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Position effect variegation in the mouse

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SUMMARY

Position effect variegation has been studied in female mice heterozygous for the *flecked* *X*-autosome translocation, T(7; *X*)Ct. Some of these carried the spotting gene (*s*) which clarifies the variegated patterns. Others carried a second *X*-autosome translocation, T(*X*; 16)16H, which suppresses the randomness of *X*-chromosome activity.

It was found the position effect variegation stems primarily from early occurring events which lead to the formation of clones of cells with different phenotypes. In this respect the phenomenon appears to parallel that found in *Drosophila*. However, in the mouse, late-occurring events are also found which can only be readily accounted for by the reactivation of previously inactive loci. They occur, not only during foetal development, but throughout the life-time of the animals and in a manner which suggests they derive from a progressive retreat of the inactivating influence of the heterochromatic *X* chromosome back along the attached autosome towards the breakpoint. It is proposed that the early occurring events do not lay down fixed programmes of gene suppression, as proposed for *Drosophila*, but that, like the later-occurring events, they represent the reactivation of previously inactivated loci. The possibility that this might also be true for *Drosophila* is discussed.

The study also provided evidence favouring the view that the *X-chromosome controlling element*, *Xce*, modifies the heterozygous phenotypes of *X*-linked genes by biasing the randomness of the *X*-inactivation process, rather than by operating through cell selection mechanisms. The data also support and extend Mintz's (1967) concept of pigment pattern differentiation.

1. INTRODUCTION

Position effect variegation is now regarded as a general phenomenon but it is in *Drosophila* that by far the largest number of cases have been described and it is from their study that the key characteristics of the phenomenon have been revealed (Baker, 1971). These include: (1) Almost invariably the variegation is associated with a rearrangement one of whose breakpoints is close to the gene whose expression is affected. (2) One of the breaks is always within a heterochromatic region of a chromosome. (3) The wild-type allele of the affected gene must be carried in the *cis* position with the rearrangement and usually with a recessive allele carried on the normal homologue. (4) The probability of a given locus being affected is dependent upon its proximity to the breakpoint; there is

what is described as a polarized spreading effect such that genes closer to the breakpoint are more likely to be affected than those more distant.

The position-effect variegation observed in the mouse obeys these rules but the situation is complicated by the fact that in all cases so far described the rearrangements comprise *X*-autosome translocations (Cattanach, 1961; Russell, 1961; Russell & Montgomery, 1970). As a result of the *X*-inactivation process (Lyon, 1961) heterozygous females have the rearranged *X* inactive and heterochromatic in approximately half their somatic cells and it is only among these cells that position effect mosaicism for rearranged autosomal loci may occur. However, because these loci are not subject to suppression of genetic activity when the rearranged *X* is active, as in males, for example, the majority of the variegation seen in females stems, not from the position effect directly, but rather from the randomness with which the rearranged and normal *X* chromosomes are inactivated. Thus, any study upon mouse position effect variegation must distinguish this complicating factor.

In *Drosophila* it has been shown that the event determining the expression of the position effect variegation, the determinative event (Baker, 1971), occurs at the end of the first larval instar and it is thought that at this time a programme of gene suppression is laid down for each cell. Thereafter, the clonal proliferation of cells with different programmes leads to variegated patterns expressed at later stages of development. An important factor is that these programmes, once established, are thought to be permanent, i.e. no reversal or further changes occur.The stability of cell lines in larval blastemas cultured through many transfer generations in adult flies has been taken to confirm this conclusion (Hadorn, Gsell & Schultz, 1970).

The situation has received far less attention in the mouse. It is, of course, known that *X*-chromosome inactivation occurs at an early stage of embryonic development and leads to clones of cells having either the maternal or paternal *X* genetically inactive and heteropyknotic. In addition, Cattanach, Wolfe & Lyon (1972) have demonstrated that the variegated-coat phenotypes of heterozygotes for the *flecked* *X*-autosome translocation resemble those of chimaeric animals produced by the aggregation of genetically different embryos. This indeed suggests that the inactivation of the autosomal loci occurs early in development but does not distinguish any position-effect clone formation from that caused by the random *X*-inactivation process. The spreading effect phenomenon observed in the mouse (Cattanach, 1961; Russell, 1963) likewise suggests the polarized inactivation of the rearranged autosomal genes but the variegations observed are generally so diffuse that it is not really possible to conclude that they derive from early determinative events and represent discrete clones of cells. Then there have also been a number of observations which seem to be totally at variance with anything known in *Drosophila*. These suggest that, in the mouse, later as well as early events or changes occur and that these may result from a reversal of the inactivating influence (Cattanach & Isaacson, 1965; Cattanach, Pollard & Perez, 1969).

