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13. A detailed description of our in utero surgical techniques has been provided in previous publications (*6, 11*). Injection parameters were manipulated (horseradish peroxidase versus wheat germ agglutinin conjugated to horseradish peroxidase, with or without 4% dimethylsulfoxide, retinorecipient nuclei versus optic chiasm injection) in order to ascertain to what extent the degree of labeling depended on procedural considerations. Peroxidase activity was demonstrated with a modified protocol based on the use of pyrocatechol and *p*-phenylenediamine [J. S. Hanker, P. E. Yates, C. B. Metz, A. Rustione, *Histochem. J.* **163**, 107 (1977); V. H. Perry and R. Linden, *Nature (London)* **297**, 683 (1982)]. For the purpose of orientation, small cuts were made in the superior retinal margin in situ before dissection.
14. Using differential interference contrast microscopy, we could observe all labeled and unlabeled cell profiles. Cells were considered labeled only if grains of reaction product were visible in the same plane of focus within the cell profile. Counts were made according to Gundersen's rule, correcting for profiles falling on two adjacent edges of the eyepiece reticule [H. J. G. Gundersen, *J. Microsc. (Oxford)* **111**, 219 (1977)].
15. The decussation line was evident after unilateral injections of horseradish peroxidase (*4*) and after injections of different fluorescent tracers into each optic tract.
16. Since there is a trend for ganglion cells in the central retina to be generated earlier than those in the peripheral retina (*10*), this could contribute to the presence of a peak density at E35. Furthermore, some retinal ganglion cell axons are still growing toward their central targets until E40 (*6*), and consequently C/P ratios obtained prior to this age may somewhat overestimate the central-to-peripheral difference in ganglion cell density. However, this overestimation would not appreciably alter the interpretation of our results.
17. The elimination of retinal ganglion cells illustrated in Fig. 2 is based on axon counts in the fetal optic nerve (*6*). The same time course is apparent in the rise and fall of labeled cell densities in the center and periphery, the peripheral density decreasing to a greater degree (Table 1).
18. For discussion of the factors that might underlie the elimination of ganglion cells in the fetal cat, see (*6*) and M. A. Kirby and L. M. Chalupa [*J. Comp. Neurol.* **251**, 532 (1986)].
19. As a first approximation, a linear function, which requires the fewest assumptions, provides a satisfactory fit to the data. The slight curvilinearity apparent in Fig. 4 could be produced by a decelerating rate of growth in the area centralis relative to the rate of peripheral growth. An overestimation of C/P ratios at the youngest fetal ages may also be a factor (*16*).
20. The ratio of nasal to temporal labeled cell densities in the peripheral quadrants of the retina changes from 1:1 at E35 to 1.6:1 at E62.
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Clonal Restriction Boundaries in *Xenopus* Embryos Shown with Two Intracellular Lineage Tracers

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It has been proposed that clonal restriction boundaries develop in *Xenopus* embryos between clones initiated at the 512-cell stage, and that these boundaries result in formation of morphological compartments, each populated by progeny of a group of ancestral cells. Although this hypothesis has gained some acceptance, it has also been criticized because the use of only one cell lineage tracer was not a conclusive test of the hypothesis. However, the critical experiment, an assessment of the extent of mingling between two labeled clones in the same embryo, has now been performed. A model of the proposed arrangement of the ancestral cell groups in the 512-cell embryo predicted that the two clones would remain separate in 49% of cases and intermingle in 51% of cases. In fact, there was a bimodal distribution, in which separation of the clones occurred in 46% of embryos and extensive interclonal mingling was observed in 54%. These results are not compatible with hypotheses in which a unimodal distribution of mingling would be predicted but are consistent with the compartment hypothesis.

