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A recombinant retrovirus encoding alkaline phosphatase confirms clonal boundary assignment in lineage analysis of murine retina

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ABSTRACT **Recombinant retroviruses encoding the histochemically detectable enzyme β-galactosidase have been used to investigate lineage in the vertebrate nervous system. Identification of the descendants of individual progenitors is straightforward when progeny cells are arranged in a reproducible, clustered pattern, but difficulties in interpretation arise when progeny migrate extensively and/or in an irregular pattern. To better resolve clonal boundaries, additional histochemical marker viruses that engender distinctive reaction products can be used in combination with *lacZ*-bearing viruses. To this end, we have created a retrovirus vector, DAP, encoding an easily assayable enzyme, human placental alkaline phosphatase. DAP was found to be at least as useful as a *lacZ*-encoding retrovirus (e.g., BAG) with respect to high viral titer, stability of expression, and in identification of infected cells *in vivo*. Moreover, it was found to be neutral with respect to postnatal rodent retinal development and offered superior staining characteristics relative to *lacZ*. Coinfection of rodent retina with DAP and BAG allowed an examination of the clonal nature of radial arrays of labeled retinal cells that previously had been described as products of a single infected progenitor. Of 1100 radial arrays examined for the presence of both DAP- and BAG-infected cells, only 1.2% were the result of infection with more than one virus.**

Lineage analysis using recombinant retroviruses has furthered our understanding of development of the vertebrate central nervous system (1–3). In both rodent and avian species it appears that progenitors are multipotent, in some cases, even through the last cell division. Multipotency has been observed within the rodent and chicken retina (4–6), chicken tectum (7, 8), rodent cortex (9–12), chicken spinal cord (13), and chicken neural crest (14). These findings are based on the ability to define clonal boundaries among siblings that bear a histochemically detectable enzyme encoded by a retrovirus vector.

The definition of boundaries of clones is straightforward in cases in which sibling cells distribute in a reproducible, obvious pattern (4, 7). In such cases, the issue of clonality has been addressed by statistical analysis of labeled cells following inoculation with serial dilutions of a virus stock. These studies, most extensively carried out in the rodent retina, suggested that independent infectious units did not frequently infect adjacent progenitors (4). However, these studies could not address whether the infectious units that were being measured were single, independent virions or were aggregates of more than one infectious virion that remained clumped during dilution of the virus stock but were able to infect adjacent progenitors. Furthermore, in tissues such as cerebral cortex, in which sibling cells migrate relatively great distances and along circuitous routes (9, 15), defining clonal boundaries with a single histochemical marker virus is difficult. As a partial solution to this problem, vectors bearing *lacZ* with an appended nuclear localization sequence have been used in conjunction with vectors encoding cytoplasmically localized β-galactosidase (β-gal; β-D-galactoside galactohydrolase, EC 3.2.1.23 (8, 16–18). However, in some cell types, such as neurons of the mammalian cortex and cerebellum, the cytoplasmically localized β-gal product cannot always be distinguished from the nuclear-localized β-gal product, and more distinctive reaction products are required.

To better define clonal boundaries, we report the development of a retrovirus vector encoding human placental alkaline phosphatase [AP; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1]. AP has a number of characteristics desirable for a histochemical reporter: The alkaline phosphatase gene, *P ALP-1*, has been cloned and sequenced (19–21). It has been transfected and expressed in cultured mammalian cells (22–24). Its natural distribution is limited primarily to the placenta in humans and the great apes (25–27). Previous reports indicate minimal AP activity within the rodent brain (28, 29) and there are several inhibitors of rodent AP activities that do not inhibit *P ALP-1*. In addition, *P ALP-1* is 100-fold more resistant to treatment with heat than is rodent AP (30, 31).

