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The clonal model of vertebral column development: a reinvestigation of vertebral shape using Fourier analysis

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SUMMARY

Little work has been done on the skeletal system of allophenic mice (chimaeras). In this paper we re-examine the evidence presented for the clonal theory of vertebral development using quantitative methods which take account of variation. We conclude that the clonal theory is either an overly simplistic approximation of the real situation, or that the evidence so far given in its support is unsubstantiated.

INTRODUCTION

Although four-parent allophenic (chimaeric) mice have been used extensively in developmental research in recent years (Le Dourain & McLaren, 1984; McLaren, 1976) the skeleton has been largely neglected. In 1972 Moore & Mintz proposed a clonal model for the development of the vertebral column and skull based upon the study of allophenic mice. They argued that the specialized cell populations comprising the skeleton are likely to be the descendents of fewer precursor cells, such an assembly of cells derived from an initiator cell being termed a clone. They therefore looked for clones resembling those seen in coat melanoblasts, hair follicles, and the retina (Mintz, 1967, 1969, 1971; Mintz & Sangal, 1970) in the vertebrae and skulls of allophenic mice. They suggested that, since clonal development of the dermatome and myotome has been demonstrated (Mintz, 1967, 1970; Mintz & Silvers, 1970; Mintz & Baker, 1967) it may well be present in the sclerotome also. They presented evidence that the vertebra is multiclonal in origin and may arise from as few as four mitotic cell lineages.

Moore & Mintz (1972) were constrained by the technology available at the time and had to base their classification of vertebrae upon visual inspection of anterior views (personal communication from W. J. Moore); they could thus take no account of variation or the full three-dimensional aspects of shape. We have repeated their study using different allophenic mice and automated methods of comparison based upon Fourier analysis of videodigitized bones (Johnson, 1985; Johnson *et al.* 1986) again viewed anteriorly in order to test their model. The

Key words: shape, mouse, vertebra, allophenic, computer, Fourier, clonal model.

present paper presents these new data, compares them with those of Moore & Mintz (1972) and discusses their bearing on the clonal theory of vertebral development.

MATERIALS AND METHODS

The mice used in this study were 16 chimaeras between a multiple dominant (DOM; C57BL×C3H) and a multiple recessive stock (REC; homozygous for non-agouti (*a*), brown (*b*), chinchilla (c^{ch}), dilute (*d*), pink eye (*p*), short ear (*se*) vestigial tail (*vt*), waved-2 (*wa-2*) and supernatant–NADP IDH type a ($ID\text{-}1^{a}$)). Individuals from the parental stocks (at least, 16DOM,25REC) were used as controls. These mice were described by McLaren & Bowman (1969) and the skeletal preparations formed part of the data of Grüneberg & McLaren (1972). These preparations were loaned by the British Museum (Natural History).

The cervical and first two thoracic vertebrae were digitized using the technique of Johnson *et al.* (1986). Essentially the Cartesian coordinates of points on the outline of the shape were first transformed to a series of 256 polar coordinates that have the centre of mass as centroid and the origin mathematically defined from the mean position of the least squares fitted (Sneath, 1967) vertebrae (Johnson *et al.* 1986). These polar coordinates were then submitted to a Fast Fourier transform producing a series of coefficients that express the relative amplitudes of a series of sinusoidal waveforms which, when summated, accurately reconstitute the original outline. There are two advantages in measuring shapes in this way; first the harmonic analysis of form considerably reduces the dependence of the measurement process on the definition of homologies, secondly, the asymmetric aspects of shape are expressed by the sine components of the Fourier series. We derived 127 Fourier components (64 cosine, 63 sine – the first sine component = 0). Selected Fourier components were then subjected to multivariate analysis.

