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Non-random spatial arrangement of clone sizes in chimaeric retinal pigment epithelium

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SUMMARY

Clonal analysis of whole-mount preparations of entire retinal pigment epithelium (RPE), using SWR ↔ C57BL/6JLac and DDK ↔ C3H/Bi mouse aggregation chimaeras in which one of the two parental components predominated, revealed a markedly non-random spatial arrangement of patch (clone) sizes. Single-cell and small patches predominated in an area around the optic nerve head while large patches occurred most frequently near the periphery. Mechanisms are discussed which may explain these results. Patch size frequency distributions were concave and skewed. Singletons were the most frequent size class, but a wide range of sizes and a smaller number of much larger patches were also always found. The results preclude the use of statistical methods previously employed to calculate clone sizes from the geometric means of observed patch sizes. Instead, the median and interquartile range may provide the best summary of the observed patch size frequency distributions. Our findings support a stochastic model of tissue growth.

INTRODUCTION

The retinal pigment epithelium (RPE) is a monolayer of cells which begins at the edge of the optic nerve head and extends peripherally to the ora serrata, overlying the neural retina (Zinn & Marmor, 1979). The RPE has received considerable attention in studies of clonal growth using chimaeric mice in which one of the component strains carries an albino marker and therefore lacks intracellular melanin granules (Tarkowski, 1964; Mintz & Sanyal, 1970; Mintz, 1971*a*; Deol & Whitten, 1972*a*; Sanyal & Zeilmaker, 1977; West, 1976, 1978). Interpretation of the observed mosaicism has, however, remained unsatisfactory (see below and West, 1978, for discussion), and led us to re-examine the issue of clonal growth in chimaeric RPE.

RPE and neural retina are derived from the retinal field which, following its induction in the neural ectoderm, evaginates from the prosencephalon as the optic vesicle (Coulombre, 1979). On contact with the head ectoderm the vesicle invaginates to form the double-layered optic cup. The inner wall of the cup is the presumptive neural retina while the outer wall, induced by periocular mesenchyme, becomes the RPE. During early embryogenesis cell proliferation is

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observed in both retinal layers, but following induction of RPE by the periocular mesenchyme, mitotic activity in the RPE ceases abruptly and almost completely. Consequently, as the neural retina continues to grow, the concurrent increase in area of the RPE is achieved predominantly by an increase in the volume and area (cell flattening) of its cells and not by cell proliferation. The timing of the drastic withdrawal from mitosis coincides with the onset of melanogenesis (Coulombre, 1979), which in the mouse is the 11th day of gestation (Deol & Whitten, 1972*a*). A small number of mitoses, however, occurs throughout the later developmental period (Coulombre, 1979) and also in the adult (Tso, 1979). Information on the spatial distribution of these mitoses is not available.

Previous studies of chimaeric RPE set out to determine the contributions of cell mingling and coherent clonal growth during development of this epithelium. A 'coherent clone' is a group of cells which are descended from a common progenitor cell through previous divisions and which have stayed together as a coherent group. A 'descendent clone', by contrast, is a group of cells related by descent from a common progenitor, but which may have separated by cell mixing and are therefore no longer contiguous. In chimaeras with balanced proportions of components, individual clones may be obscured by aggregation into larger patches, but in chimaeras in which one component predominates, each patch may be regarded as a single coherent clone, and clusters of patches may possibly be recognized, which can be interpreted as single descendent clones (West, 1975; Whitten, 1978; Schmidt, Garbutt, Wilkinson & Ponder, 1985*a*; Schmidt, Wilkinson & Ponder, 1985*b*). Using an algebraic solution of the relationships between the number of equal-sized clones in a patch and the proportions of the two components (West, 1975), West (1976) estimated the mean size of coherent clones in the RPE from the observed geometric mean of patch sizes, assuming that coherent clone sizes conform to a geometric distribution and that coherent clones are randomly distributed. Mintz & Sanyal (1970) and Mintz (1971*a*), inspecting patchiness in whole-mount preparations of RPE, suggested that 10 RPE precursor cells give rise to 10 clonally distinct sectors by rapid proliferation from the centre of the RPE. Sanyal & Zeilmaker (1977) suggested that their data partly supported the idea of 20 alternating sectors. West (1978) used Sanyal & Zeilmaker's (1977) data on sector numbers with the proportions of each genotype to estimate the number of descendent clones in the RPE, but found large discrepancies between results for different eyes. The underlying assumptions in any of these studies of

Table 1. *Sources of RPEs and percentage contributions of component strains*

Chimaera No.	Strain combination	Age	Percentage of minority component
87	SWR↔B6	10 days	5·5 (B6)
88	SWR↔B6	15 days	4·7 (B6)
97	SWR↔B6	2 days	5·0 (B6)
106	DDK↔C3H	4 months	8·8 (C3H)

chimaeric RPE were not critically examined. A random distribution of clones on the one hand, and a number of sectors derived from a fixed number of progenitors which assumes a deterministic mode of tissue growth on the other, are in contrast to results from our previous quantitative clonal analyses of chimaeric intestinal epithelium (Schmidt *et al.* 1985*a*,*b*). We therefore decided to re-examine chimaeric RPE, to see whether the previous assumptions and the conclusions based on them were justified.