MATERIALS AND METHODS

DAP Plasmid Construction and Virus Preparation. All procedures used in cloning were essentially as described (32). Plasmid CMV-PLAP (J. Flanagan and P. Leder, personal communication), containing the cDNA of *P ALP-1* (generously provided by S. Udenfriend; refs. 22 and 23), was digested with *Kpn* I and a 1930-base-pair fragment encoding AP was isolated. The ends of this fragment were blunted with T4 DNA polymerase and ligated to a *Sal* I linker (no. 1027; New England Biolabs). The linked fragment was digested with *Sal* I and inserted into the *Sal* I site of the Moloney murine leukemia virus retroviral vector DO-L (11, 33) to generate DAP.

DAP plasmid was transfected with calcium phosphate (34) into ψ-CRIP cells, an amphotropic packaging line (35). Transiently produced virus from these cells was harvested 18 hr after glycerol shock and was used to infect ψ-2 cells, an ecotropic packaging line (36). Infected ψ-2 cells were selected with G418. Resulting G418-resistant colonies were isolated and expanded. Virus produced by ψ-2-DAP lines was titered for the ability to confer G418 resistance and/or alkaline phosphatase activity to NIH 3T3 fibroblast cells, and was tested for the presence of helper virus (37). At all points in this process, cells were stained for AP activity as detailed below.

To produce stocks containing BAG and DAP virus at a variety of ratios, ψ-2-BAG and ψ-2-DAP cells were trypsinized

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Abbreviations: AP, alkaline phosphatase; β-gal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; X-P, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; CFU, colony forming units; P0, postnatal day 0.

to single cells and plated together on the same dishes at ratios of 12:1 to 1:4, respectively. The final number of cells plated was $\approx 5 \times 10^6$ cells per 10-cm dish. Viral supernatant was gathered several days after cultures reached confluency and stocks were concentrated by centrifugation (38, 39). The titer of the concentrated stocks averaged 10^7–10^8 colony forming units (CFU) per ml, as measured by the ability to confer G418 resistance and/or AP or β-gal activity to NIH 3T3 cells. The ratio of DAP to BAG was assessed by first selecting infected NIH 3T3 cells in G418 and then staining the resulting colonies for β-gal and AP activity.

Enzyme Histochemistry. Cell lines transfected or infected with DAP and/or BAG were washed once with phosphate-buffered saline (PBS) and fixed for 5–10 min in 0.5% glutaraldehyde in PBS at room temperature. Fixed cells were washed three times for 5–10 min in PBS. The culture was then incubated in 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (1 mg/ml), 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2–5 mM $MgCl_2$ in PBS for 4–16 hr at 37°C. Cells were washed twice for 5 min in PBS and then incubated at 65°C for 10–30 min. Cells were washed for 10 min in AP buffer (100 mM Tris·HCl, pH 9.5/100 mM NaCl/5–50 mM $MgCl_2$ (buffer 3 detailed in the Genius kit, Boehringer Mannheim) and then incubated in 5-bromo-4-chloro-3-indolyl phosphate (X-P) (0.1 mg/ml), nitroblue tetrazolium (NBT) (1 mg/ml), levamisole [L(−)-2,3,5,6-tetrahydro-6-phenylimidazo(2,1-*b*)thiazole] (0.24 mg/ml) in AP buffer in the dark at room temperature for 1–24 hr (40). The following AP inhibitors were also tested at concentrations recommended by Zoellner and Hunter (41): 2 mM mercuric chloride, 5 mM L-leucylglycylglycine, 1 mM EDTA, 1 mM L-phenylalanylglycylglycine, 0.2 M lysine hydrochloride, or 0.3 mM sodium arsenate. Stained cells were washed with 20 mM EDTA in PBS and either mounted in gelvatol or stored at 4°C.

