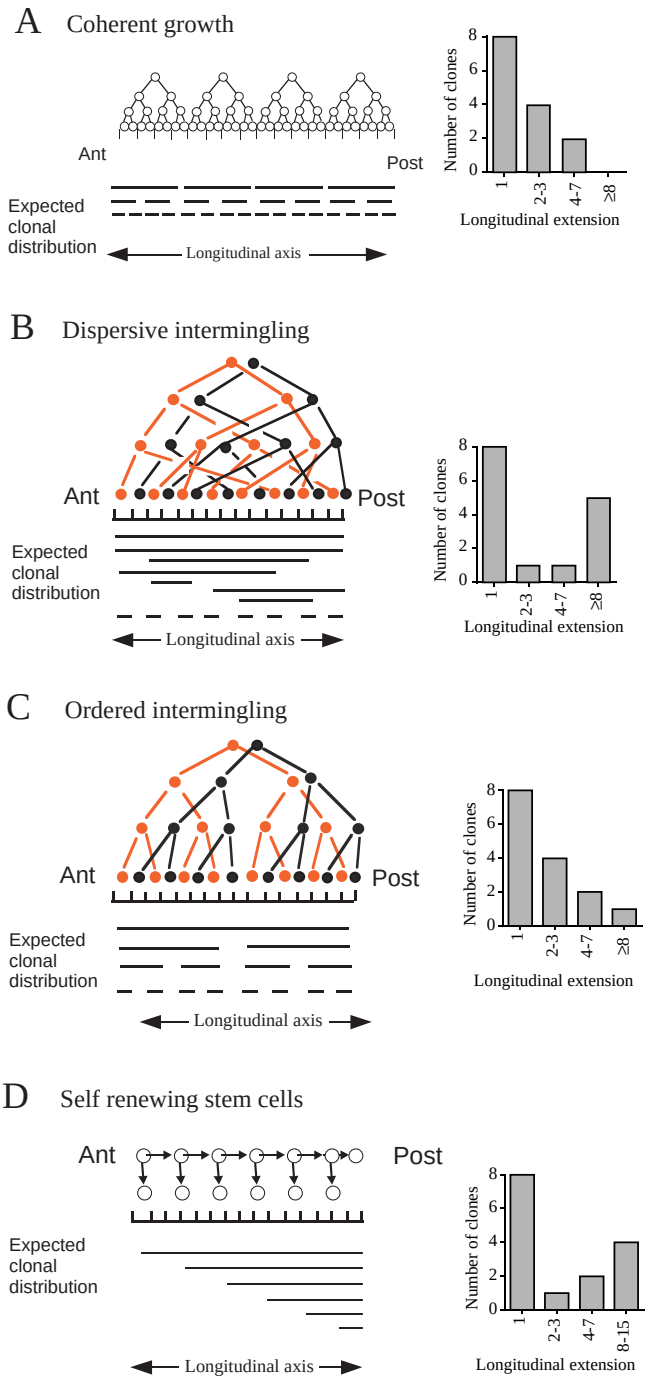

Fig. 8. Models of growth and dispersion of the precursors of a longitudinal structure. Growth in a single dimension (longitudinal axis formation) can be described by a few general characteristics, including: (1) The origin of the epithelium, which can be a single progenitor (monoclonal origin) or a pool of precursors (polyclonal origin). In the mouse, embryonic structures have a polyclonal origin. (2) The mode of precursor recruitment, which can occur in a restricted (closed) precursor pool or in an unrestricted (open) pool, if some precursors have a separate origin. We have assumed that there is no external recruitment. (3) The mode of cell division, which can be symmetrical (proliferative mode) or asymmetrical (stem cell mode). (4) The mode of cell dispersion, which may or may not involve cell intermingling. Assuming a polyclonal origin and a closed precursor pool, the growth of an epithelium can be entirely described by a few models reflecting the different modes of cell division and dispersion. In each model, a schematic lineage tree of the relationship between cells and the expected clonal distributions if each precursor is labeled once are shown. The expected distributions of L values, represented on the right, have been calculated for a fixed number of clones (n=15), which correspond to three symmetrical cell divisions of a single precursor. In these different models, if the number of cell generations increases or, if more unrelated cells intercalate between clonally related cells, longer clones are expected but the shape of the distributions remains the same. (A-C) Production by proliferative mode. (A) Coherent growth: cells remain close to one another after each division, and there is no change of neighbour. Therefore, the longitudinal extensions of the clones decrease at each cell generation. (B) Dispersive intermingling growth: cells can rearrange with spatially distant cells. Long clones can be produced at any cell generation. Therefore, an increase of the frequency of clones with exponentially increasing longitudinal extensions is expected. (C) Ordered intermingling growth: cells intermingle only with their closest neighbors. The longitudinal extensions decrease at each cell generation and, therefore, a decrease of the frequency of clones with