The purpose of this communication is therefore to bring together the various pieces of evidence concerning mouse position effect variegation and present new material obtained under more controlled and defined conditions which confirm and extend the earlier observations. The data demonstrate that the position effect variegation is largely determined early in development and that this does indeed lead to the production of clones differing with regard to the activity of the rearranged autosomal genes. However, they also provide evidence of late-occurring events which are most easily interpreted in terms of reversal of the inactivation process. The implication of these findings for the understanding of position effect variegation in general are considered.

2. MATERIALS AND METHODS

Variegation was studied only in animals heterozygous for the unbalanced duplication form, Dp(7; *X*)Ct, of the *flecked* (*fd*) *X*-autosome translocation, T(7; *X*)Ct (Cattanach, 1961). Hereafter, Dp/+ will signify this genotype. Dp heterozygotes possess a normal autosomal complement plus a piece of chromosome 7 inserted into one *X* chromosome. This *X* will be called the X^T and the normal *X*, X^N. The autosomal insertion carries the wild-type alleles of three known coat and eye colour genes, *albino* (*c*), *pink-eye* (*p*) and *ruby-eye-2* (*ru-2*) and, thus, variegation can be observed for any one when the appropriate recessive alleles are present on both normal chromosome 7's (Cattanach, 1961; Eicher, 1970). The average amounts of *c* and *ru-2* in the coats of *c*-variegated and *ru-2*-variegated animals are about the same but *p*-variegation is more limited. This constitutes evidence for the concept of a spread of inactivation entering both sides of the insertion, for the *p* locus is the central marker (Cattanach, 1970).

Two groups of Dp heterozygotes were employed. One is homozygous for the Xce^b allele (Cattanach, 1972) (formerly known as Xce^i; Cattanach, 1970) of the *X-chromosome controlling element*, i.e. Dp $Xce^b/+Xce^b$. The other is heterozygous at the *Xce* locus, carrying the Xce^a allele (formerly known as Xce^c) in the X^T, i.e. Dp $Xce^a/+Xce^b$. The latter animals show higher levels of translocation-induced variegation and their heterozygous phenotypes for *X*-linked genes are similarly modified (Cattanach *et al.* 1969). It was hoped that by using animals of the two genotypes evidence might be obtained which would assist in distinguishing the mechanism by which the modification is accomplished. The two mechanisms at present under consideration are a bias in the randomness of *X*-inactivation, and cell selection operating differentially upon the two cell populations differentiated by *X*-inactivation (Cattanach & Williams, 1972). The end effect of either mechanism is that non-random *X*-chromosome activity is observed in *Xce* heterozygotes, *X* chromosomes carrying Xce^a tending to be the more likely to be inactive.

Three experiments have been carried out: the first concerns the position effect variegation in animals in which *X*-inactivation is random. It has therefore to be distinguished from the variegation resulting from *X*-inactivation mosaicism and this can be done by studying the variegation for one rearranged locus relative to

that for another. The *p* and *c* loci were selected for this purpose since only with this combination can the variegating colours readily be distinguished.

All animals studied in this experiment were homozygous for *non-agouti* (*a*), an allele at the *agouti* locus which reduces the *agouti* (yellow tipped black hair) pattern of the coat to a solid black colour. This aided the distinction of the variegating colours. The *p* and *c* locus variegations were studied in animals either homozygous for *p* or c^{ch} (an allele of *c*) or carrying *p* and *c* on one chromosome and *p* and c^{ch} on the other, i.e. Dp/+ ; pc^{ch}/pc^{ch} or Dp/+ ; pc/pc^{ch}. Most animals studied were of the latter genotype, this being the more preferable since the cc^{ch} areas produced (*c* locus only inactivated) are a light fawn colour and, hence, more readily distinguishable from the black or wild-type (+) areas (neither locus inactivated) than the $c^{ch}c^{ch}$ areas of Dp/+ ; pc^{ch}/pc^{ch} animals which are dark brown to near-black. The third colour is white (pc^{ch}/pc^{ch} or pc/pc^{ch}) in both genotypes (both loci inactivated); the fourth possible colour, *p* (*p* locus only inactivated) is not found in our experience (cf. Eicher, 1970). For the sake of clarity and convenience of presentation, all the *c* locus phenotypic and genotypic symbols used will be described c^{ch}, e.g. c^{ch} refers to both cc^{ch} and $c^{ch}c^{ch}$ colours.