Discrimination between groups

We have shown (Johnson *et al.* 1986) that the first 30 sine and cosine components in the Fourier series reconstruct vertebral shape well and the first 15 cosine components almost as well, but without information concerning asymmetry. These components also allow good discrimination between dissimilar groups of bones (e.g. the parental strains). It occurred to us on investigating the frequency of significantly different components, and the size of significant differences between groups, that better discrimination might be obtained by using components selected in order of their discriminating ability (F ratio) rather than in order of their appearance in the Fourier series. (The F ratio (Steel & Torrie, 1980) is superior to the usual *t*-test in that it computes the ratio of the variances for each sample rather than assuming them to be equal.) This was indeed so and was demonstrated by multiple iteration of a computer program where tenths of the sample, chosen at random, were tested against the rest and the number of misclassifications noted (Johnson *et al.* 1986). The number of variates chosen was that which gave the least number of misclassifications.

Individual chimaeras were then tested for their fit to parental strain means by multivariate statistical analysis. This yielded a table of generalized distances. In such a table based on the analysis of n variates an individual has a 95 % chance of belonging to a group if it lies within $\pm 2\chi$ standard deviations of the mean, χ being chosen with $n-1$ degrees of freedom. Individual chimaeras could thus be assigned to one or other of the following groups; like the DOM parent, like the REC parent, like both parental types, or like neither parental type.

Asymmetry about the line startpoint–centroid–(startpoint+180°) in the Fourier series is expressed in the sine components (Zahn & Roskies, 1972; Lestrel, 1974). Asymmetry was thus tested by compiling an asymmetry index. This consisted of the sum of the absolute values of the sine components divided by the number of sine components derived (63). This statistic was computed for each parental strain and for each individual chimaera.

RESULTS

The vertebrae analysed here differ from each other in both size and shape. Since we have equalized the areas the results that follow deal with the residual shape information. Information regarding shape is contained in a series of Fourier coefficients each dealing with a particular aspect of the shape. In some cases a particular component, which has a distribution and hence a mean and a standard deviation, will not differ between shapes in a statistically significant manner. In others the difference will be highly significant. The significance of the difference in each coefficient (expressed as an F ratio) between dominant and recessive parental strains (DOM and REC respectively) for C1 and C6 is illustrated in Fig. 1. For each vertebra some coefficients show highly significant differences whilst others do not. Which coefficients differ is a function of vertebral shape. The lower coefficients represent 'simple', lower frequency, aspects of the shape, the higher coefficients more 'complex', higher frequency aspects. It is not surprising that the significant coefficients for a simple shape, such as C1 are at the low end of the range whilst those for the more complex C6 include much higher coefficients. There is also a marked harmonic relationship between significant coefficients. This is again not surprising – a shape which is distinctive because of its 4 lobedness might be expected also to be distinctive in features relating to 2 lobedness.

The coefficients with the highest F values will be the best shape discriminators. The number of discriminators necessary to give the best distinction between shapes depends on the complexity of their outlines. In practice we found that when we tested discrimination between parental strains by repeatedly removing 10% of the sample at random and classifying them against the bulk of the data, the curve of number of misclassifications versus number of variates used was with a minimum at around six variates. The sixth cervical vertebra, the most complex in outline, needed twelve (Table 1). In practice most of the best discriminators were cosine components.

When the best discriminators for each shape had been determined, classification was repeated testing individual chimaeras against the parental groups. Multivariate analysis showed that some chimaeric vertebrae classified with one or other

Table 1. *Data used for classification of vertebrae*

Vertebra	% misclassified	No. of variates used	Fourier components (in decreasing order of significance) c = cos, s = sin
C1	1·7	7	c2,c6,c13,c4,c7,c25,c19
C2	3·6	5	c4,c3,c2,c7,c1
C3	4·9	5	c14,c4,c6,c7,c2
C4	12·5	5	c16,c57,c55,s6,c48
C5	4·9	5	c13,c8,c18,s44,s46
C6	13·4	12	c31,c20,c30,c23,c28,s16,c38,s59,c21, c18,s57,c10
C7	0·0	6	c3,c13,c18,c29,c26,c31
T1	2·8	7	c3,c4,c12,c8,c33,c15,c35
T2	11·8	6	c21,c17,c25,c13,c29,c5

parental group, some were within 2χ standard deviations of both parental group centroids and others were far away. Examples of these data are given as a table of generalized distances (Table 2) and as a dendrogram (Ward, 1963; Fig. 2). The