exponentially increasing longitudinal extensions is expected. (D) Self-renewing stem cells. Growth is polarized and, therefore, the clonal complexity (the number of time a given axial domain is populated by clones) increases in the same direction as the progression of the pool of self-renewing stem cells. In this mode, the different longitudinal extensions of long clones appear with the same frequency and, by consequence, an increase of the frequency of clones with exponentially increasing longitudinal extensions is expected. Ant, Anterior; Post, posterior.

suggests that clones of the SC and brain are produced by the descendants of the longest clones. Therefore, at least part of the precursor pool for these domains derives from a non-regionalized pool of CNS founder cells. To further analyze the possibility of a unique clonal origin for the CNS, we compared the characteristics of very long clones and long clones restricted to the SC (since clonal arrangement is more homogeneous along the A-P dimension in the SC). Both the distance between the D-V clusters (Fig. 10A) and the number of cells comprising each D-V cluster (Fig. 10B) were similar for the two categories of clones. Furthermore, similar values of about 16 D-V clusters (corresponding to four cell generations before the production of D-V cluster founders) were observed in the SC of very long clones and in the long clones restricted to the SC (Fig. 10C). These data show that the precursors of long clones either restricted or non-restricted to the SC derive from cells with very similar properties of A-P dispersion and growth. This is compatible with the idea that SC-restricted precursors derive from a pool of precursors that give rise to descendants dispersed along the entire CNS. If we follow this hypothesis, the allocation of cells in the pool of CNS founder cells, which generates about 36 D-V clusters, and the allocation of cells in the pool of SC founder cells, which generates about 16 D-V clusters, are separated by only 1 or 2 cell generations.

Restriction of clonal crossing between brain and spinal cord

The analyses presented above allowed us to deduce the different modes of dispersion in the SC and in the brain, but a remaining question concerns the site of the transition between these modes of dispersion. To get an insight into the