MATERIALS AND METHODS

C57BL/6JLac (B6) and SWR mice were obtained from the National Institute for Medical Research, Mill Hill, London, U.K. SWR↔B6 chimaeras were constructed at this Institute by aggregation of 4- to 8-cell embryos according to methods described by Mintz (1971*b*). The aggregated embryos were brought to term and reared by B6 × DBA/2Lac F_1 hybrid foster mothers (F_1 hybrids were also obtained from the National Institute for Medical Research). The DDK↔C3H/Bi (C3H) chimaera was kindly provided by Dr M. Buehr, MRC Mammalian Development Unit, London.

The ages of the mice and the relative proportions (see below) of the parental components are given in Table 1. In order to avoid the effects of patch aggregation (see Introduction), we restricted our analysis to mice with highly unbalanced proportions. The animals chosen were the most unbalanced out of 32 chimaeras examined, with the pigmented parental genotype being in each case the minority component.

Preparations

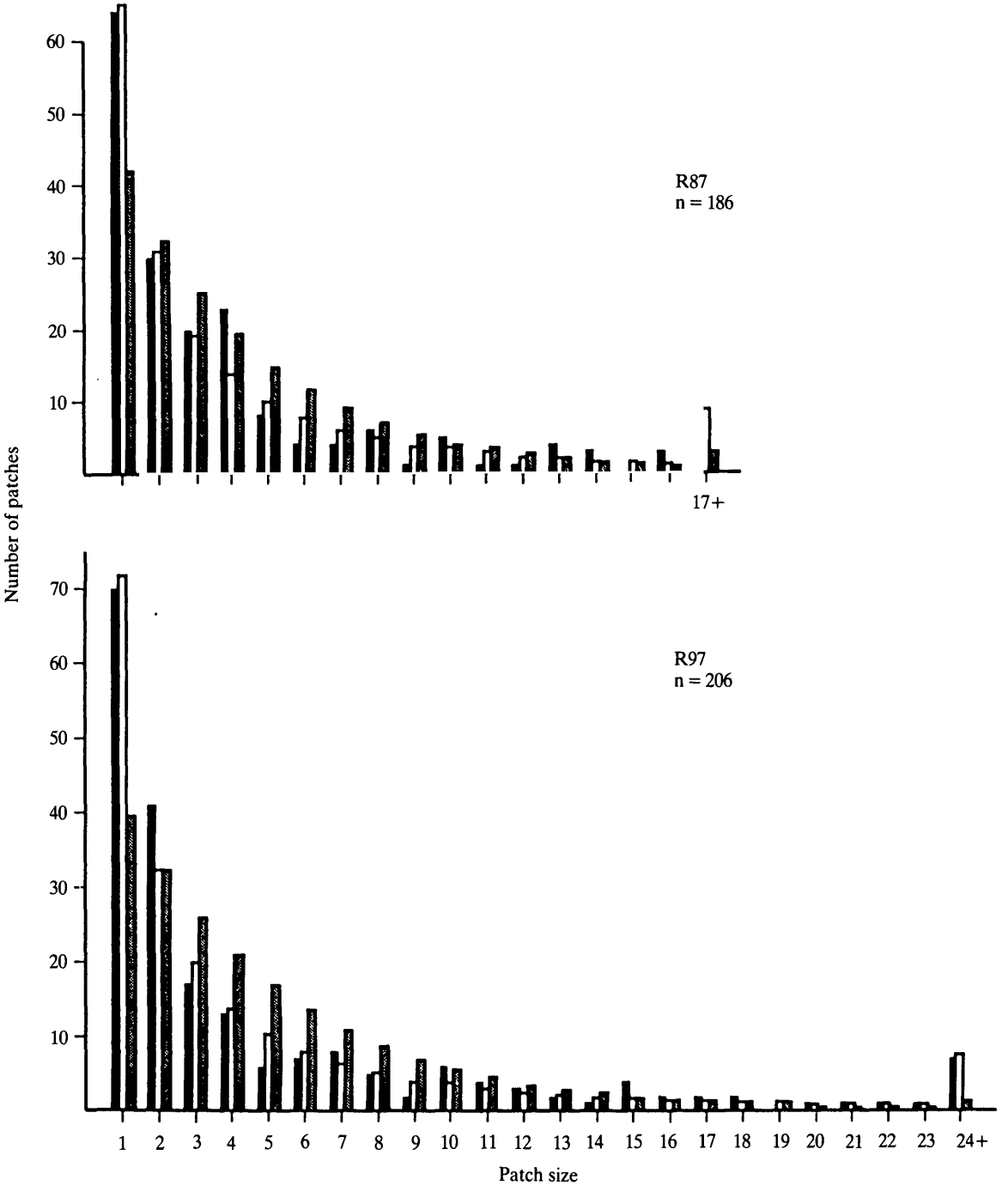
Animals were killed by ether overdose. Eyes were excised and an incision made in the cornea with a scalpel. The eyes were then fixed in 10 % formol saline overnight (or for longer periods), which detaches the neural retina from the RPE (Tso & Friedman, 1967). Subsequent micro-dissections were carried out using a wax-based Petri dish and a Kyowa binocular microscope; the eye was cut along the ora serrata (referred to as the 'periphery' in the text) using a pair of fine scissors (Prof. Kinmoth's scissors, Macarthy's Surgical Ltd, Dagenham, Essex). Cornea, lens and vitreous body were discarded, and the choroid membrane cleared from attaching muscles. The neural retina was carefully peeled away with the aid of fine dissecting forceps (Micro-Surgery, Serr. 4″, Macarthy's Surgical Ltd) and scissors (see above), exposing the RPE. Following four or five equally spaced radial cuts it was possible to obtain flat mounts of RPE.