The primary object of the first experiment was simply to determine whether c^{ch} cells occur together in discrete clusters, indicating a closely related cell lineage and, if so, whether the patches are large or small, this perhaps giving an indication of the stage of development when the cells are programmed for their eventual activity, i.e. the timing of a determinative event.

Coat colour in the mouse is produced by the melanocytes present in the hair follicles. These cells are of neural crest origin and reach their position in the follicles by migrating laterally down the sides of the body during the 8th–12th days of foetal life (Rawles, 1947). Thereafter, they colonize the hair follicles and their location is then fixed (Reed & Henderson, 1940; Chase, 1949). From her studies on chimaeric mice produced by the fusion of embryos differing with respect to coat colour genes Mintz (1967, 1970) has deduced that there are 17 pairs of sites from which single melanoblasts migrate and multiply, producing transverse bands representing clones of cells. The *X*-inactivation mosaicism shows the same kind of patterning. Hence, it is in the context of these cell lineage relationships that the patch size and distribution caused by position effect variegation must be studied.

One other component must be added before this becomes a practical proposition, however. In both the chimaerics and inactivation mosaics the transverse bands are generally obscure in most areas of the body. This results primarily from the intermingling of cells and leads to what may often appear to be a pepper-and-salt mosaicism. To reduce this problem we utilized the approach of Schaible (1969), who attempted to define the 'pigmentation centres' of the coat by combining 'spotting genes' thought to reduce the migration and/or multiplication of the melanoblasts. For our purposes, we introduced one of these genes, *recessive spotting* (*s*), into all our Dp heterozygotes in the hope that cell intermingling would be reduced and that this would allow cell lineages to be more easily traceable.

The second experiment concerns the study of the position effect variegation when the randomness of *X*-chromosome activity is suppressed, i.e. when the rearranged *X* is in the inactive, heterochromatic condition in all cells. Any variegation then seen is due entirely to the position effect and is directly comparable to the *Drosophila* situation. Non-random *X*-activity was achieved by introducing Searle's *X*-autosome translocation, *T*(*X*; 16)16*H* or *T*16*H* (Lyon *et al.* 1964), marked with the *X*-linked gene, *Greasy* (*Gs*), into the system. Heterozygotes for this translocation have the normal *X*-inactive in virtually all cells, this resulting either from non-randomness in the *X*-inactivation process or from death of those cells which have the rearranged *X*-inactivated. Cytological data have indicated that a small fraction of cells may not behave in this way (Ohno & Lyon, 1965) but genetical studies with *X*-linked genes have consistently failed to demonstrate any indication of a 'variegated' phenotype (Grüneberg, 1967; Lyon, 1966; Lyon *et al.* 1964). For the present purposes, therefore, it seems justifiable to assume that any variegation observed in the double translocation heterozygote should result from the position effect associated with the *fd* translocation, not from any 'leakiness' in the non-randomness of *X*-inactivation brought about by Searle's translocation. In this system, the *fd X* should play the role of the normal *X* and generally be the inactive chromosome in all cells.

Mention of the *c*-variegation found in such double translocation heterozygotes has been made in previous communications (Cattanach, 1966; Cattanach *et al.* 1969). Further such animals will be described here and also those showing variegation for both *p* and c^{ch}. None of these animals carried the spotting gene, *s*, and because a series of crosses have had to be made to produce them, not all were homozygous for *non-agouti*. The full genotypes of these animals are therefore Dp+ +/+ T16H *Gs*; pc^{ch}/pc^{ch}; +*a* or *aa*.

The final experiment only represents an extension of the previous two. The aim was to determine if, and in what way, the variegation changed as the animals aged. Therefore, the procedure was simply to keep a number of variegated animals from these and other experiments and check for changes in the coat colours over a period of up to 18 months.

3. RESULTS

(i) *Banding patterns*

The combination of *s* with the translocation-induced variegation proved to be extremely effective in demonstrating the existence and location of the transverse bands proposed by Mintz (1967, 1970). Although by no means all bands were clearly defined in all animals, all animals showed clear bands in some areas of the body. Thus, from the examination of many animals it was possible to draw up a chart of the band positions.

Fifty-one low-group animals and 37 high-group animals were studied and the derived chart is shown in Fig. 1. There is broad agreement with the pattern derived from the study of chimaeric mice (Mintz, 1967, 1970) in so far as three bands can be allotted to the head (and neck) and perhaps seven, rather than six, to the body.