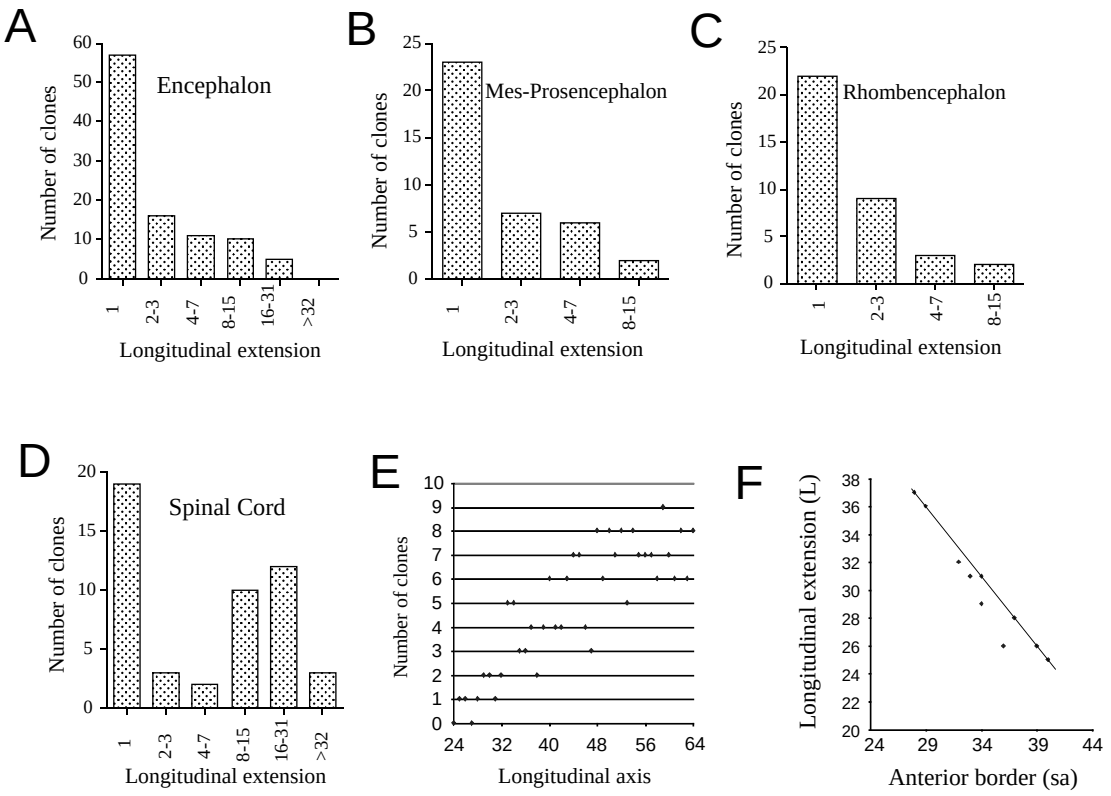

Fig. 9. Mode of growth, dispersion and production of the progenitor cells in the SC and brain. (A-D) Relationship between number of clones and length. Distribution of the number of clones (*y* axis) grouped by exponentially increasing longitudinal extensions (*x* axis) for clones restricted to the indicated domains. (E) Clonal complexity. Number of times (*y* axis) that the different axial levels of the spinal cord from subdivision 24 to 64 (*x* axis) are populated by L>21 clones restricted to the SC. (F) A-P polarity. Longitudinal extension (L) as a function of the anterior border in the L>21 clones restricted to the SC.

Table 2. Distribution of clonal borders at the junction between rhombencephalon and SC

Axial level (subdivision numbers)	15-23	24-32	33-41
Number of clonal borders (sa+sp)	23	20	22

We determined the number of time the indicated axial levels contain a clonal border (sa or sp) in clones with L>2. The observed values are not statistically different (χ^2 test, 2 d.f., P=0.6, n.s.).

axial level at which the transition occurs, we reasoned that when the caudally oriented growth is initiated in the SC, and because growth is coherent in the brain, clones should have a lower probability of giving descendants into both the anterior and posterior domains. We have defined a parameter, ΣC, which measures the probability that a clone will disperse across the different axial levels (Fig. 11A). This parameter is a characteristic of clonal cell dispersion, which is independent of the genealogical relationships between precursors. ΣC was calculated for clones populating more than half the axis (L≥32) and exhibited the expected distribution for clones displaying overlapping distributions (Fig. 11B). In contrast, ΣC calculated for clones populating less than half the axis (2<L<32), displayed a marked minimum value at the level of subdivisions 24-32 (Fig. 11C). To determine whether the minimum ΣC was not simply due to a lower proportion of clones in this sub-region, we calculated the number of clonal borders in domains 24-32 and in the adjacent domains of similar A-P extensions (Table 2). Since similar numbers of clonal borders were observed, this demonstrates that the density of clones is homogeneous along the A-P axis. Therefore, the minimum ΣC reflects a lower probability of crossing by the clones, which indicates that the transition between the two modes of cell dispersion in the anterior and posterior CNS is near subdivision 24 and that the stem cell-based mode of dispersion is acquired in the most anterior part of the SC.