THE COMPARTMENT HYPOTHESIS OF *Xenopus* development was proposed as an explanation for observations on the positions of labeled cells in embryos of *Xenopus* after injection of a lineage tracer, horseradish peroxidase (HRP), into single blastomeres at the 2- to 512-cell stages of development (*1, 2, 3*). This hypothesis, which has achieved only partial acceptance to date (*4, 5*), proposes that ancestral cell groups are formed in the embryo at the 512-cell stage, and that cell mingling is subsequently restricted between the progeny of different ancestral cell groups but not among progeny of cells comprising a single ancestral cell group (*1, 3, 6*). As a result of this clonal restriction of cell mingling, the progeny of each ancestral cell group form a separate morphological compartment. Each compartment contains many different cell types. The patterns of histological differentiation and compartmentalization are not congruent (*6*). Evidence in support of the hypothesis comes not only from lineage tracer studies but also from the observation that, when excised and cultured together, cells of different ancestral cell groups are restricted in their ability to intermingle in comparison with the mingling between cells derived from the same ancestral cell group (*7*).

Within the *Xenopus* central nervous system (CNS), seven compartments (three rostral and four caudal), derived from seven ancestral cell groups at the 512-cell stage of

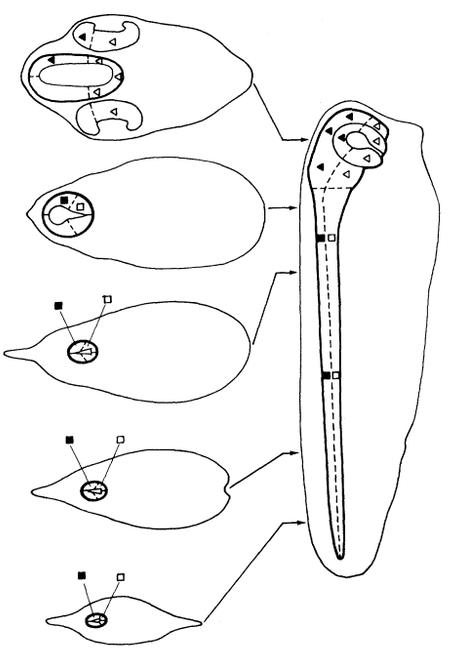

Fig. 1. Diagram of compartments in the central nervous system and retina of tailbud stage *Xenopus* embryo shown in lateral view (**right**) and at five cross-sectional levels (**left**). Compartmental boundaries are shown by dashed lines. Anterior is to the top. Symbols showing compartments on one side only: (▲) anterior-dorsal; (△) anterior-ventral; (■) posterior-dorsal; and (□) posterior-ventral. See text for details.

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development, have been defined (*2, 3, 6*) (Fig. 1). The anterior-posterior (rostral-caudal) boundary is at the level of the isthmus, between mesencephalon and rhombencephalon. The three anterior compartments are divided as follows: in a horizontal plane separating dorsal and ventral, and in the dorsal midline, separating right and left. Thus there are one anterior-ventral (anterior-median) compartment and two anterior-dorsal compartments. The posterior (caudal) compartments are demarcated horizontally and in the dorsal and ventral midlines. Thus there is one posterior-dorsal and one posterior-ventral compartment on each side of the midline.

Compartmentalization during development has also been proposed as an important morphogenetic factor during invertebrate development (*8*). Although the same name has been chosen for what appear to be similar phenomena in flies and frogs, there is no reason why the mechanism, controlling factors, and functional significance of the two should be the same.

Introduction of fluorescent cell lineage tracers (*9*) has made it possible for us to perform a crucial experiment to establish whether the apparent clonal restriction boundaries are real in frog embryos. This experiment involves the injection of two near neighboring cells with different lineage labels at the 512-cell stage, followed by analysis of the extent of mingling of differently labeled cells (*10*). If the compartment hypothesis were true, the two populations of labeled progeny would mingle freely within a compartment if their injected ancestors were in the same ancestral cell group. If the injected cells were in different ancestral cell groups, then we would expect to see labeled progeny in separate compartments with little or no mingling between the two populations of labeled cells.