However, there is also much in common with the pattern deduced by Schaible (1969). His observations also indicated the existence of three bands on the head and neck, plus a head spot, but he found evidence of only three, rather than six or seven, bands on the body. The shortage almost certainly stems from the use of combinations of spotting genes. This is less effective than chimaerism or *X*-inactivation mosaicism for distinguishing adjoining bands and, in addition, causes complete band elimination in some regions of the body.

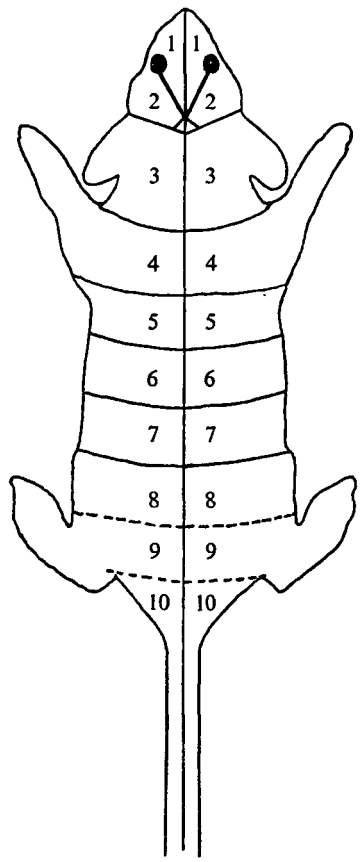

Fig. 1. Chart depicting dorsal view of a mouse and indicating positions of bilateral transverse bands in the coat which represent regions populated by distinct clones of melanocytes.

On the head and forequarters the band positions can be fairly accurately placed with respect to the anatomical landmarks – eyes, ears, limbs. Most on the body cannot be so precisely defined although those in the thoracic area are fairly clear, with band 7 lying just anteriorly to the hindquarters. Only on the rump is there any consistent difficulty in band recognition, a finding that has also been made by B. Mintz (personal communication). In agreement with Schaible (1969) a head spot is also to be found. Like the transverse bands this shows a dorsal mid-line

effect such that areas to either side of the mid-line may be of different colours. As Mintz (1967, 1970) has concluded, this indicates that each band has two components, one for each side of the body. Thus, including the head spot, there are at least 10 and more probably 11 pairs of sites on the head and body which become colonized by single clones of cells.

(ii) *Position effect variegation with X-inactivation mosaicism*

The tendency for clear bands to be produced in the presence of *s* permitted some immediate answers to the questions posed for the position effect variegation. In addition to + and pc^{ch} bands, clear c^{ch} bands were found (see Fig. 2). This

Table 1. *Composition of bands in Dp* $Xce^a/+Xce^b$ *and Dp* $Xce^b/+Xce^b$ *animals*

Band no.	1	2	3	4	5	2–5	6	7	8	9	10
						Total					
						High-variegation group (Dp $Xce^a/+Xce^b$)					
Colour ...											
+	50·0	40·5	51·4	44·6	51·4	47·0	40·5	28·4	43·2	32·4	50·0
pc^{ch}	28·3	29·7	8·1	10·8	10·8	14·9	4·1	2·7	1·4	5·4	0
c^{ch}	6·8	5·4	12·2	5·4	10·8	8·5	9·5	1·4	8·1	4·1	0
Mixed	13·5	24·3	27·0	21·6	12·2	21·3	6·8	8·1	32·4	54·1	50·0
s	1·4	0	1·4	17·6	14·9	8·5	39·2	59·5	14·9	4·1	0
						Low-variegation group (Dp $Xce^a/+Xce^a$)					
+	60·8	46·1	53·9	41·2	47·1	47·1	32·4	28·4	44·1	33·3	39·2
pc^{ch}	18·6	18·6	3·9	9·8	7·8	10·1	2·0	6·9	0	2·9	0
c^{ch}	1·0	0	1·0	3·9	8·8	3·4	7·8	4·9	4·9	2·9	0
Mixed	19·6	35·3	41·2	39·2	17·7	32·4	17·7	4·9	46·1	60·0	60·0
s	0	0	0	5·9	18·6	6·1	40·2	54·9	4·9	0	0

The data show the frequency (%) and distribution of the +, pc^{ch}, c^{ch}, mixed and *s* bands at each band position in 37 high-group and 51 low-group animals.

indicates that the position-effect variegation in the mouse is indeed clonal as it is in *Drosophila* and that for these bands the determinative event must have occurred early in development. The time cannot be pin-pointed exactly but must lie between the time of *X*-inactivation (about 4½ days; Lyon, 1972) and that when the 11 pairs of sites are colonized by the melanoblasts (about 8 days).