DISCUSSION

In this study, we have used the laacZ labeling method (Eloy-Trinquet et al., 1999) to perform a systematic clonal analysis of the distribution of cells in the mouse CNS at E12.5, a developmental stage at which the primordia of most CNS structures are already established (Altman and Bayer, 1995; but note that E12.5 in the mouse corresponds roughly to E11 in the rat). The random character of the cell labeling event and the targeting of *laacZ* transgene expression to the CNS by the NSE promoter (Forss-Petter et al., 1990; Mathis et al., 1999) have allowed us to visualize cellular clones whose dates of birth span the entire period of CNS development. These characteristics have generated data regarding the mode of cell growth, dispersion and production of the precursors of the regions of the CNS that complement data obtained from fate maps (Lawson et al., 1991; Tam and Behringer, 1997). Our interpretation of the pattern of clones is partially based on the hypothesis that there is no late recruitment of cells and that the longitudinal extension of the clones is correlated with their date of birth. This latter hypothesis is plausible if cell behavior exhibits a certain degree of coherence, which is suggested by the fate maps of the mouse CNS primordium and further confirmed by our results. The retrospective interpretation of the clonal patterns has allowed us to deduce the successive patterns

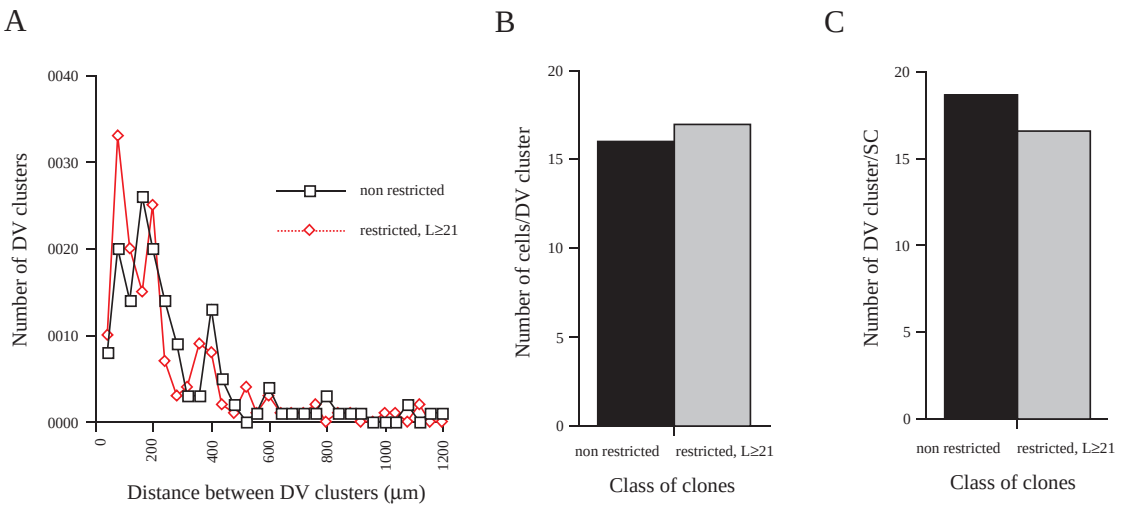

Fig. 10. Comparison of the characteristics of clones restricted or not to the SC. (A) Distribution of the distances between a D-V cluster and its closest caudal neighbour (*x* axis) for the long clones restricted (*n*=10 clones, 161 clusters) or non-restricted (*n*=11 clones, 164 clusters) to the SC. The middle of the clusters was used for the determination of the distance between clusters. (B) Mean cell numbers per D-V cluster determined for the long clones restricted (L>21, *n*=10), or very long clones non-restricted, to the SC (*n*=11). (C) Mean number of D-V clusters in the SC for the longest clones restricted to the SC (*n*=7 clones) or very long non-restricted clones (*n*=10 clones).