Analysis of Infected Retinas. Retinas were inoculated with DAP and/or BAG virus at postnatal day 0 (P0), as described (4). Mature (>P21) infected retinas were harvested and fixed, also as described, and stained for 2–4 hr in X-gal staining solution contained 0.02% Nonidet P-40 and 0.01% sodium desoxycholate. X-gal-stained retinas were then washed with PBS, heated to 65°C for 30 min, and incubated for 1–3 hr in X-P staining solution as described above. After PBS/EDTA quenching, retinas were dehydrated with ethanols and xylene, embedded in paraplast X-TRA (tissue embedding medium; Oxford), and cut into 10-μm sections on a Reichert Jung Biocut 2030. Sections were dried onto gelatin-coated slides, washed in xylene, and mounted in Permount (Fisher Scientific).

Sections were examined for the presence and morphology of labeled cells. To aid in clonal reconstruction, a camera lucida drawing, containing the location and identity of each labeled cell, was made of each section. Radial arrays contained within one section were defined as a clone. Occasionally, a radial array appeared to occupy more than one section. Superimposition of the camera lucida drawings and examination of the sections at high magnification were then used to determine whether individual labeled cells within such an array were contiguous across more than one section.

RESULTS

Characterization of DAP Virology and Histochemistry. The cDNA of human *P ALP-1* was cloned into the retroviral vector, pDO-L, to create DAP (DO-L encoding AP). *P ALP-1* is transcribed from the 5′ long terminal repeat, while the neomycin-resistance gene, Tn5 *neo*, is transcribed from the internal simian virus 40 early promoter. Expression of histochemically detectable AP activity was first assessed by transient expression assays following Ca_2PO_4-mediated transfection of cultured fibroblasts (data not shown). These assays indicated that DAP could express *P ALP-1* activity transiently and thus production of DAP virus by the ecotropic retroviral packaging line ψ-2 (36) was undertaken. The viral titers of clonal, G418-resistant, ψ-2-DAP producer lines ranged from 10^5 to 10^6 CFU/ml. Infected NIH 3T3 cells were selected for the presence of the *neo* gene by using G418 and were stained for AP activity. Greater than 90% of such colonies were found to be AP positive.

Cultured cells infected with DAP virus or transfected and expressing DAP transiently were tested for AP activity. The histochemical assay used resulted in formation of a purple precipitate after cleavage of X-P and reduction of NBT, hereafter referred to as X-P/NBT. Regardless of the method of introduction, the staining was rapid, well localized, and easily distinguishable from faint background staining. Cultures of mouse fibroblasts were then infected with both viruses at low multiplicity of infection and conditions for histochemical detection of both β-gal and AP were devised (Fig. 1*A*). As controls for the specificity of the enzyme reaction conditions, some cultures were infected with a single type of virus. Such cultures were tested for enzyme activity of the virus that was not used, as well as under conditions for both enzyme activities. In all cases, no enzyme activity was observed unless the cognate virus had been used for the infection. Interestingly, the X-P/NBT precipitate produced by DAP-infected cells was somewhat more granular than the X-gal product but consistently filled processes more completely and was more intense.

Although earlier reports, utilizing naphthol-phosphate conjugates with fast dyes, indicated negligible AP activity in rodent neural tissue (28, 29, 42), rat brain and mouse retina produced a considerable amount of reaction product when stained with X-P and NBT. A variety of AP inhibitors were tested. Levamisole proved to be most effective at reducing background while not inhibiting the human placental enzyme. Furthermore, 65°C heat treatment of tissue and certain cell lines, such as NIH 3T3, for up to 30 min greatly reduced background staining while not interfering with human placental enzyme activity. The necessity of using heat treatment defined the order of staining cells and tissue coinfected with BAG and DAP. β-gal staining must be done prior to heat treatment as this enzyme is not heat resistant.

Comparison of AP and β-gal Staining in DAP- and BAG-Infected Mouse Retina. Retinas of newborn mice were infected with cogrown stocks of DAP and BAG virus, or with a single type of virus, and were harvested upon reaching maturity. They were stained for the presence of either β-gal, AP, or both activities, and subsequently embedded and sectioned. Retinas infected with BAG virus alone contained only blue cells, while DAP-infected retinas contained only purple cells, even after reaction under conditions for both enzyme activities. Uninfected control retinas did not contain either blue or purple cells. The number and composition of blue and purple radial arrays in coinfected retinas were catalogued based on classical descriptions of retinal cell morphology. Radial arrays composed of a small number of cells were generally found within one section. Larger arrays, particularly those containing Muller glia, often spread across two to five sections.