Whether the timing of the determinative event is fixed cannot be stipulated with certainty. In fact, earlier observations suggest that it is not. As in previous experiments (Cattanach *et al.* 1969) the variegated patterns frequently seemed to indicate late-occurring events, i.e. the appearance of subclones of cells of a different colour within an otherwise apparently clearly defined band and the direction of change almost invariably appeared to be towards the wild type (+), e.g. pc^{ch} to c^{ch} or +. The results of this experiment thus clearly demonstrate that early determinative events occur but they further suggest that later determinative events or reversals of the inactivating process may also take place.

In addition to providing some answers to the principal questions regarding the

position effect variegation it was hoped that with the clearer banding obtainable with the use of *s* an assessment of the frequencies and distribution of the three types of clone (+, c^{ch} and pc^{ch}) might be possible. The results for a series of animals of both *Xce* groups are shown in Table 1 and several points may be noted:

(1) The data provide quantitative evidence for the general observation that band definition is most obscure in the rump area. Thus, the frequencies of mixed bands (mostly + and pc^{ch}) are highest in this region. The data also show that the *s* gene affects the dorsal part of the body primarily in the lumbar regions at band positions 6 and 7. The gene is also known to affect the nasal area but the apparent lack of such an effect at band 1 almost certainly stems from a greater difficulty in distinguishing the white *s* and pc^{ch} areas in this region. On the basis of these two sets of observations it may be concluded that an analysis of the frequencies and distribution of the different coloured clones of cells is only practicable in the band 2–5 area of the head and body. This area offers the optimum conditions for band identification in the presence of *s*.

(2) Within the band 2–5 area the frequency of mixed bands (mostly diffuse mixtures) is higher (32·4%) for the *Xce* group of animals which show the lower levels of variegation than for those which show higher levels (21%). This difference is in accord with expectation, for the two principal clonal types (+ and pc^{ch}) are present in more equal numbers in the low group and therefore more liable to intermix. Such a greater equality of numbers would be consistent with the fact that these animals are homozygous for one *Xce* allele. In the high group, the *Xce* heterozygosity leads to an excess of cells with the X^T inactive.

(3) The frequency of c^{ch} bands is higher in the high *Xce* group (8·5%) than in the low group (3·4%). This is also consistent with an excess of cells with the X^T inactive, i.e. the more cells there are with the X^T inactive, the more there are likely to be with the *p* locus active and the *c* locus inactive, the conditions necessary for c^{ch} colour. The implications of this finding for interpreting the mechansim of action of *Xce* will be discussed later.

(4) The sum totals for pc^{ch} plus c^{ch} bands in each of the two *Xce* groups of animals is much lower than the number of + bands. For the low *Xce* group in particular this contrasts with the 50:50 ratio of X^N-active to X^T-active cells expected from random *X*-inactivation. Undoubtedly c^+ activity in inactive X^T chromosomes accounts for part of this discrepancy as may also the apparent loss of some pc^{ch} bands in the presence of *s*. However it is also probable that the exclusion of the mixed bands from these figures is responsible. It may be that the majority of the mixed bands were originally pc^{ch} (or c^{ch}) and were subsequently colonized by + cells by invasion from adjoining + areas. As already noted, the probability of this happening seems to be higher in the low *Xce* group of animals.

The data from the analysis of regions 2–5 also provide material for studying the distribution of the different coloured clones. Table 2 presents the results of analyses to determine if the clones in equivalent bands on the right and left sides of the body tend to be the same and, hence, liable to be of related cell lineage. It can be seen that for the high *Xce* group of animals there was no indication of a right–left

correlation whether considering *p*-variegation (pc^{ch} v. + plus c^{ch}) or *c*-variegation (pc^{ch} plus c^{ch} v. +). However, a statistically significant correlation was indicated among the low *Xce* group animals and this stemmed from an excess of paired + bands. In view of the negative high-group results it may be that the excess of paired + bands in the low group derives only from the elimination of many pc^{ch} or c^{ch} bands as mixed bands as proposed above, this being the greater in the low *Xce* group of animals. A right–left correlation is therefore not clearly indicated.