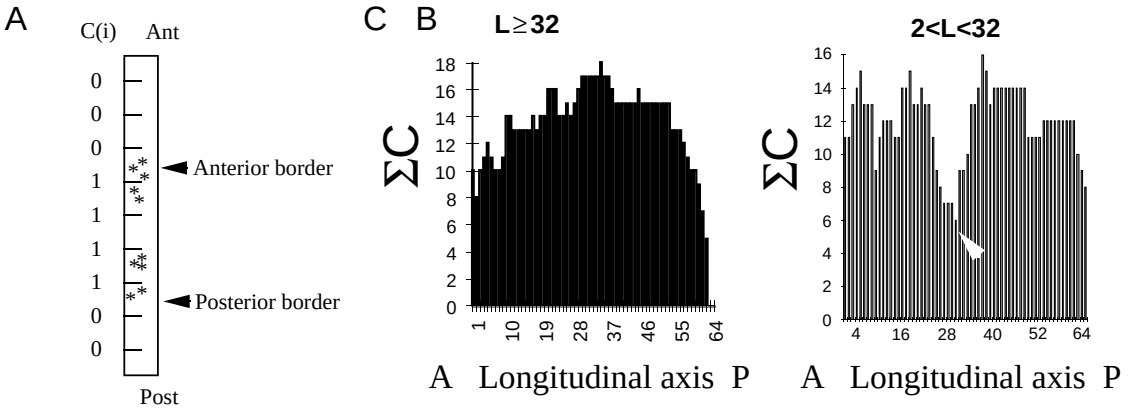

Fig. 11. Distribution of the crossing index (ΣC) along the CNS. (A) Definition of ΣC. For a given subdivision, i, and a given clone, if the clone crossed the limit between subdivisions i and i+1, then C(i) =1. If it did not cross, then C(i) =0. ΣC(i) is the sum of C(i) for all the clones. Clonally related cells are indicated by asterisks. (B) Clones with L≥32. (C) Clones with 2<L<32 clones. A minimum of ΣC is observed at the level of subdivisions 24-32 (white arrowhead).

of cell dispersion during neural tube morphogenesis and the mode of growth on which they are based.

The pool of CNS progenitor cells undergoes progressive regionalization until a general arrest of A-P cell dispersion occurs

The pool of CNS founder cells is not regionalized and its initial expansion is accompanied by widespread A-P clonal dispersion

One remarkable result is the identification of a pool of CNS ancestor cells in the embryo that produce descendants dispersed along the entire A-P axis of the CNS. Because the organization into separate D-V clusters and the density of D-V clusters of the very long clones are very similar to those of the shorter clones, the precursors of very long clones are probably the founder cells of the CNS (or their ancestors during their period of non-clonal growth). Therefore, the properties of these clones reveal the properties of CNS founder cells and demonstrate that these founders form a non-regionalized pool of cells (Fig. 12).

When is this non-regionalized pool of CNS founder cells present in the embryo? We have shown previously that the D-V cluster founders are born before the formation of the neuromeres. For example, in the rhombencephalon, D-V cluster founders are born before expression of Krox 20, that is E8 (Mathis et al., 1999). Our present analysis of the number of D-V clusters in the very long clones suggests that 5-6 cell generations separates the CNS founder cells and the D-V cluster founders. With a cell cycle length of about 7-12 hours in the epiblast (Lawson et al., 1991), this suggests that the CNS founder cells are born at around E5-E6.5. This conclusion is also supported by the data concerning clonal bilaterality. In the early streak-stage mouse epiblast (E6.5), about 50% of the ectoderm precursors give rise to bilateral clones (Lawson et al., 1991). In our study, a comparable ratio of about 50% of bilateral clones was observed for the very long clones (L>42). These data suggest that the non-regionalized pool of CNS founder cells is initially located in the epiblast (Fig. 12).

We found that half of the very long clones are unilateral. Obviously, these clones disperse less along the left-right axis