We made a model (Fig. 2), using the published (*2*) representation of the approximate dimensions of the ancestral cell groups on the surface of the 512-cell embryo. From this model we calculated that any pair of cells, when chosen at random from the region of the presumptive nervous system, would have a line of clonal restriction between them in 49% of cases if those two cells were separated by one cell diameter, regardless of the orientation of the pairs (Fig. 2). This prediction would apply only to the model shown; the numbers of ancestral cells and shapes of the ancestral cell groups (ACGs) are shown in Fig. 2. If a significantly different proportion of mingled and unmingled clones were found, it would invalidate this model and suggest that either the ACGs contain a different number of cells or they are a different shape at the surface from that shown, or both, or it would invalidate the compartment hypothesis. Because it was already our intention to separate injected cells by one uninjected cell to preclude cross-labeling, we adopted this method and predicted clonal separation in approximately 49% of embryos successfully labeled with two tracers.

In this series of experiments, lissamine-rhodamine-dextran-lysine was injected into one blastomere and fluorescein-isothiocyanate-dextran-lysine into another at the 512-cell stage of development in 286 embryos (*10*). Cell pairs were chosen at random from the region of the presumptive nervous system as indicated in Fig. 2. Of these, 70 developed abnormally or were not labeled, and 93 were labeled with either fluorescein or rhodamine, but not with both. Embryos with only a single labeled clone always mimicked closely our previous results (*2*) where HRP was the sole lineage tracer, and these embryos are not included in the further analysis of these experiments. One hundred and twenty-three embryos developed successfully to the early tailbud stages 20 to 29 (*11*) and had two labeled clones approximately equal in cell number.