Table 2. *Comparison of bands on left and right sides of the body at band positions* 2–5

		Colour: left–right					
Variegation	Genotype	W–W	W–B	B–B	W:B	Same:different	χ^2
p locus	High group (Dp $Xce^a/+Xce^b$)	5	26	49	36:124	54:26	0·20
	Low group (Dp $Xce^b/+Xce^b$)	7	14	71	28:156	78:14	5·39
c locus	High group (Dp $Xce^a/+Xce^b$)	11	34	35	56:104	46:36	0·29
	Low group (Dp $Xce^b/+Xce^b$)	9	23	60	41:143	69:23	3·76

W = pc^{ch} and B = + plus c^{ch} for *p*-variegation.
W = pc^{ch} plus c^{ch} and B = + for *c*-variegation.

Table 3. *Comparison of adjacent bands*

		Colour					
Variegation	Band numbers	W–W	W–B	B–B	W:B	Same:different	χ^2
p locus	2 *v.* 3	2	17	23	21:63	26:17	0·08
	3 *v.* 4	2	8	15	12:38	17: 8	0·21
	4 *v.* 5	1	8	21	10:50	22: 8	0·01
c locus	2 *v.* 3	0	10	32	10:74	32:10	0·20
	3 *v.* 4	0	7	27	7:61	27: 7	0·10
	4 *v.* 5	0	15	23	15:61	23:15	1·07

W = pc^{ch} and B = + plus c^{ch} for *p*-variegation.
W = pc^{ch} plus c^{ch} and B = + for *c*-variegation.

A similar type of analysis showed no indication of a correlation between the colours of adjoining bands (Table 3). It therefore seems probable that cells of the three phenotypic classes are randomly mixed at the time the 11 pairs of clonal sites are colonized by single cells.

(iii) *Position effect variegation without* X*-inactivation mosaicism*

As has been reported in previous publications, the presence of Searle's translocation T16H, does not completely suppress the *c*-variegation caused by the *fd* translocation as it does the heterozygous phenotypes of *X*-linked genes. Wild-type

areas are found in the coats of Dp+/+T16H; *c/c* animals but the amounts are greatly reduced, well below the lowest levels found in ordinary *Dp* heterozygotes, and this is seen as small pigmented areas scattered throughout the whole animal (Fig. 3). Over 40 such mice have been studied and all have shown this phenotype. Assuming the randomness of *X*-inactivation is indeed completely suppressed, the presence of pigmented areas shows that the rearranged c^+ allele can be genetically active even though the X^T is inactive. The frequency with which this happens would appear to be less than 5%.

An important and regular feature of the *c*-variegation observed in these double translocation heterozygotes is the small size of the pigmented areas. Whole + bands have never been seen even on the head where the bands are small. Four explanations can be offered and all are based on the established fact that the pigment-forming cells do not move after they are established in the hair follicles (Reed & Henderson, 1940; Chase, 1949).

(1) The small size of the + sectors may result from selection operating against pigment-forming cells during the migrating period. These cells could be of two types; X^T inactive, c^+ active or as a result of some 'leakiness' in the non-randomness of *X*-chromosome activity, X^T active and hence c^+ also active.

(2) Cell death rather than cell selection may be operating. The scattered small + areas would then represent pockets of surviving pigment-producing cells. Since extensive cell death would not be expected for X^T inactive c^+ active cells, this explanation implies X^T activity (and hence c^+ activity) in the pigmented areas. Cell death has been proposed as a possible mechanism to account for the non-random *X* activity in heterozygotes for Searle's translocation.

(3) The + areas could be due to the action of the position effect with the determinative event for the *c* locus occurring late, i.e. during the time when the melanoblasts are multiplying and migrating down the sides of the body. This would contrast with the earlier finding in animals variegating for *p* and c^{ch} (Dp/+; pc^{ch}/pc^{ch}; *s/s*) that the determinative event of the *p* locus (giving c^{ch} patches) occurs much earlier. The timing of the determinative events for the two loci would have to be different.

(4) The *c* locus in the rearranged *X* may indeed be inactivated early, but during the time of melanoblast migration, reversal of the inactivation process occurs to produce isolated clones of cells with the c^+ allele genetically active. This and interpretation (3) are consistent with the earlier observations which suggested that either late determinative events or reversals of inactivation occur in ordinary *Dp* heterozygotes.

Further information about this position effect variegation comes from the study of double translocation heterozygotes variegated for both *p* and c^{ch} (Dp+/+ T16H; pc^{ch}/pc^{ch}). In accord with expectation for the operation of the position effect, animals of this genotype showed extensive amounts of pigmentation, this being of the c^{ch} colour (Fig. 4). Small + areas might also be expected but to date none of the six animals studied have been of the background genotype necessary to facilitate the distinction of any small + areas in the c^{ch} regions. Since the large c^{ch}

Fig. 2. Colour photograph of Dp/+ ; pc/pc^{ch}; s/s; a/a mouse demonstrating the presence of + (black), pc/pc^{ch} (white) and cc^{ch} (brown) areas in the coat. Note the twin $cc^{ch} - pc/pc^{ch}$ head spot.