Examination of a large number of labeled cells revealed differences in the appearance of cells stained for β-gal and AP activity. The X-gal reaction product was most prominent in cell bodies and less well-defined in cell processes. Process staining was apparent in the more darkly X-gal-stained cells, but more faintly stained cells sometimes failed to display process staining. Cells that stained for AP revealed the opposite pattern: processes were heavily stained and cell bodies often exhibited a ring pattern, suggesting that AP staining was membrane localized (Fig. 1). Occasionally, cells

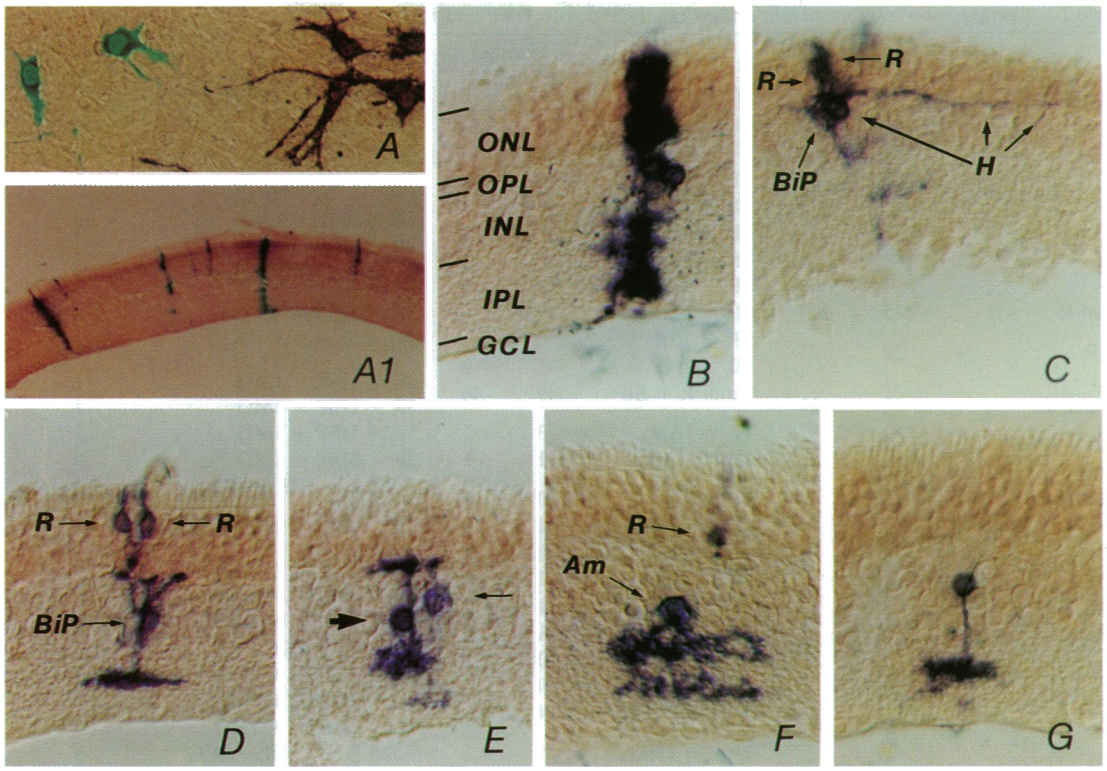

FIG. 1. AP staining of DAP-infected cells. (*A*) NIH 3T3 cells were coinfected with BAG and DAP viruses and stained for the presence of β-gal (blue) and AP (purple) 48 hr later. (A1-K) P0 mouse retinas were injected with BAG and DAP viruses. Mature mice were sacrificed and injected retinas were removed and stained for the presence of β-gal (blue) and AP (purple). Photomicrographs are of sections of retina displaying different retinal cell types. (*A1*) Two blue and five purple radial arrays. (*B*) Clone containing Muller glia. (*C*) Clone containing two rods, one horizontal cell, and one bipolar cell. (*D*) Clone containing two rods and one bipolar cell. (*E*) Neighboring cells with cone bipolar (thick arrow) and rod bipolar (thin arrow) morphology. (*F*) Amacrine cell and rod cell body. (*J*) Single amacrine cell. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; R, rod; BiP, bipolar cell; Am, amacrine cell; H, horizontal cell.

would be observed with both the nuclear and plasma membrane staining (Fig. 1*F*). Enhanced X-P/NBT process staining revealed details that were not obvious by using X-gal. For example, in some cases, two types of bipolar cells could be distinguished that may represent rod and cone bipolar cells (Fig. 1*E*). Furthermore, we also observed several examples of horizontal cells (Fig. 1*C*), a rare cell type that was not observed in BAG-infected retinas generated from postnatal infection in this or other studies (4).

Over 300 X-gal$^+$ and 300 X-P/NBT$^+$ radial arrays were examined and reconstructed. The analysis is enumerated in Table 1. These data show that the number and percentage of labeled cell types and the composition of single and multiple cell type arrays are very similar for both X-gal$^+$ and X-P/NBT$^+$ arrays. Both viruses labeled various cell types in essentially the same ratios after infection at P0. Small differences can be seen in the numbers of unidentified inner nuclear layer cells and Muller glia, which are probably a reflection of the different staining properties discussed above.