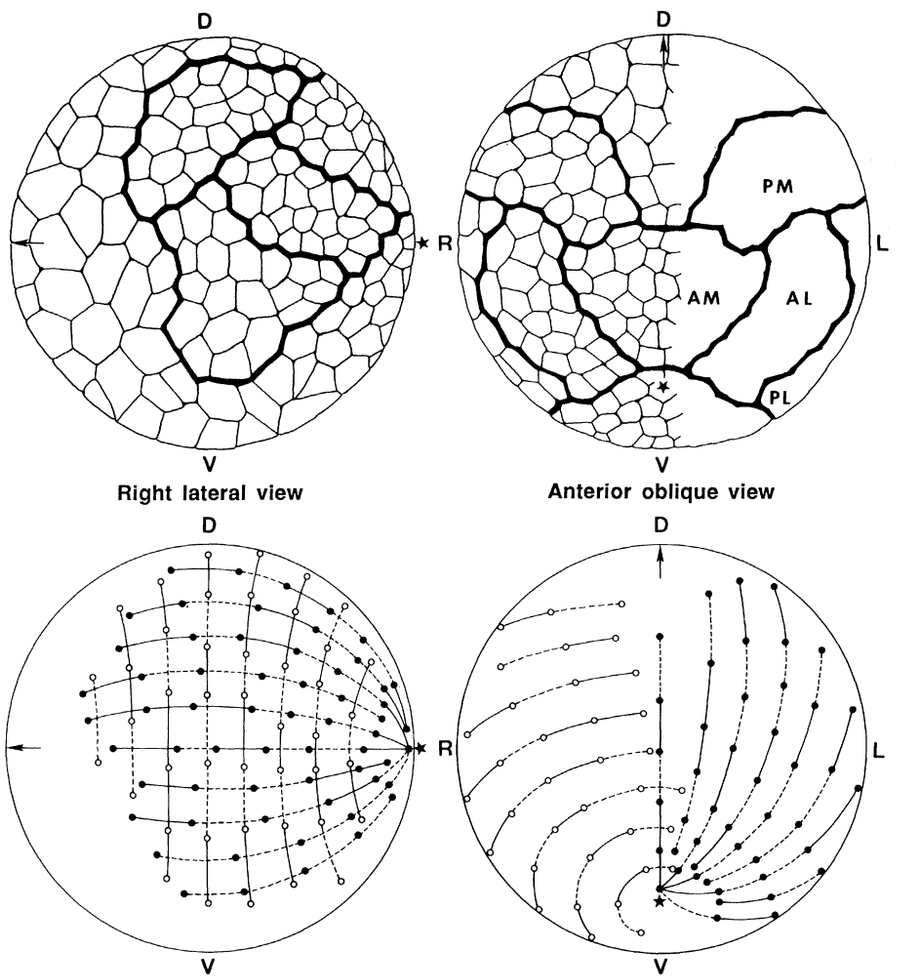

Fig. 2. Method of calculating the probability that a clonal restriction boundary will develop between clones originating from pairs of ancestral cells in the 512-cell *Xenopus* embryo. Two views of the 512-cell embryo are shown. (**Upper diagrams**) Cell outlines and boundaries (thick lines) between different ancestral cell groups (ACGs) as deduced from earlier results (*2*). (**Lower diagrams**) Positions of a small selection of pairs of labeled cells (marked by dots) separated by one unlabeled cell, oriented along meridians (●) or along parallels (○). Dashed lines (---) connect cell pairs in the same ACG (not crossing a clonal restriction boundary) (51 ± 2%); continuous lines (——) connect cell pairs in different ACGs (crossing a clonal restriction boundary, 49 ± 2% . The frequency of cell pairs crossing or not crossing a clonal restriction boundary is dependent upon the exact cells chosen. In this model the proportions vary by approximately 2%, depending on the particular sample taken. When this ratio is calculated for all possible pairs of surface cells in this region, 49% have a boundary between them and 51% do not. Only a small number of possible combinations is shown here for clarity. The model predicts that regardless of the positions of pairs of labeled ancestral cells (when chosen at random from the areas enclosed by the thick lines), clones originating from them will either be mingled or will be separated by a clonal restriction boundary, with equal probability. AL, anterior-lateral ACG; AM, anterior-median ACG; D, dorsal; L, left; PL, posterior-lateral ACG; PM, posterior-medial ACG; R, right; V, ventral. Star is at animal pole, arrow points toward vegetal pole.

In 56 of the 123 successfully labeled embryos clonal separation was 99% complete. The hypothesis allows for 1% overlap along the line of clonal restriction as a form of developmental noise in which the aberrant cells have no significant influence on morphogenesis and do not appear to have had any functional effect (*2, 3, 12*). The 46% (56 of 123) of cases in which clonal separation was complete is consistent with the 49% predicted by the model described above. When clones did not mingle, they were often in contact over an extensive boundary, either between the dorsal and ventral (Fig. 3, D and E) or between anterior and posterior compartments (Fig. 3C) as those compartmental boundaries have been defined earlier (*2, 3, 6*) (Fig. 1). Extensive intermingling of clones was observed in 54% (67 of 123) of embryos containing both labels (Figs. 3, A and B, and 4). These cases show that the experimental method did not preclude normal cell mingling. There was no tendency for anterior clones to intermingle more or less frequently than posterior clones.

A frequency distribution histogram of percent interclonal mingling is shown in Fig. 5. As predicted by the hypothesis, the data form a bimodal distribution with mingled and unmingled specimens present in almost equal proportions. The degree of interclonal mingling was estimated in two ways. First, in sections containing a large number of both types of labeled cells, the percentage of the total domain of one clone that was also occupied by the other clone was calculated with an image analyzing computer (Fig. 6). This method was also used for specimens with significant numbers of labeled cells in the epidermis, where they could be easily visualized prior to sectioning (Fig. 3, A, B, and C). The results obtained with this method correlated closely with the more subjective analysis used previously (*7*), which assigned a "mingling index" to the two clones in comparison with a set of standard models in which black and white discs, initially separated, encroached into one another's domain one rank at a time. This second method was used for specimens in which the two clones did not have significant numbers of cells in the same sections (when an anterior and a posterior clone were labeled and the specimen was sectioned coronally, or when a left and a right clone were labeled and the specimen was sectioned sagittally). The amount of mingling was independent of the orientation of the pairs of injected cells. "Intermediate" mingling values (2 to 99%) were observed (Fig. 5). This is a result of the partial mingling of