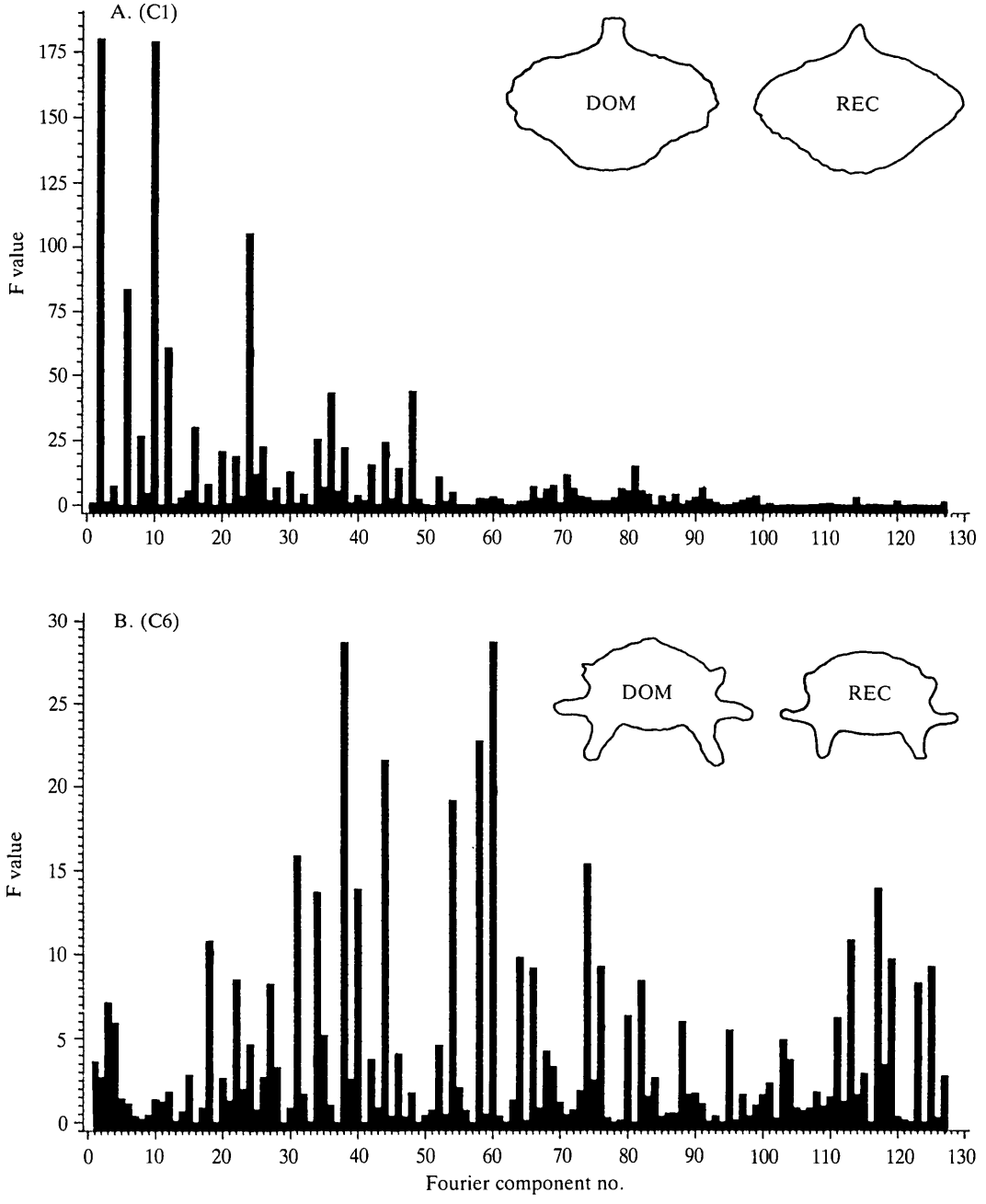

Fig. 1. Plots of Fourier component number (x axis) against significance of difference between DOM and REC strains (F value; y axis) for vertebrae C1 and C6. The 5% probability level of F for 41 individuals in two groups is 4·08. The Fourier components (1–127) alternate between cosine and sine thus – c1,s1,c2,s2 ... c63,s63. c0 and s0 are omitted.