Analyses

One eye from each chimaera was used in the quantitative analyses. Pigmentary differences served as a chimaeric, strain-specific marker: DDK or SWR (unpigmented: albino), B6 or C3H (pigmented). Numbers of B6 or C3H cells per patch were counted (patch size) for entire RPE samples. Binucleate cells were frequent; however, cells were scored by their outlines and a binucleate cell was therefore scored as a single cell. Patchy pigmentation did not occur in control, non-chimaeric RPE.

The percentage contribution of the minority component (B6 or C3H) was calculated by the line interception method (Aherne & Dunhill, 1982). The entire preparation was scanned under ×80 magnification (10/0·25 objective) and the number of interceptions of a 10×10 eyepiece graticule overlying pigmented cells was scored. This number, divided by the total number of points sampled (500–600 for each RPE), ×100, yielded an estimate of the percentage of the minority component.

The size frequency distribution of patches (in tissues in which one chimaeric component largely predominates; see Introduction) should conform to geometric (cf. Schmidt *et al.* 1985*a*) if clones were derived through proliferation of randomly spaced progenitors under conditions where each cell had an equal probability of dividing in any given time period (non-differential proliferation), and there was no disruption of patches by cell mingling. This model was assumed


R87
n = 186
17+
R97
n = 206
Number of patches
Patch size
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24+