Confirmation of Clonal Identity. To determine what frequency labeled radial arrays were the result of the infection of neighboring progenitors by viral aggregates, BAG and DAP were cogrown and coconcentrated (at a ratio of 1:3) rather than being mixed after separate preparation and concentration as in previous studies (8, 13). This would enable the detection of the formation of viral aggregates, regardless of when they might form during the preparation of the inoculum. The stock was injected at P0 into mouse retina and mature infected retinas were processed for AP and β-gal enzyme activity. The retinas were examined for the presence of radial arrays composed of single blue cells, multiple blue cells, single and multiple purple cells, and arrays containing both blue and purple cells. In all cases, the color of individual cells within arrays was unambiguous and easily distinguished (Fig. 1*A1*). Of 1100 radial arrays, only 5 contained both blue and purple cells.

DISCUSSION

Characterization of Human Placental AP as a Retroviral-Encoded Reporter Gene for Lineage Mapping. Viral titers generated by stable ecotropic ψ-2 producer lines of DAP were relatively high for this class of vector (10^5–10^6 CFU/ml) and comparable to those generated by ψ-2-BAG lines. DAP viral titers are similar when measured by assays of G418 resistance or AP activity in infected NIH 3T3 cells. These observations indicate that the presence of *P ALP-1* in DO-L does not significantly interfere with any aspect of vector transmission. Moreover, as >90% of G418-resistant colonies were observed to possess AP activity, the simian virus 40 early promoter and the Moloney murine leukemia virus long terminal repeat are active in the same cells, much as was observed in BAG-infected cells.

A feature of AP staining that is of interest is the apparent membrane association of AP. In the human placenta, the enzyme leads to intense staining in membranes of the microvilli (43). In DAP-infected cells, staining is most intense around cell bodies as well as in processes. In terms of determining cell type and other interesting features of infected cells (e.g., projection patterns of neurons), this can be an improvement over the staining observed in BAG-infected cells.