Table 2. *Generalized distance matrix (square root of Mahalanobis distance) for C3 vertebrae*

	DOM	REC	X1	X8	X9	X10	X13	X14	X16	X18	X19	X21	X22	X27	X28	X29	X31
DOM	0																
REC	3·23	0															
X1	**1·97**	**2·65**	0														
X8	5·01	5·18	4·60	0													
X9	5·48	4·30	5·23	4·60	0												
X10	19·18	18·83	20·09	17·41	15·92	0											
X13	11·91	11·50	13·04	11·49	10·29	9·15	0										
X14	5·38	4·34	3·81	3·95	4·21	19·6	13·77	0									
X16	7·15	6·99	8·27	8·05	6·66	14·00	6·12	9·55	0								
X18	**1·67**	**2·62**	2·66	4·77	5·31	18·42	10·83	5·62	6·46	0							
X19	3·26	4·53	4·68	5·39	4·40	16·17	9·32	6·63	5·18	3·24	0						
X21	**2·26**	4·77	2·88	5·18	7·20	20·40	13·09	6·15	8·68	2·88	5·37	0					
X22	3·55	5·82	3·97	5·32	8·11	20·80	13·48	6·77	9·16	3·95	5·99	1·51	0				
X27	2·82	3·90	4·18	5·11	4·02	16·54	9·52	6·05	4·66	2·98	1·31	4·88	5·80	0			
X28	6·82	8·19	7·76	4·98	7·73	15·39	9·44	8·74	4·66	6·31	5·87	6·53	6·34	6·32	0		
X29	9·63	9·73	10·38	8·14	6·33	10·50	7·60	9·95	7·84	9·33	6·74	10·98	11·64	7·25	7·50	0	
X31	**2·54**	4·20	3·51	6·00	6·39	19·68	11·87	6·64	6·53	2·86	4·56	3·21	3·66	3·78	7·46	10·83	0

Values in **bold** type are considered to be members of DOM or REC parental groups since they lie within 2·78 standard deviations of the parental group mean (2χ S.D.).