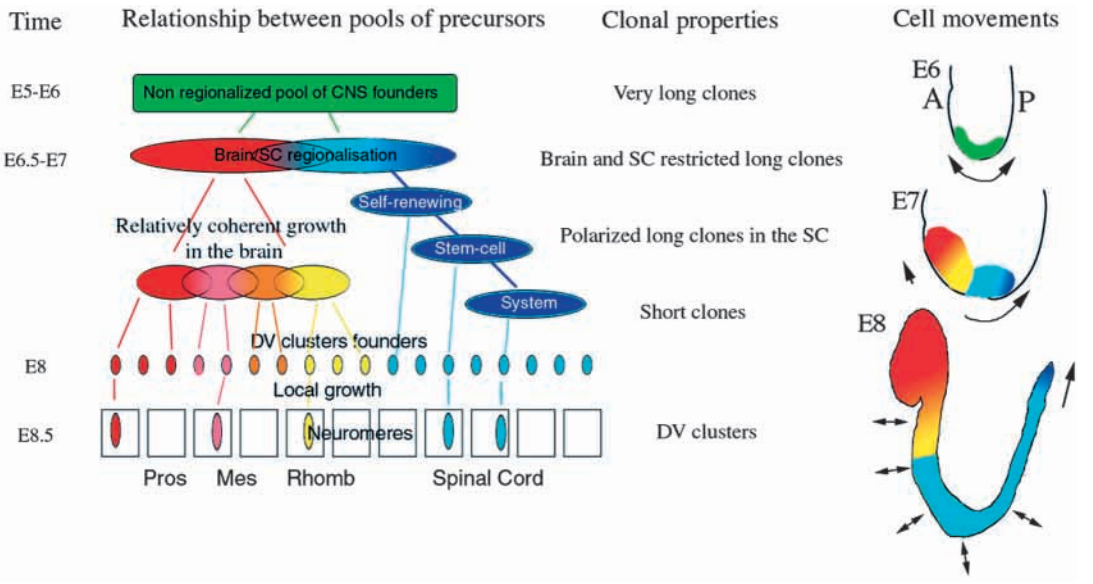

Fig. 12. A model for cellular events during development of the neural tube. The model concerns the organization of territories and global cell movements, as well as the development of individual cell lineages. Before the onset of gastrulation (E6), the pool of CNS founders (in green) is not regionalized, and due to the important cell intermingling occurring before gastrulation and during neural plate elongation (E5-E7), individual precursors produce descendants distributed along the entire neuraxis. From the onset of gastrulation (E7), individual clones have a lower probability of producing descendants in both anterior and posterior CNS, due to the posterior oriented cell movements of the self-renewing pool of cells and an anterior coherent growth. Consequently, the pools for spinal cord (in blue) and brain (red-yellow) become separated (earliest regionalisation). Subsequently (E7-E8), important cell intermingling continues in the SC primordium, due to an anterior to posterior regression of the self-renewing pool of stem cells. In contrast, due to coherent growth in the anterior neural plate, the A-P order of precursors is rapidly established, leading to an early regionalisation of brain territories (red-to-yellow gradient). After E8, A-P cell mixing in the brain and anterior SC becomes more limited and is followed by a phase of D-V cell dispersion.

than along the A-P axis. This demonstrates a degree of coherence of the pool of CNS founder cells in one of its dimensions. However, it is difficult to define which axes exhibit coherence as the relationship of the orientation of the axes in the epiblast compared to neurectoderm is not known. Thus, certain authors have suggested that the initial A-P order in the epiblast prefigures the final longitudinal arrangement of cells in the neurectoderm (Quinlan et al., 1995), but others have proposed that the initial A-P dimension of the pool corresponds to the final D-V arrangements in the neural plate (Lawson and Pedersen, 1992). Additional analyses are required to clarify this problem but, clearly, the very long unilateral clones show that the expansion of the pool of CNS founder cells is not isotropic.

A separation between brain and spinal cord precursors due to different modes of production

Our results indicate a separation between anterior and posterior neural system precursors, which occurs about two cell divisions after the formation of the pool of CNS founder cells, i.e. probably between E6 and E7. In principle, this separation could reflect a restriction of cell dispersion at the interface between the two clonal domains (due to a clonal boundary or to differences in adhesive properties of the precursors of adjacent domains) or to cell movements in opposite directions. Our analysis suggests that this separation is due to the regression of a pool of self-renewing cells and to a relatively coherent growth in the brain (Fig. 12). Therefore, complementing other mechanisms such as cell sorting (Mellitzer et al., 1999), slow cell intermixing (Wetts and Fraser, 1989), an arrest of cell dispersion (Mathis et al., 1999) or the restriction of cell movements at boundaries (Fraser et al., 1990), a differential mode of growth is a possible mechanism for the separation of pools of founder cells in the embryo.