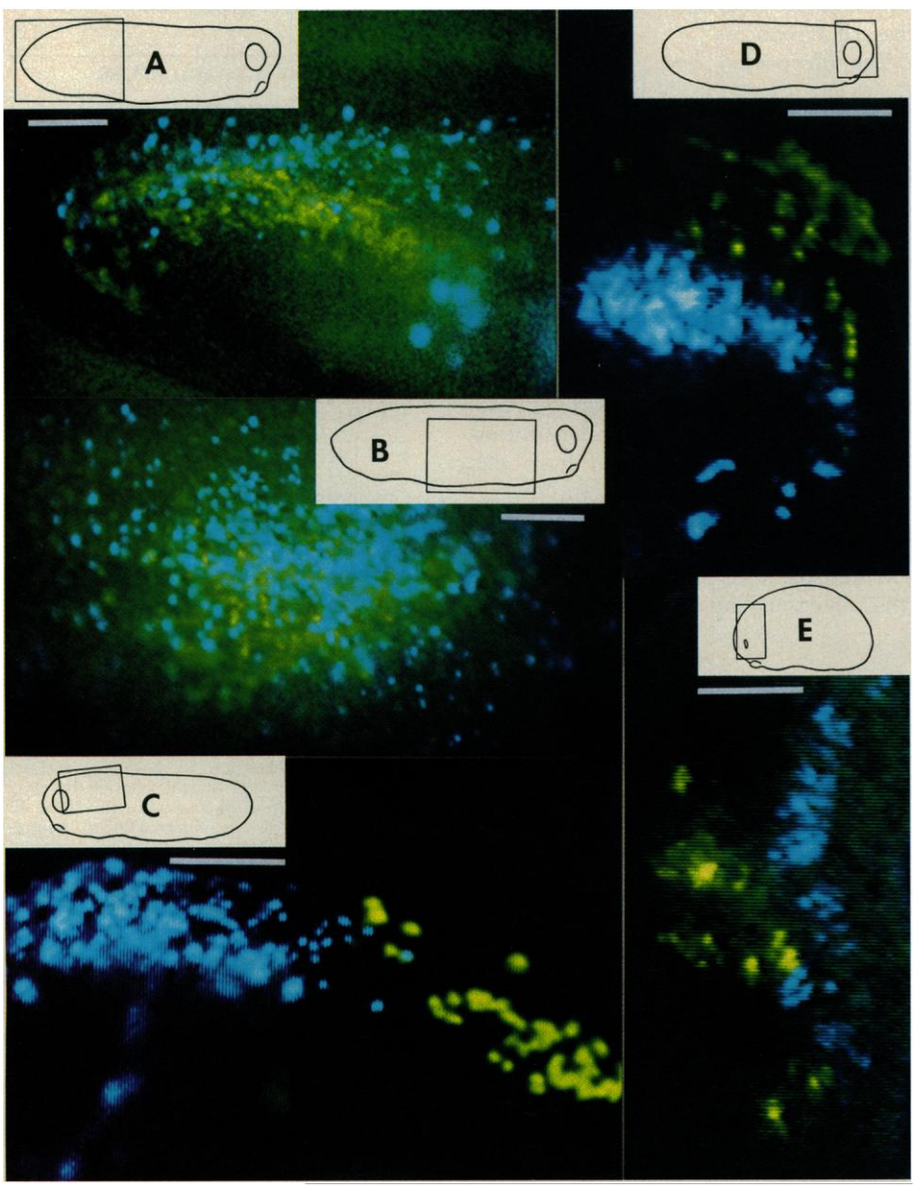

Fig. 3. Photographs of fluorescein-labeled cells (blue) and rhodamine-labeled cells (yellow) in embryos at tailbud stages in the region shown by the rectangle in the outline view of each embryo. (**A** and **B**) Intermingled clones are seen in the epidermis. (**C**) Unmingled clones are seen in the epidermis. (A), (B), and (C) are shown whole before fixation, and the results remained the same after fixation. (**D** and **E**) are sagittal sections of fixed, frozen embryos showing unmingled clones. Rhodamine-labeled cells (yellow) are seen in the anterior-dorsal CNS in (D) and (E), and in the dorsal head epidermis in (D), while fluorescein-labeled cells (blue) are located in the anterior-ventral CNS in (D) and (E). Scale bars, 100 μm.

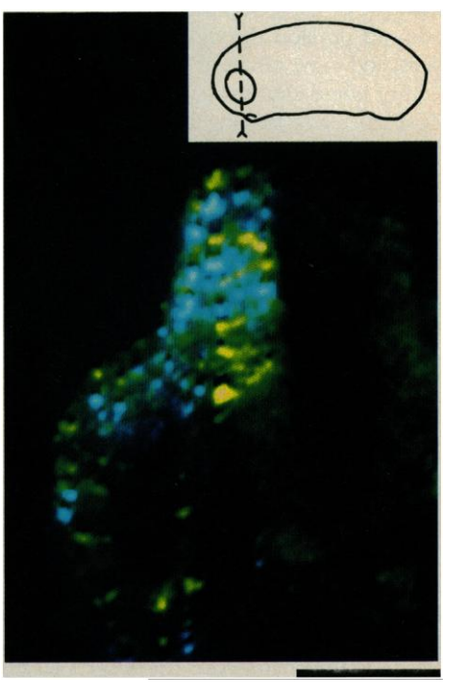

Fig. 4. Coronal (transverse) section of a fixed, frozen stage 25 *Xenopus* embryo. Fluorescein-labeled cells (blue) are intermingled with rhodamine-labeled cells (yellow) in the anterior-dorsal part of the CNS and retina on one side of the embryo. The two labeled ancestral cells were in the anterior-lateral ACG. The inset shows an outline of the embryo, with the approximate plane of section marked. Scale bar, 100 μm.

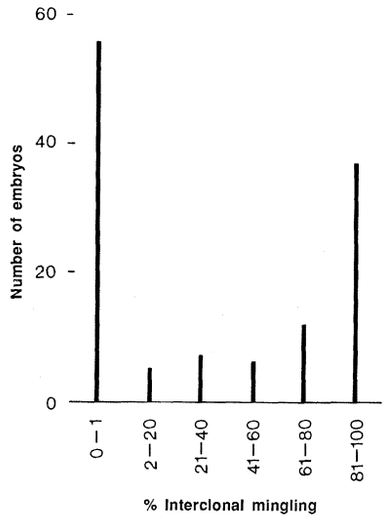

Fig. 5. Histogram showing measures of interclonal mingling. Methods of measurement are given in the text and Fig. 6.

clones within a compartment and is consistent with the compartment hypothesis. The compartment hypothesis makes no predictions concerning the extent of mingling between clones derived from the same ancestral cell group, except to state that they may become "freely intermingled."

Each labeled clone included cells of many different types, and the clonal restriction boundary often extended across different tissues (Fig. 3D). Compartmentalization appears to develop throughout the frog embryo, and some details of compartmentalization of non-neural structures are known (*6, 7*). Particularly striking in this study were lines of clonal restriction observed in the epidermis (Fig. 3C).

These results do not support the notion that the amount of mingling is a consequence of directed cell movements during gastrulation. This is because these movements are mainly in one axis only, the boundaries are not related to patterns of morphogenetic migrations during gastrulation (*13*) and in the neural plate (*14*), and because the difference in behavior of labeled clones when initiated at the 256- as compared to the 512-cell stage (*2*) cannot be accounted for by mechanisms that exert their influence only at much later stages, as would be the case with directionally selective cell movements.