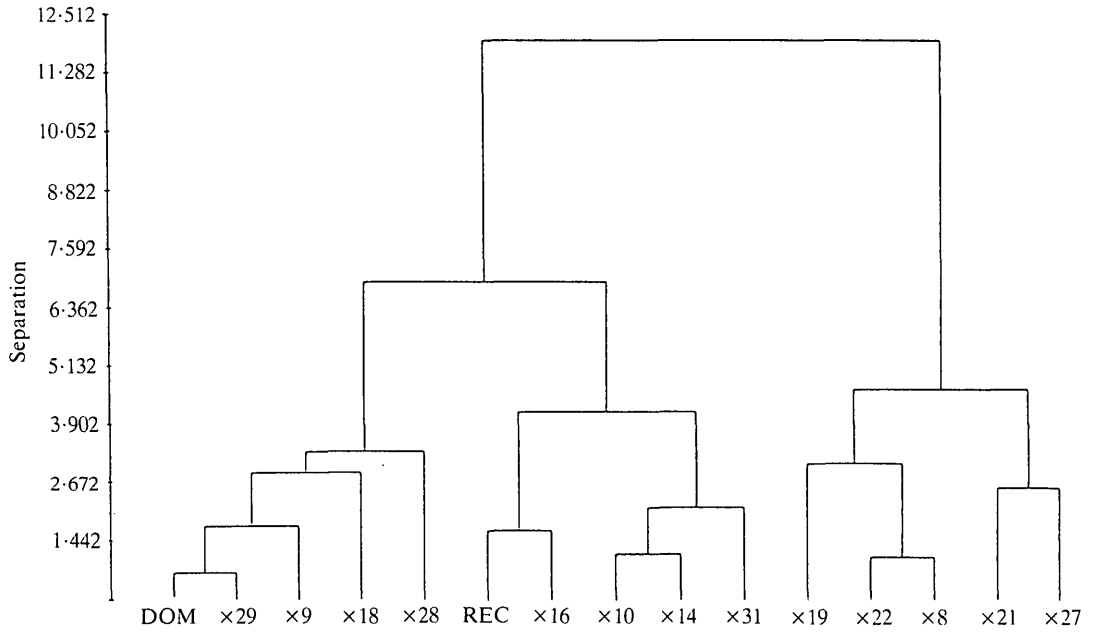

Fig. 2. Dendrogram (Ward's method – error sum of squares) of the distribution of the shapes of C4 vertebrae. The vertebrae fall into three groups, two clustered about the DOM and REC mean shapes and a third dissimilar group.

latter shows some chimaeric vertebrae uniting with each parental type before linking with a third group of more distant chimaeras. The classification of individual chimaeric vertebrae is presented in Table 3 and the number of individuals falling into each group in Table 4.

Asymmetry in the bones was examined by calculating the sum of absolute values of the sine components/number of sine components. Table 5 shows the value of this index for parental stocks and chimaeras. There is, of course, some asymmetry in the parental stocks and roughly the same mean amount in the chimaeras. This measure of asymmetry is distributed similarly in parents and their chimaeric offspring. The means and standard deviations are similar and there is no consistent skewing of the distribution in chimaeras.

DISCUSSION

The clonal theory of vertebral development is most clearly demonstrated by a figure from Moore & Mintz (1972) which is reproduced here as Fig. 3. This shows a dorsal (or ventral) view of part of a vertebral column and suggests that each vertebra is formed from four clones of cells derived from left and right caudal and cranial sclerotomites respectively. The theory assumes:

(a) that each half somite (left or right) is composed of cells of the same genotype;

Table 3. *The classification of individual chimaeric vertebrae*

Mouse number	Vertebra								
	C1	C2	C3	C4	C5	C6	C7	T1	T2
X1	D/R	D	D/R	—	R	X	R	R	X
X8	X	X	X	X	D/R	X	X	D	X
X9	D	X	X	D	X	X	X	D	R
X10	X	D	X	D/R	X	X	D	D/R	D
X13	X	X	X	—	R	X	D	D	X
X14	X	X	X	D/R	D/R	X	X	R	X
X16	R	D/R	X	R	R	X	—	—	D/R
X18	X	X	D/R	D	D	D/R	—	—	R
X19	X	D/R	X	X	D	D	D	—	D
X20	—	—	—	—	—	D	D	D	—
X21	R	D/R	D	X	D	X	R	R	X
X22	X	—	X	X	X	D	X	D	X
X27	X	D	X	X	X	X	X	D	X
X28	X	D	X	D	R	X	R	X	X
X29	—	D	X	D	D	D	X	D	X
X31	D	D/R	D	D	D	X	D	D	X

D = dominant; R = recessive; D/R = parental (see text); X = chimaera.

Table 4. *Occurrence of various types of offspring in chimaeras*

Type of vertebra	Vertebra									
	C1 (14)*	C2 (14)	C3 (15)	C4 (13)	C5 (15)	C6 (16)	C7 (14)	T1 (13)	T2 (15)	Tot (129)
D	14 %	36 %	15 %	38 %	33 %	25 %	36 %	62 %	13 %	29 %
R	14 %	0 %	0 %	8 %	27 %	0 %	21 %	23 %	13 %	12 %
D/R	7 %	29 %	13 %	15 %	13 %	6 %	0 %	7 %	6 %	12 %
All parental	35 %	65 %	28 %	53 %	73 %	31 %	57 %	92 %	32 %	53 %
X	65 %	35 %	72 %	47 %	27 %	69 %	43 %	8 %	68 %	47 %

* No. of vertebrae.