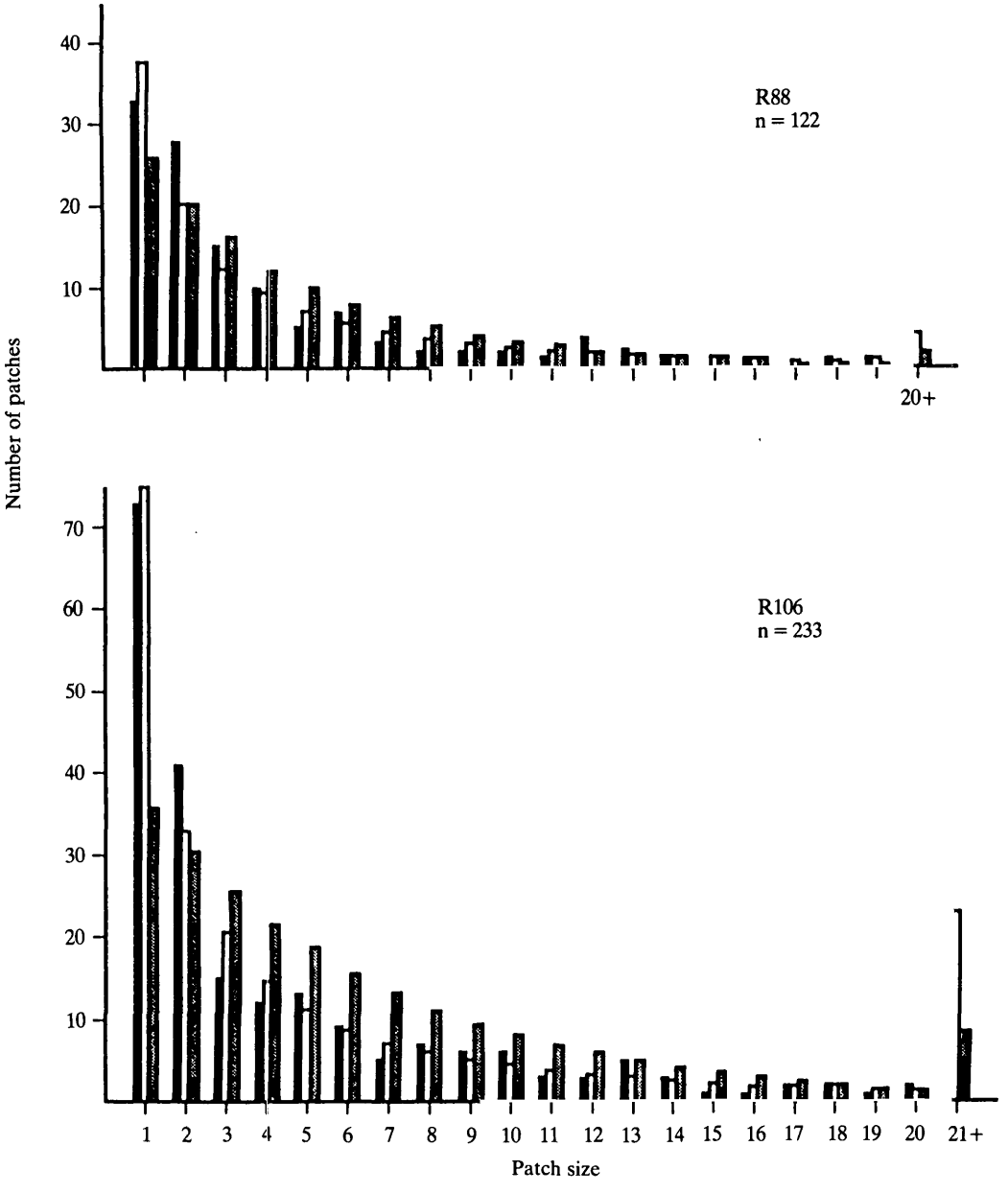


Fig. 1. Patch size frequency distribution of discrete patches of the minority component (cf. Table 3) in four chimaeric RPEs. The observed distributions (solid bars) departed significantly from fitted geometric distributions (dotted bars) (significance tested at the 5% level; χ^2 values of all fitted distributions are given in Table 2). The lack of fit is due to the considerable skewness of the distributions at both extremes, yielding highly concave curves and good fitted values to a negative binomial model (open bars).

in previous statistical analyses of chimaeric RPE (West, 1976, 1978). If there was differential growth of some clones, for example because of local environmental differences, or cell mixing, causing fragmentation of clones, a more skewed distribution of clone sizes would be obtained. In this case the observed data would no longer fit a geometric, but might conform to a negative binomial model (cf. Schmidt *et al.* 1985*a*). Either result would be compatible with a stochastic but not deterministic mode of tissue growth. Using the method of maximum likelihood, geometric and negative binomial distributions were fitted to the size frequency data and tested for goodness of fit by means of χ^2-test (Ross, 1980).

The relationship between patch size and position in the RPE was analysed with the aid of an eye piece graticule and a Leitz microscope (Laborlux 12; Wetzlar), by scoring the linear distance of the patch centres from the edge of the optic nerve head together with the sizes of each patch. A computer program, 'Minitab' (Ryan, Joiner & Ryan, 1981), was employed to divide the linear distances into 4, and to establish patch size frequency distributions for each resulting concentric zone. Results were summarized in box-plot diagrams to facilitate comparisons (Ryan *et al.* 1981) (see legend to Fig. 3 for explanation).

Photographs were taken on a Zeiss photomicroscope using Ilford Pan F, 50 ASA film.

RESULTS

The range of patch sizes in all specimens was considerable (Fig. 1). All distributions revealed a preponderance of singletons. The curves were markedly concave. The skewness, reflected by the high numbers of observations at both extremes of the distributions, was greater than predicted by a geometric while conforming to a negative binomial model, the latter consequently giving a better χ^2-fit (Table 2).