The observation that the two labeled clones intermingled freely in 54% of cases is in accordance with the prediction from the model. The most likely explanation for this result is that the two injected cells were from the same ancestral cell group, and their progeny were therefore not restricted in their movements among the cells of other clones derived from the same group. This result parallels the observation that when a single ancestral cell is injected with HRP, its progeny intermingle with unlabeled cells from the same ancestral cell group (*3*). Also completely consistent with the compartment hypothesis and the prediction from the model is the observation that, in 46% of cases, the two labeled clones did not intermingle, despite being adjacent. This result is irreconcilable with any type of model in which intermingling of the progeny of two ancestral cells is an inverse function of the distance between those cells at the time of labeling. In our experiments injected cells were separated by one cell diameter. With a model in which the likelihood of the two clones mingling is a function only of the distance between their labeled ancestors one would expect the labeled progeny in these experiments always to be intermingled in similar proportions, commensurate with the consistent spacing between the injected cells. What we observed was a bimodal distribution of amounts of interclonal mingling as predicted by the compartment hypothesis and not a unimodal distribution as predicted by other hypotheses.

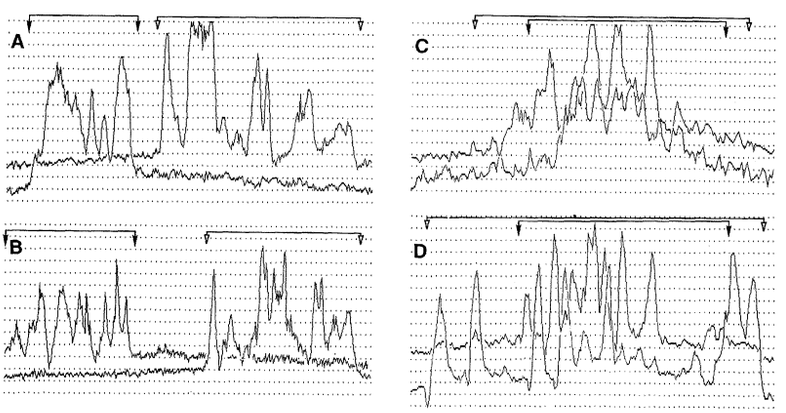

Fig. 6. Computer-generated plots of fluorescence brightness levels (vertical axis of plot) at points along a line (horizontal axis of plot) crossing the domains of two clones, one labeled with fluorescein, the other with rhodamine. Peaks represent fluorescent labeled cells. The plots have been separated slightly from one another vertically. Arrows show spatial limits of each clone. The clonal domains do not overlap in (**A**) and (**B**) but one clone is completely overlapped by the other in (**C**) and (**D**). From such traces measurements of percentage overlap of clonal domains were made in 46 different embryos in which interclonal mingling was also assayed by another method (*7*), which was used in all 123 embryos.

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10. Clones were labeled and analyzed as follows: *Xenopus* embryos at the 512-cell stage were injected (*2*) with 2% (weight to volume) solutions of the fluorescent lineage tracers lissamine-rhodamine-dextran-lysine (RDA) and fluorescein-isothiocyanate-dextran-lysine (FDA) synthesized according to the method of Gimlich and Braun (*9*) with 10,000-dalton dextran to prevent passage of molecules between cells. Single blastomeres in the region of the presumptive nervous system (*2*) were selected at random. After intracellular injection of one blastomere with FDA, a second blastomere was chosen for RDA injection; this blastomere was separated from the FDA-labeled blastomere by one unlabeled cell. This precaution eliminated the possibility of cross-labeling daughter cells should injection be done before completion of division. At stage 20 to 29 (*11*) living embryos were observed under a fluorescence microscope fitted with an ISIT high sensitivity video camera and a videotape recorder. Fluorescence images were recorded on videotape before and after fixation and cryostat sectioning of the embryos. Pseudocolor photographs were made from the TV monitor image using color filters and double exposure.
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