Table 1. Composition of clones from retinas coinfected with DAP and BAG

Composition	Number B	Number P
1R	159	153
2R	34	41
3R	12	15
4R	6	5
5R	2	2
6R	1	1
10R	1	0
Total R	215	217
1BiP	22	25
2BiP	1	2
Total BiP	23	27
1Am	5	8
3Am	0	1
Total Am	5	9
MG	4	6
1UINL	8	4
2UINL	2	0
3UINL	1	2
4UINL	1	0
5UINL	0	1
Total UINL	12	7
1R + 1UINL	2	1
2R + 1UINL	2	0
Total R + UINL	4	1
1R + 1BiP	29	23
2R + 1BiP	8	10
3R + 1BiP	5	7
4R + 1BiP	3	1
5R + 1BiP	0	1
7R + 1BiP	0	1
1R + 2BiP	1	0
2R + 2BiP	2	0
3R + 2BiP	0	1
4R + 2BiP	1	0
Total R + BiP	49	44
1R + 1Am	3	1
1Am + 1BiP	1	1
1R + 1MG	0	1
2R + 1MG	1	0
3R + 1MG	0	1
4R + 1MG	1	3
5R + 1MG	1	1
6R + 1MG	1	3
7R + 1MG	0	1
12R + 1MG	0	1
Total R + MG	4	11
1BiP + 1MG	1	1
2UINL + 1MG	1	0
6UINL + 1MG	0	1
Total UINL + MG	1	1
1R + 1Bip + 1Am	0	1
3R + 3BiP + 1Am	1	0
Total R + BiP + Am	1	1
3R + 1BiP + 1UINL	0	1
1R + 1Am + 1UINL	1	0
1R + 1BiP + 1MG	1	0
3R + 1BiP + 1MG	1	0
5R + 1BiP + 1MG	1	0
7R + 2BiP + 1MG	0	1
Total R + BiP + MG	3	1
2R + 1BiP + 1Am + 1MG	1	0
2R + 1UINL + 1MG	0	1
3R + 2UINL + 1MG	0	1
4R + 3UINL + 1MG	1	0
5R + 1UINL + 1MG	0	1
6R + 1UINL + 1MG	1	0
6R + 2UINL + 1MG	1	1
7R + 3UINL + 1MG	0	1
Total R + UINL + MG	3	5
Total clones	331	336
UIPLS	8	20

Tabulations indicate the number of clones composed of the listed combinations of cell types. B, blue (identifies BAG-infected cells); P, purple (identifies DAP-infected cells); R, rod; BiP, bipolar cell; Am, amacrine cell; MG, Muller glia; UINL, unidentified inner nuclear layer cell; UIPLS, unidentified inner plexiform layer staining. Due to extensive staining of processes, MG figures indicate the number of clones containing one or more Muller glia.

The Presence of DAP Does not Interfere with Retinal Development. An initial concern was that a phosphatase could alter or interfere with development or function of infected cells. As an initial test of the neutrality of *P ALP-1*, we compared, in a quantitative fashion, retinal clones generated by BAG and DAP infection of postnatal mice. If the presence of *P ALP-1* was toxic to one or more populations of retinal cells or induced aberrant development, differences between BAG- and DAP-infected clones in the ratio and type of cells labeled would have been observed. However, both viruses labeled the same cell types in essentially the same percentage, ratio, and combination. Small differences were observed in the numbers of Muller glia and unidentified inner nuclear layer cells, which can be largely accounted for by the staining characteristics of BAG- and DAP-infected clones.

Definition of Clonal Boundaries. In previous studies of the retina, assignment of clonal boundaries was made on the basis of the strictly radial arrangement of labeled cells. As a test of this method, a dilution analysis was performed (4). The relationship of the number of blue radial arrays to the virus dilution after injection of serial dilutions of BAG virus was determined and was found to be first order. Clone size was approximately the same regardless of dilution. These data eliminated the scenario of independent virions infecting adjacent progenitors as a frequent phenomenon but did not address the possibility of infection of adjacent progenitors with viral aggregates. Coinfection of retinas with BAG and DAP allowed an assessment of the frequency of this latter phenomenon, together with the frequency of independent virions infecting adjacent progenitors.