A general arrest of A-P cell dispersion and a transition from A-P to D-V cell dispersion

The systematic clonal organization of cells in separate D-V clusters demonstrates an important A-P dispersion of the descendants of CNS founder cells, even after the segregation between brain and SC. At around E7.5-E8, cells have very limited A-P dispersion so that they become the founders of the D-V clusters (Fig. 12). It is not yet clear what mechanisms are responsible for the arrest of A-P cell dispersion, but it is reminiscent of the final period of convergent extension in the neuroepithelium (Keller and Danilchik, 1988; Keller and Tibbetts, 1989; Kimmel et al., 1994). Given the results presented above, a surprising finding is that a similar arrest of A-P dispersion occurs in the spinal cord, the rhombencephalon and the mesencephalon (Mathis et al., 1999; this article). This indicates that the transition from A-P and D-V cell dispersion is independent of the mode of A-P cell dispersion preceding the genesis of the D-V cluster founders. This conclusion is consistent with our previous hypothesis that the initiation of the D-V cell dispersion may be initiated by external inductive events (Mathis et al., 1999). Importantly, this observation suggests that the subsequent phase of D-V cell dispersion is a crucial event and may be related to the fact that the basic D-V organisation of the neural tube is similar from the SC to midbrain, with a subdivision into alar and basal domains. These results also indicate independent and successive antero-posterior and dorso-ventral deployment of cells, reminiscent of the independent A-P and D-V genetic patterning events (Lee and Jessell, 1999; Simon et al., 1995). This conclusion further

supports the idea that the phase of D-V cell dispersion may be a necessary event for the D-V organization of the neural tube.

Comparison with the fate maps in the mouse and other vertebrate species

Our results are in general agreement with the fate maps of the neural plate in the mouse. These fate maps have revealed cell rearrangement in the mouse epiblast (Lawson et al., 1991; Lawson and Pedersen, 1992) and a dissipation of coherent growth before gastrulation (Gardner and Cockroft, 1998). This period is likely to correspond to that of non-clonal growth of the ancestors to the CNS founder cells (Fig. 12). Previous analyses have also suggested a progressive limitation of cell movements, beginning at E6.5 (Quinlan et al., 1995; Tam, 1989) and this is likely to correspond to the segregation of the anterior and posterior neural system reported here.

A dispersive cell behavior has also been described in the chick embryo at the onset of gastrulation (Hatada and Stern, 1994) and during neural plate formation (Schoenwolf and Smith, 1990; Stern et al., 1991). In contrast, in amphibian, lineage analyses of blastomeres have shown that cells maintain their relative order during axis formation (Jacobson, 1983; Wetts and Fraser, 1989). Therefore, there are differences in clonal dispersion in different vertebrate species. Because the acquisition of regional differentiation is likely to differ, depending on whether clonal growth is coherent or dispersive, certain aspects of the cellular mechanisms and/or the timing of induction and early patterning preceding gastrulation may differ in these different species. Nonetheless, other characteristics of cell behavior described in the mouse embryo, such as the early regionalisation of the spinal cord and brain (Quinlan et al., 1995; our results), the more coherent cell behavior in the brain, and the caudal polarity of cell dispersion in the SC (this study) are consistent with descriptions of neurulation movements in other species (Kimmel et al., 1994; Schoenwolf and Smith, 1990; Woo and Fraser, 1995). Together, these results illustrate the significant conservation of the cell movements of gastrulation and neurulation during vertebrate evolution.

Relationship between the patterns of cell behavior and the genetic organisation of the CNS

Analysis of the distribution of clones along the axis shows several transitions in the mode of growth and dispersion. The main transition is observed between the brain and the SC. This dual organization further suggests important differences in the mode of development of the brain and spinal cord, possibly associated with different genetic programs.