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(*Facing p.* 300)

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Fig. 3

Fig. 4

Fig. 3. Black and white photograph of Dp + +/+ T16H *Gs*; *c*/*c*; *a*/*a* mice at 10 days of age. The phenotype is predominantly *c* (white) but there are many small + (black) areas.

Fig. 4. Black and white photograph of a Dp +/+ T16H; pc^{ch}/pc^{ch}; +/*a* mouse at 10 days of age. The coat is extensively variegated. pc^{ch}/pc^{ch} (white) areas predominate but whole bands of $c^{ch}c^{ch}$ (pigmented) hair are found. Small + areas may also exist.

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Fig. 6

Fig. 5

Figs. 5 and 6. Black and white photographs of a DP +/+ T16H; *c/c*; *a/a* mouse at 3 and 8 months of age, respectively. The amount of hair pigmentation increases with age. Note the difference between the old hair (rump) and new hair in the older animal shown in the midst of a change of coat.

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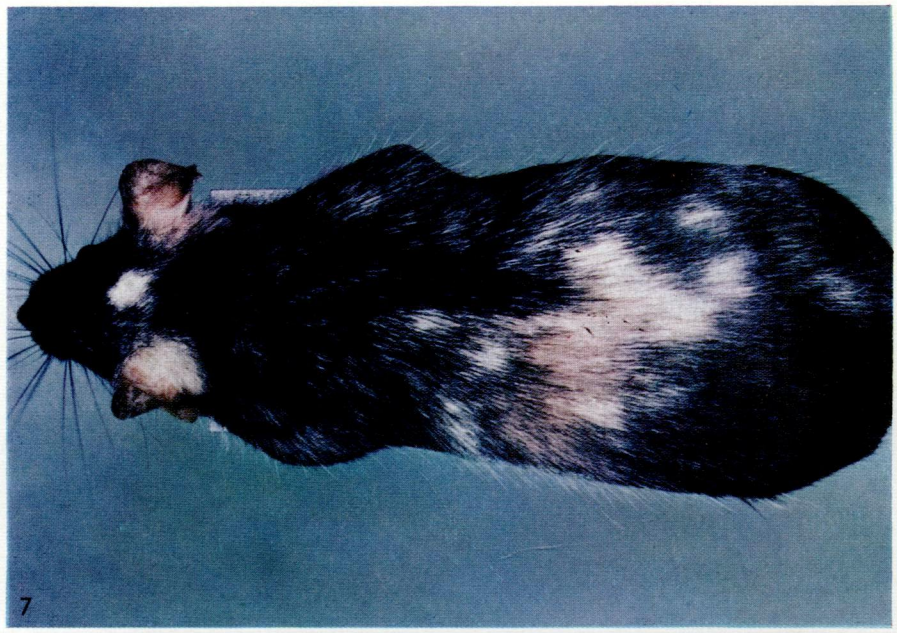