Table 3 shows that the geometric mean of patch sizes is greatly affected by the extreme values of the skewed distributions and consequently the geometric mean may vary considerably between retinas (cf. retina 106). Moreover, the wide spread of the data is apparent from the large standard deviations, but not from the standard errors (which are often given in quantitative studies of chimaeric tissues – cf. West, 1976; Oster-Granite & Gearhart, 1981; Weinberg, Howard & Iannaccone, 1985) because the standard errors are small when the number of observations is large. Our analysis suggests that the median patch size and the interquartile range may provide a representative summary of the observed size frequency distributions of patch sizes, if a summary is sought, while retaining some indication of the range of values observed (Table 3).

Table 2 *Goodness of fit of observed patch size frequency distributions (Fig. 1) to theoretical models*

Chimaera No.	χ^2 (d.f.)	
	Negative binomial	Geometric
87	22·72 (14)	50·85 (15)*
88	12·47 (17)	25·97 (18)
97	14·16 (21)	67·87 (22)*
106	9·49 (18)	85·67 (19)*

* Deviation at the 5% level.

Table 3. *Statistical details of observed patch size frequency distributions*

	Chimaera			
	87	88	97	106
Number of patches	194	122	206	233
Patch sizes				
minimum value	1	1	1	1
maximum value	82	79	55	150
Median	3	2·5	2	3
Lower quartile	1	1	1	1
Upper quartile	6	6	7	8·5
Geometric mean	5·5	5·6	5·5	8·9
Standard deviation	9·3	9·5	8·0	17·0
Standard error	0·7	0·9	0·6	1·1

The quartile points mark the 75 % (lower quartile) and 25 % (upper quartile) points of the distributions shown in Fig. 1.

The range of patch sizes found was not randomly distributed over the entire RPE; instead, single cell patches predominated in the area adjacent to the optic nerve head (zone 1; Figs 2, 3). Although single cell patches also occurred in other parts of the RPE, the particularly high numbers of singletons together with the exclusion of large patches were a marked and consistent distinguishing feature between zone 1 and the remaining areas of the RPE. There was a tendency for the median and the range of patch sizes continuously to increase with increasing distance from the optic nerve head, with the exception of retina 88 (Fig. 3).

The shapes of the larger patches were irregular although longitudinal orientations, i.e. expansions perpendicular to the ora serrata, were apparent in the peripheral region (cf. Fig. 2), as observed by others (Mintz, 1971*a*; West, 1976).

DISCUSSION

Our results revealed a striking non-random spatial distribution of sizes of clones in the RPE. A similar distribution pattern of pigment cells in mosaic RPEs was reported by Deol & Truslove (1981, 1983) for non-chimaeric mice which were heterozygous for autosomal unstable genes (p^{un}; c^{m}) affecting melanogenesis. Possible explanations for the observed pattern include a higher frequency of cell death around the optic nerve head than elsewhere in the RPE, and less cell mixing or more proliferation during morphogenesis at the periphery giving rise to larger clones in that region. Reports in the literature indicate that the periphery is the zone of greatest growth, which supports the latter interpretation, although no reports on the spatial distributions of cell labelling indices are available (Mund & Rodrigues, 1979). There is no information regarding cell mixing or cell death in the RPE.