A progressive cellular and genetic regionalisation in the anterior CNS

Growth is relatively coherent throughout the anterior neural system, a mode which suggests organized properties. In fact, following this mode, the fate of cells may become progressively limited, simply due to this limitation of cell movements. Such an hypothesis is compatible with a cell intrinsic patterning process in the formation of the brain. Interestingly, genetic patterning is thought to occur progressively in the anterior neural plate, with the formation of smaller and smaller domains (Rubenstein et al., 1998). Therefore, our results raise the possibility that the progressive limitation of A-P cell dispersion and its arrest before neuromere formation (Mathis et al., 1999) may facilitate the acquisition of A-P positional information and/or the specification of genetic domains. It is even possible that the acquisition of this mode of growth is under the genetic control of developmental genes.

We observed that the boundary between the mesencephalon and the rhombencephalon (isthmic organizer) does not appear as a transition in the modes of A-P cell dispersion. This is one of the earliest genetic boundaries in the growing neuroepithelium, defined by the caudal limit of expression of otx-2 (Acampora et al., 1995; Broccoli et al., 1999; Matsuo et al., 1995) and the rostral limit of Gbx-2 (Millet et al., 1996). Therefore, this boundary may be essentially a genetic one and may not be involved directly in the control of A-P cell dispersion. This is consistent with the onset of expression of Fgf8 in the isthmus at the 5-somite stage (Shamim et al., 1999) a time at which A-P cell dispersion has already become limited (Mathis et al., 1999).

What controls this mode of growth? One possibility is that the signals controlling anterior CNS development, such as otx-2 (Acampora et al., 1995; Rhinn et al., 1998) noggin (Smith and Harland, 1992) or chordin (Sasai et al., 1994), may endow cells with more ordered cell behavior. However, we suggest that the axial level of the transition between ordered cell mingling and the self-renewing progenitor cell system is posterior relative to the boundary defined by the head and trunk organizers. Still, the topological relationship between the initial genetic boundaries (of otx-2 and gbx-2) at E6-E7 and the subdivisions at E12.5 are not known and the exact position of the most anterior cell produced by the pool of self renewing stem cells is not firmly established. Thus, further studies are needed to elucidate the relationship between neural induction and the initiation of morphogenetic movements.

Pools of self-renewing progenitor cells produce the trunk domain

We show that a pool of SC precursors disperses extensively along the A-P axis and this observation is compatible with either a dispersive intermingling or polarized self-renewing stem cell mode. However, the polarity of the clones in the spinal cord is a strong indication of a self-renewing stem cell mode (Nicolas et al., 1996). These results suggest that the ancestors of the D-V cluster founders are distributed sequentially by self-renewing cells regressing from rostral to caudal. So far, it is difficult to delimit the most anterior border of the territory formed by this system because this limit can only be defined by the longest polarized clones observed. The generation of more clones is needed to learn where in the rhombencephalon this limit is.

On the mouse fate map, the SC precursors are located lateral to the node and, in the chick, the cellular and genetic organization of this territory appears to remain constant during the regression of the node (Catala et al., 1996; Henrique et al., 1997). Our results add to this notion by suggesting that this territory contains self-renewing stem cells that establish a clonal continuity in the development of the SC. A possibility is that posterior developmental signals such as bFGF (Lamb and Harland, 1995) and retinoic acid (Blumberg et al., 1997) may endow posterior CNS founder cells with this mode of growth. In a polarized self-renewing system, the clonal history

(i.e. the number of cell generations preceding the arrest of cell dispersion) is longer for the caudal part of the trunk than for the rostral part. This correlates with the fact that the number of expressed *Hox* genes increases from rostral to caudal (Duboule and Dollé, 1989; Graham et al., 1989). A possibility previously discussed (Nicolas et al., 1996; Stern, 1992) is that some A-P positional information could be delivered by this temporal system, as it is in simpler organisms (Martindale and Shankland, 1990). Pools of self-renewing progenitor cells also participate in the longitudinal development of the notochord and floor plate (precursors located within the node; Beddington, 1994), as well as the somites (precursors located in the anterior primitive streak; Nicolas et al., 1996; Psychoyos and Stern, 1996). Our present results suggest that at least part of the area surrounding the node is composed of precursors with self-renewing properties, and that the development of the trunk is dependent on pools of polarized self-renewing systems regressing from A to P.

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