(b) that sclerotomites uniting to form a vertebra may be of the same or different genotypes according to the genotype of the somite from which they are derived;

(c) that caudal sclerotomites influence vertebral phenotype more than cranial ones: a vertebra with two C57BL caudal sclerotomites for instance, becomes C57BL-like in shape;

(d) that a vertebra whose caudal sclerotomites differ on right and left sides becomes asymmetrical.

We feel that the Moore–Mintz hypothesis and the evidence which they offer in support of it is open to criticism on the following three counts.

(1) The theory is based on the concept of the formation of vertebrae by somite resegmentation (Neugliederung). The concept of resegmentation has itself been recently questioned (Verbout, 1976).

(2) Moore & Mintz examined only the anterior aspect of the vertebrae. If we allow that each vertebra was made up of two anterior and two posterior clones (the

posterior, caudal ones being most important) then it must follow that any outline perceived superiorly is made up of variable contributions from anterior and posterior clones. Our own findings, since we followed Moore & Mintz in using this view, are open to the same criticism.

(3) Moore & Mintz compared each chimaeric vertebra with a bone from an animal of the same sex from each of the parental strains, which were selected as

Table 5. *Asymmetry of vertebrae as expressed by sum of sine components/no. of components*

Vertebra	Strain	No.	Mean	S.D.	S.E.M.	Skew
C1	DOM	24	62·03	19·75	4·03	−0·16
	REC	33	46·42	12·09	2·10	−0·16
	X	14	56·68	15·78	4·22	−0·21
C2	DOM	16	63·82	13·06	3·26	0·00
	REC	25	64·87	15·16	3·03	0·35
	X	14	60·63	15·42	4·12	−0·33
C3	DOM	16	85·51	22·65	5·66	−0·25
	REC	25	67·97	25·66	5·13	0·50
	X	15	87·69	24·14	6·23	−0·16
C4	DOM	16	86·69	31·51	7·88	0·22
	REC	24	90·66	31·44	6·41	−0·07
	X	13	100·94	31·82	8·83	−0·34
C5	DOM	16	118·23	34·65	8·66	−0·52
	REC	24	107·48	42·16	8·43	−0·51
	X	15	115·68	25·59	6·61	−0·12
C6	DOM	16	220·92	89·75	22·43	−0·55
	REC	25	226·82	76·00	15·20	−0·76
	X	16	241·21	73·28	18·31	−0·06
C7	DOM	16	108·71	32·29	8·07	−0·12
	REC	23	95·01	37·26	7·77	0·42
	X	14	93·50	45·59	12·19	0·96
T1	DOM	22	87·09	26·12	5·57	0·20
	REC	32	77·26	29·93	5·29	0·66
	X	13	84·39	16·59	4·60	−0·11
T2	DOM	14	142·95	40·90	10·93	0·53
	REC	20	147·55	40·47	9·05	−0·27
	X	15	146·26	46·50	12·00	0·00

DOM = dominant; REC = recessive; X = chimaeras.

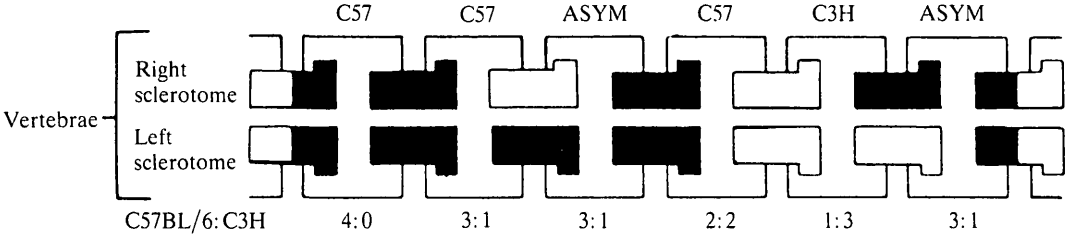

Fig. 3. The Moore & Mintz clonal hypothesis of vertebral origin (reproduced from Moore & Mintz, *Devl Biol.* **27**, 55–70, with permission).

standards. This method of analysis can take no account of intrastrain variation in vertebral shape. Our findings are based upon a method that allows for variation in shape between bones and, we submit, is therefore more reliable.