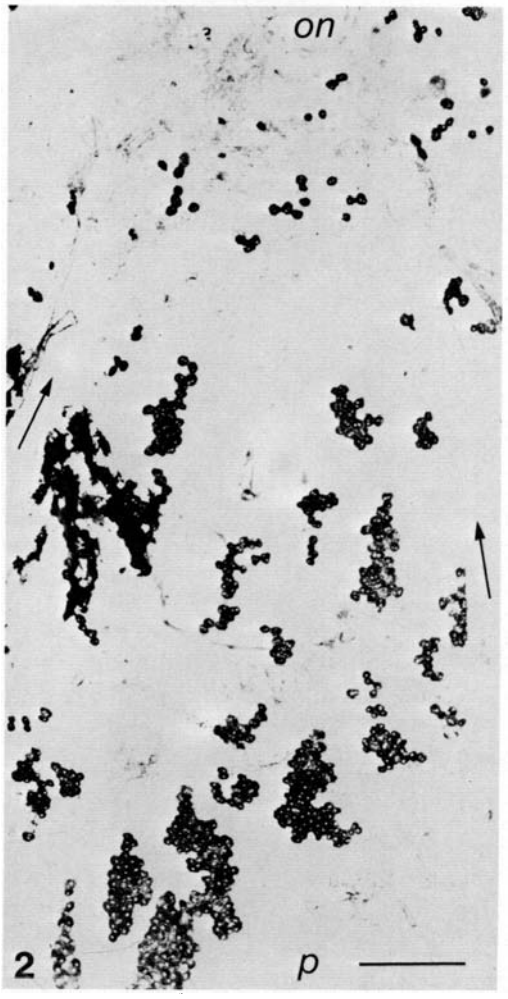


Fig. 2. Part of whole-mount preparation of RPE 106 (DDK↔C3H), showing discrete coherent patches of C3H cells. Singletons and small patches predominate in the zone adjacent to the optic nerve head (*on*) where large patches are not found (cf. Fig. 3). Large patches often show an oblong shape, perpendicular to the periphery (*p*). The arrows indicate radial incisions which were necessary to obtain flat preparations. Some patches near the arrow on the left are obscured by pigmentation from the choroid membrane. This was, however, rarely found in the preparations. The largest patch at the bottom may comprise two coherent clones. Bar equals 0·5 mm.

The 'sectors' described by Mintz & Sanyal (1970), Mintz (1971*a*), and Sanyal & Zeilmaker (1977) imply a deterministic model of growth, particularly if they are regarded as spatially fixed geometric patterns with a basic plan of 10 sectors derived from 10 progenitor cells (Mintz, 1971*a*). Our results, however, favour a highly variable, stochastic mode of tissue growth (see below) compatible with the idea that the 'sectors', or stripes, may simply represent descendent clones of variable sizes, as suggested by West (1978). There is no need to invoke more than incomplete mixing ('limited coherent growth') and a tendency for 'outwards'