If infectious virions frequently formed aggregates, if two independent particles frequently infected adjacent cells, and/or if helper virus were present in the inoculum, which could lead to infection of adjacent cells, then radial arrays of retinal cells would display both blue and purple cells at a significant rate. A low frequency of bicolored arrays is not unexpected at higher multiplicities of infection, as two independent viral particles in a helper-free inoculum may sometimes infect adjacent progenitors. In retinal infections, we observed a frequency of bicolored arrays of 0.44% (5/1100). This value will be an underestimate of the true frequency of incorrect assignment of clonal boundaries as sometimes >1 BAG or >1 DAP virions (rather than 1 BAG and 1 DAP virion) will infect adjacent cells. A closer approximation of the true frequency of multiple infection events that lead to a radial array can be calculated by the following formula (see *Appendix* for derivation):

$$\%\ \text{errors in assignment} = \frac{(\text{No. of bicolored arrays})/[2\,p(\text{B})p(\text{P})]}{\text{Total no. scored}},$$

where $p(\text{B})$ and $p(\text{P})$ refer to the proportion of the inoculum consisting of BAG and DAP. The relative titer of BAG and DAP used in the coinfection was 1:3 [$p(\text{B}) = 0.25$ and $p(\text{P}) = 0.75$], there were 5 bicolored arrays observed, and 1100 arrays were scored. Using these values, the percentage errors in clonal assignments was 1.2%. This value also places an upper limit of 1.2% as the frequency of aggregation, since this figure will include errors due to both aggregation and independent virions (including helper virus-mediated spread) infecting adjacent progenitors. An error rate of 1.2% does not affect the interpretation of clones that are frequently found in a large data set, such as has been generated for the retina. However, as with any experimental procedure that relies in

some way on statistical analysis, rare associations of cell types must be interpreted with some caution.

Since both multiplicity of infection and the local architecture at an injection site can affect viral distribution (i.e., cause pooling or trapping of virions), the error rate for each tissue that is lineage mapped must be independently derived. In addition, although the 1.2% error rate indicates that aggregation does not occur frequently in the preparations tested here, inocula prepared by different methods may vary in this parameter.

APPENDIX

The proportion of radial arrays that are generated by two viruses (hits) can be calculated from the first principles of probability.

$$\text{Proportion of 2-hit arrays that are blue} = p(\text{B})p(\text{B})$$

$$\text{proportion of 2-hit arrays that are purple} = p(\text{P})p(\text{P})$$

$$\text{proportion of 2-hit arrays that are bicolored} = p(\text{B})p(\text{P}) + p(\text{P})p(\text{B}) = 2p(\text{P})p(\text{B}),$$

where

$$p(\text{B}) = \frac{\text{no. of blue arrays}}{\text{no. of blue arrays} + \text{no. of purple arrays}},$$

and

$$p(\text{P}) = \frac{\text{no. of purple arrays}}{\text{no. of blue arrays} + \text{no. of purple arrays}}.$$

Since arrays that are generated by two viruses will only be scored as such if they are bicolored, the observed number of such 2-hit arrays will be only a proportion of the total number (T) of 2-hit arrays.

$$\text{No. observed} = 2p(\text{B})p(\text{P})T.$$

Thus,

$$T = \frac{\text{No. observed}}{2p(\text{B})p(\text{P})}.$$

The error rate in assigning clonal boundaries is then

$$\frac{T}{\text{total no. of arrays}}.$$

In the case presented here, the ratio of DAP to BAG virus was 3:1, and there were 1100 arrays scored, with 5 blue + purple arrays

$$p(\text{P}) = 0.75,\ p(\text{B}) = 0.25,\ \text{observed} = 5,$$

$$\text{total arrays scored} = 1100.$$

The percentage error in assignment of clonal boundaries is 1.2%.

This analysis is true if we assume that the formation of aggregates, or the infection of adjacent progenitors, is not affected by the nature of the genome carried within the virion. This assumption is reasonable as both BAG and DAP virions are produced by ψ-2 packaging cells, cogrown on the same dish, and ψ-2 produced capsids are identical, regardless of which genome is carried within them.

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