Although we cannot apply our results to the full clonal theory for the reason stated above we can compare our findings with those of Moore & Mintz, and thus contrast methodologies. They found that some chimaeric bones seen in anterior view were indistinguishable from parental strains, some were 'indeterminate' and some were markedly asymmetrical.

Our sample of chimaeric vertebrae (more limited than that of Moore & Mintz in both number and range) contained parental types (i.e. vertebrae falling within the normal distribution of the parental strains). Some of these resembled the DOM parent, some the REC and those which we classified D/R fell into the same no-man's land as misclassified parental vertebrae (i.e. the overlap between DOM and REC). Both we and Moore & Mintz found an excess of one parental type over the other in chimaeric vertebrae resembling parental strains, suggesting perhaps that the potence (Wigan, 1944) of each strain was not equal.

A simplified clonal theory of vertebral development can be tested by both our results and those of Moore & Mintz. This supposes that each half vertebra (left or right) is made up of the descendents of a number of clones of precursor cells. This assumes left–right independence, a reasonable assumption in the light of other chimaeric investigations and normal embryology, but an unproven one. In its simplest form, with one clone of cells making up each vertebral half (Fig. 4, Table 6) the possible types of chimaera are parental (1aa:1bb) and nonparental (1ab:1ba). In this case parental types would make up half the offspring, and the other half might be expected to be asymmetrical. If we postulate two clones per side the number of parental types falls to 2/9, with three it becomes 2/16 and with five 2/36, i.e. the number of parental types falls exponentially. Moore & Mintz found 80·5 % parental types, too high even for one clone per side: we found 53 %, a reasonable fit to an expected 50 % but too high for more than one clone per side.

Our data show that the 'indeterminate' nonparental chimaeric vertebrae are not intermediate between parental shapes, but often lie at considerable distances from parental centroids. This implies the generation of new and different shapes. Although the meaning of intermediacy in a genetical as opposed to a mathematical sense awaits proper assessment common sense suggests that a conventional cross would yield an F_1 which was intermediate between parental strains and consistent in shape between individuals. We might argue that the 'indeterminate' vertebrae are those that are positioned between parental types in the vertebral column and differ in shape due to the constraints of mechanical fit. A cursory examination of Table 3 shows that this is not the case.

Moore & Mintz assessed symmetry within chimaeric vertebrae by comparing right and left halves with parental stocks. They found 10 % of C1–T2 (5·8 % of C1–S1) to be asymmetrical. We found that the distribution of symmetry in chimaeras (Table 5) differed little if at all from that in parental strains. The means, standard deviations and standard errors of our index of symmetry are similar and