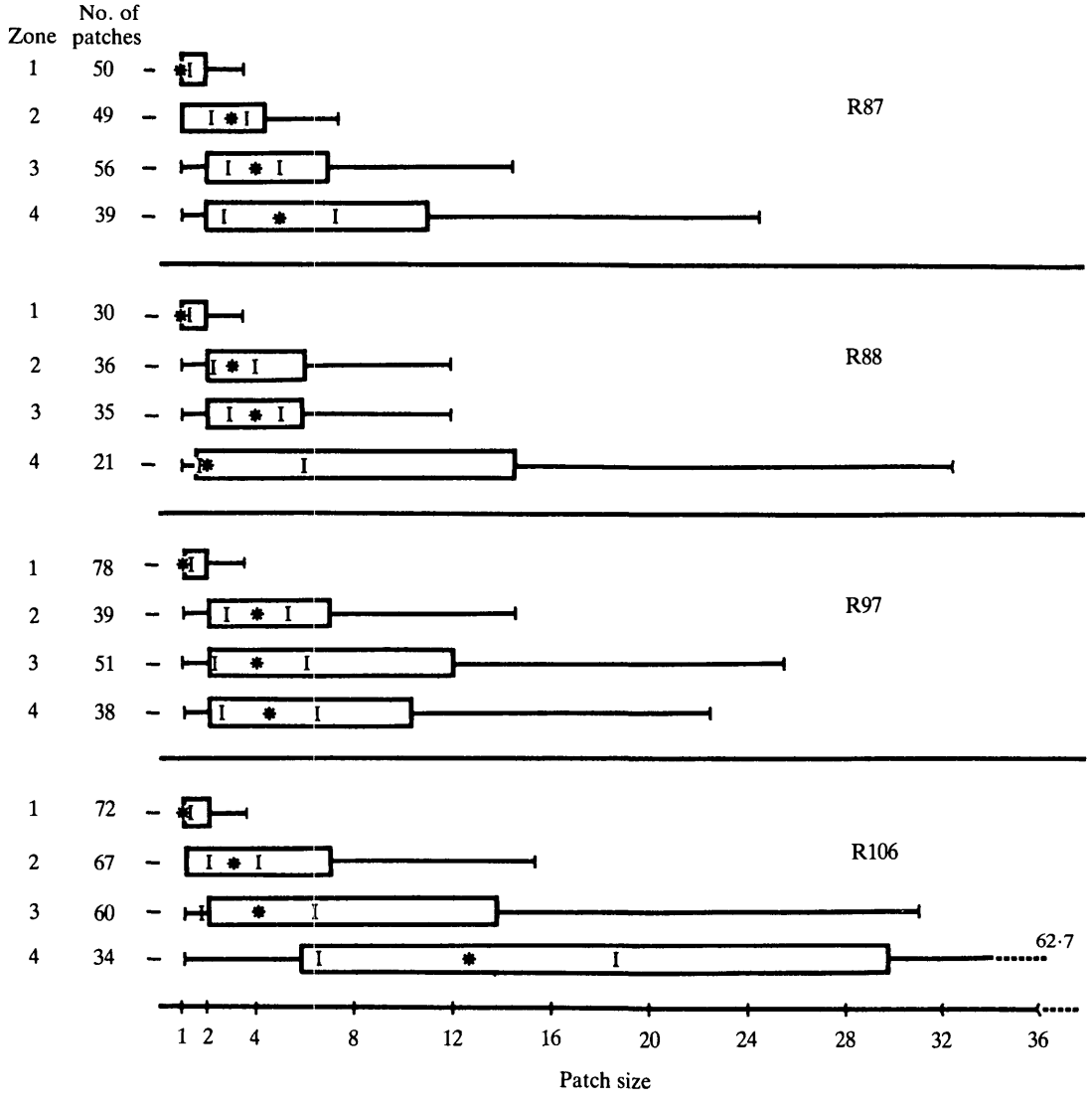


Fig. 3. Box plots of patch size frequency distributions for each of four different zones of the chimaeric RPEs, zone 4 being the most peripheral (towards the ora serrata). The interquartile range (cf. Table 3) is given by the box, the 90 % spread of the data is indicated by box + horizontal bars; *, median; I ... I, 95 % confidence interval of the median. A consistent pattern is seen: singletons predominate in zone 1 (yielding a median patch size of 1), and there is a tendency for patch size ranges to increase distally, the largest patches occurring in zones 3 and 4.

growth to be more pronounced than circumferential growth to create the impression of striping.

Previous statistical analyses and estimations of mean clone sizes in the RPE (West, 1976, 1978) were made on the assumption of regular-shaped, randomly arranged clones with a range of sizes conforming to a geometric distribution

(West, 1978). In fact, none of these assumptions is probably true. In particular, our data show that there is a great range of coherent clone sizes, and size frequency distribution which conforms to negative binomial. The negative binomial fit precludes the use of the algebraic solution which has previously been used to calculate coherent clone sizes from observed patch sizes, because this was based on the geometric mean, and on the assumption of a geometric distribution of patch sizes. The data in Table 3 and Fig. 3 demonstrate, moreover, that the geometric mean is greatly affected by the extreme values of a skewed distribution, and so is unsuitable as a summary of the patch sizes in a tissue. The range of patch sizes is also obscured by the standard error, but is apparent from the standard deviation. Our data suggest that if a summary of the patch size frequency distributions is required, e.g. for comparison between different tissues, the median and the interquartile range may be most representative.

While the great range of patch sizes is consistent with a stochastic rather than a deterministic mode of tissue growth, the negative binomial distribution might have arisen in a variety of ways (Ross, 1980), and is therefore, on its own, of relatively little value in identifying the causes for the observed distribution of patch sizes. In this context, the general appearance and the spatial arrangement of patches may prove to be more informative. For example, any of the following factors may have contributed to the good fit to negative binomial: (1) Cell mingling. This may disrupt small clones to a greater extent than larger ones (differential cell mixing) (Lewis, 1973). Although such a process could only act during early embryogenesis when permanent contacts between RPE and neural retina have not yet been established (Coulombre, 1979), some cell mingling and fragmentation of patches is suggested by the appearance of the patches in all preparations (cf. Fig. 2). Fragmentation would, however, have reduced the range of patch sizes and is therefore unlikely to have played a prominent role in generating negative binomial as opposed to geometric patch size frequency distributions. (2) Clone aggregation. Although we examined only RPEs in which unpigmented cells predominated, some patches of pigmented cells may have constituted more than one coherent clone: particularly so as the spatial arrangement of patches was non-random. Such potential aggregation of clones could have contributed to the variation in patch sizes. This may be especially true for chimaera 106, which had the highest percentage of pigmented cells and also the greatest range of patch sizes. (3) Unequal probabilities of cell proliferation or cell death within the whole population. Our observation of smaller patches in the centre and larger patches at the periphery of the RPE is consistent with regionally determined, differential cell proliferation or cell death. This could yield highly skewed patch size frequency distributions, such as those observed in the present study.

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REFERENCES

AHERNE, W. A. & DUNHILL, M. S. (1982). *Morphometry*. London: Arnold.

COULOMBRE, A. J. (1979). Roles of the retinal pigment epithelium in the development of ocular tissues. In *Retinal Pigment Epithelium* (ed. K. M. Zinn & M. F. Marmor), pp. 53–57. Cambridge, MA: Harvard University Press.

DEOL, M. S. & TRUSLOVE, G. M. (1981). Non-random distribution of unpigmented melanocytes in the retina of chinchilla-mottled mice and its significance. In *Pigment Cell*, vol. 6 (ed. M. Seiji), pp. 153–157. University of Tokyo Press.