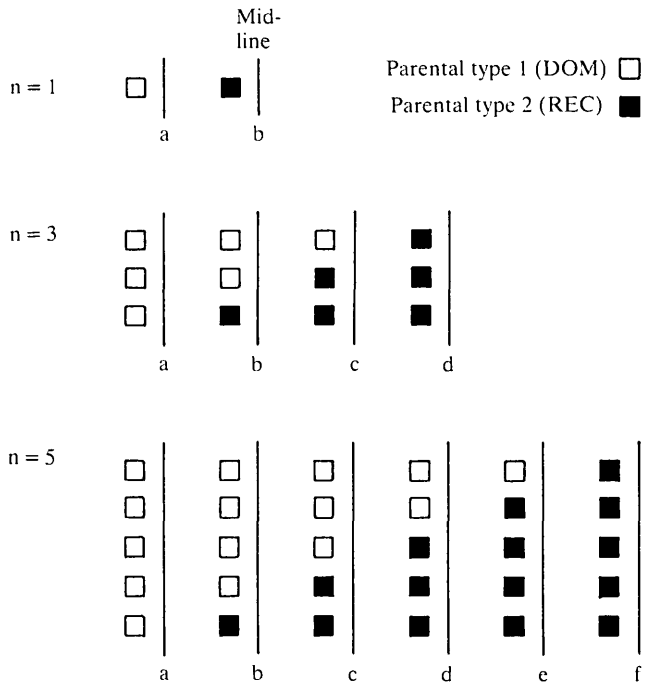

Fig. 4. Possible makeup of vertebrae with differing numbers (n) of clones per side.

show no significant trends. An increase in the variability of symmetry in chimaeras would be reflected in changes in the mean or standard deviation. A clonal model of vertebral development demands an excess of asymmetrical vertebrae and should thus impart a positive skew to the distribution.

Moore & Mintz attribute the finding of a smaller number of asymmetrical vertebrae than they expected to the blurring effect produced by factors such as the mechanical constraints acting in bone growth and remodelling and possible cell migration during early development. Many mutant genes are known in which abnormal, often asymmetrical vertebrae are produced as a result of defects in development from the primitive streak onwards (Johnson, 1986; Grüneberg, 1963). In some of these one or more abnormal vertebrae is found interpolated into an otherwise normal series. We have no evidence to support the view that the deformity so caused is ameliorated during the subsequent life of the animal; a kink in the vertebral column persists. In man a small kyphosis in the spine tends to be exaggerated by muscle action and weightbearing. We suspect that the asymmetry seen by Moore & Mintz was no more extensive than that seen in parental strains, a conclusion borne out by our results.

Our finding that c.50% of vertebrae are of parental type accords well with a modified clonal hypothesis i.e. that left and right vertebral halves may be of different origin and that each half vertebra represents the descendents of one clone. If we accept the concept of one clone per side, however, then the 50% nonparental vertebrae should be of mixed phenotype with the left half derived

Table 6. *Makeup of chimaeric vertebrae with 1, 3 and 5 clones per side (see also Fig. 3)*

n = 1

Half vertebral type			*% DOM parent*		
	a	b		a	b
a	aa	ab	a	100	50
b	ba	bb	b	50	0

n = 3

Half vertebral type					*% DOM parent*				
	a	b	c	d		a	b	c	d
a	aa	ab	ac	ad	a	100	83	67	50
b	ba	bb	bc	bd	b	83	67	50	
c	ca	cb	cc	cd	c	67	50		
d	da	db	dc	dd	d	50			

n = 5

Half vertebral type							*% DOM parent*						
	a	b	c	d	e	f		a	b	c	d	e	f
a	aa	ab	ac	ad	ae	af	a	100	90	80	70	60	50
b	ba	bb	bc	bd	be	bf	b	90	80	70	60	50	
c	ca	cb	cc	cd	ce	cf	c	80	70	60	50		
d	da	db	dc	dd	de	df	d	70	60	50			
e	ea	eb	ec	ed	ee	ef	e	60	50				
f	fa	fb	fc	fd	fe	ff	f	50					

n, no. of clones.

from one parent and the right from the other. We feel that such vertebrae should be asymmetrical, and that the methods used here are sufficiently sensitive to detect the asymmetry. In fact no such asymmetry was detected and this must weaken the one clone per side hypothesis, just as it weakens that of Moore & Mintz.

We suggest that the clonal model of vertebral development as proposed by Moore & Mintz, and which is currently gaining acceptance (McLaren, 1976; Le Dourain & McLaren, 1984) must be regarded as unproven. Any cloning theory of

vertebral development requires rigorous testing taking account both of the variability that exists between vertebrae and the fact that a vertebra is a three-dimensional structure: whether or not methodology to do this with the required precision is yet available is an open question.

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