DEOL, M. S. & TRUSLOVE, G. M. (1983). The effects of the pink-eyed unstable gene on the retinal pigment epithelium of the mouse. *J. Embryol. exp. Morph.* **78**, 291–298.

DEOL, M. S. & WHITTEN, W. K. (1972*a*). Time of X chromosome inactivation in retinal melanocytes of the mouse. *Nature, New Biol.* **238**, 159–160.

DEOL, M. S. & WHITTEN, W. K. (1972*b*). X-chromosome inactivation : does it occur at the same time in all cells of the embryo? *Nature, New Biol.* **240**, 277–279.

LEWIS, J. (1973). The theory of clonal mixing during growth. *J. theor. Biol.* **39**, 47–54.

MINTZ, B. (1971*a*). Clonal basis of mammalian differentiation. In *Control Mechanisms of Growth and Differentiation, Symposia of the Society for Experimental Biology,* **25** (ed. D. D. Davies & M. Balls), pp. 345–370. London : Cambridge University Press.

MINTZ, B. (1971*b*). Allophenic mice of multi-embryo origin. In *Methods in Mammalian Embryology* (ed. J. Daniel), pp. 186–214. San Francisco: Freeman.

MINTZ, B. & SANYAL, S. (1970). Clonal origin of the mouse visual retina mapped from genetically mosaic eyes. *Genetics* **64** (Suppl.), 543–544.

MUND, M. L. & RODRIGUES, M. M. (1979). Embryology of the human retinal pigment epithelium. In *The Retinal Pigment Epithelium* (ed. K. M. Zinn & M. F. Marmor), pp. 45–52. Cambridge, MA: Harvard University Press.

OSTER-GRANITE, M. L. & GEARHART, H. (1981). Cell lineage analysis of cerebellar Purjinke cells in mouse chimaeras. *Devl Biol.* **85**, 199–208.

ROSS, G. J. S. (1980). MLP (*Maximum Likelihood Program*). Harpenden: Rothamstead Experimental Station.

RYAN, T. A., JOINER, B. L. & RYAN, B. F. (1981). *Minitab*. Pennsylvania: Statistics Department, Pennsylvania State University.

SANYAL, S. & ZEILMAKER, G. H. (1977). Cell lineage in the retinal development of mice studied in experimental chimaeras. *Nature, Lond.* **265**, 731–733.

SCHMIDT, G. H., GARBUTT, D. J., WILKINSON, M. M. & PONDER, B. A. J. (1985*a*). Clonal analysis of intestinal crypt populations in mouse aggregation chimaeras. *J. Embryol. exp. Morph.* **85**, 121–130.

SCHMIDT, G. H., WILKINSON, M. M. & PONDER, B. A. J. (1985*b*). Detection and characterisation of spatial pattern in chimaeric tissue. *J. Embryol. exp. Morph.* **88** (in press).

TARKOWSKI, A. K. (1964). Patterns of pigmentation in experimentally produced mouse chimaeras. *J. Embryol. exp. Morph.* **12**, 575–585.

TSO, O. M. (1979). Developmental, reactive and neoplastic proliferation of the retinal pigment epithelium. In *The Retinal Pigment Epithelium* (ed. K. M. Zinn & M. F. Marmor), pp. 267–276. Cambridge, MA: Harvard Universal Press.

TSO, O. M. & FRIEDMAN, E. (1967). The retinal pigment epithelium. I. Comparative histology. *Arch. Ophthalmol.* **78**, 641–644.

WEINBERG, W. C., HOWARD, J. C. & IANNACCONE, P. M. (1985). Histological demonstration of mosaicism in a series of chimaeric rats produced between congenic strains. *Science, N.Y.* **227**, 524–527.

WEST, J. (1975). A theoretical approach to the relation between patch size and clone size in chimaeric mice. *J. theor. Biol.* **50**, 153–160.

WEST, J. D. (1976). Clonal development of the retinal epithelium in mouse chimaeras and X-inactivation mosaics. *J. Embryol. exp. Morph.* **35**, 445–461.

WEST, J. D. (1978). Chimaeras and Mosaics. In *Development in Mammals*, vol. 3 (ed. M. H. Johnson), pp. 413–460. North Holland: Elsevier.

WHITTEN, W. R. (1978). Combinatorial and computer analysis of random mosaics. In *Genetic Mosaics and Chimaeras in Mammals* (ed. L. B. Russell), pp. 445–463. London: Plenum Press.
ZINN, K. M. & MARMOR, M. F (eds) (1979). *The Retinal Pigment Epithelium.* Cambridge, MA: Harvard University Press.

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