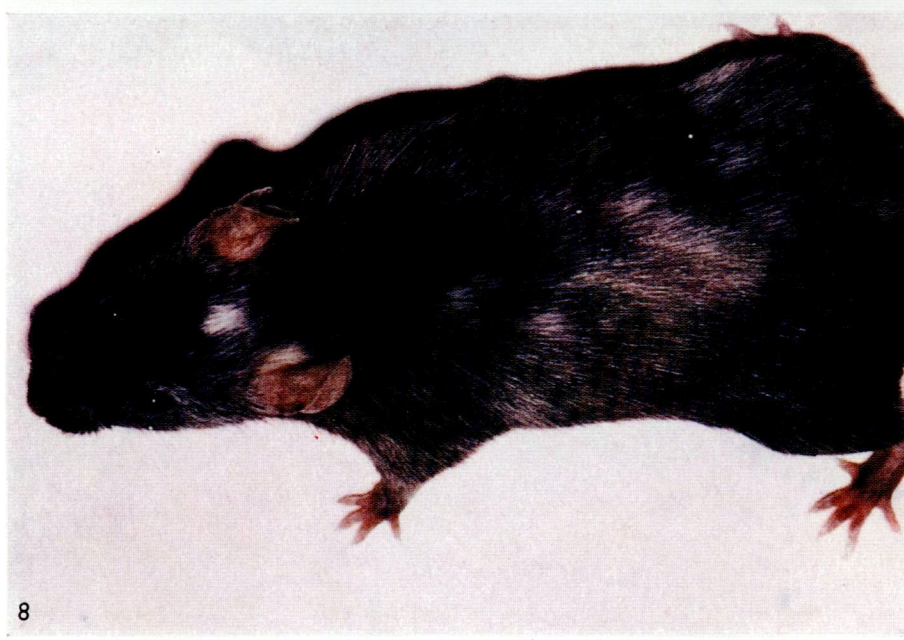

Figs. 7 and 8. Colour photographs of a Dp/+ ; pc/pc^{ch}; a/a mouse at 4 months and one year of age, respectively. The pc/pc^{ch} (white) areas become first cc^{ch} (brown) and then + (black.

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areas in these animals can hardly be ascribed to a different order of 'leakiness' in the non-random *X*-chromosome activity in these animals, they demonstrate instead the frequent activity of the rearranged *p* locus in the inactivated X^T chromosome.

It is important to note that in these double translocation heterozygotes the c^{ch} areas regularly occupy whole band positions such that the variegated phenotypes closely resemble those of orindary *Dp* heterozygotes. This observation has an important bearing on the interpretations offered for the small size of the + sectors seen in Dp+/+T16H; *c*/*c* animals. As already indicated, 'leakiness' of the non-random *X* activity is unlikely to be concerned since the c^{ch} areas of pc^{ch} variegating animals (Dp+/+T16H; pc^{ch}/pc^{ch}) are much larger than the + areas of *c*-variegating animals (Dp++/+T16H; *c*/*c*). If small + areas could also be detected in animals of the former genotype, this conclusion would be beyond question. If 'leakiness' is not concerned, cell death operating on X^T active cells cannot play a part. The different sizes of the c^{ch} and + areas also makes cell selection unlikely for this would have to operate differentially against cells with only different amounts of the autosomal insertion genetically active, i.e. the more autosomal material active, the more selection against. The conclusion offered is therefore that we are observing the consequences of genetic events; the small + areas are derived either by late determinative events or by reversals of the inactivating process.

(iv) *Age effect*

The effect of ageing upon the *c*-variegated phenotype has been reported several times (Cattanach & Isaacson, 1965; Cattanach *et al.* 1969). The observation is that the white (*c*) areas gradually become darker until reaching a dark grey or lead colour (on a *non-agouti* background). The darkening is uniform throughout the white areas and bears no relation to any surrounding + areas, i.e. there is no indication of any spread of pigment-precursor or even cells (were this possible) from the + to the *c* areas. The uniformity of the darkening is most clearly observable in animals showing extensive *c* areas and limited + regions and this is the phenotype characteristic of the double translocation heterozygote, i.e. Dp+/+T16H; *c*/*c*. Photographs of such an animal taken at 3 and 8 months of age are shown in Figs. 5 and 6. In the second photograph the animal can be seen to be in the midst of a new hair cycle. Not only is the posterior part of the animal darker than it was at 3 months, but the new wave of hair growth moving posteriorly is darker still. In seeking an explanation for this phenomenon only genetic events would seem to be possible. The effect is one that might be expected if reversal of inactivation was repeatedly occurring until the rearranged c^+ allele was genetically active and capable of transcription and translation in most originally *c* melanocytes.

An even more intriguing aspect of the ageing effect is observed when variegation for both *p* and c^{ch} are considered. Thus, although the Dp+/+T16H; pc^{ch}/pc^{ch} animal has not yet been studied in this respect a clear picture emerges from the investigation of the ordinary Dp heterozygote. It is regularly found that the white (pc^{ch}) areas first tend to go brown (c^{ch}) and then black (+), (see Figs. 7 and 8). Such a

sequential change requires that the process be of a genetic nature. The change is thus one of a sequential retreat of the inactivating influence of the heterochromatic *X* back along the autosomal material towards the breakpoint(s). The *p* and the *c* loci are, in effect, sequentially reactivated. It is perhaps also of note that because of the age effect the pc^{ch} and *s* areas of Dp/+; pc^{ch}/pc^{ch}; *s*/*s* animals can readily be distinguished in older animals; the *s* areas remain pure white.

4. DISCUSSION

The primary aim of the first two experiments described in this paper was to test whether mouse position effect variegation results from some early determinative event in which the cells are programmed for their eventual activity, and, if so, whether this leads to the formation of clones of cells with different programmes. A clear answer is available for the second part of this question; the c^{ch} bands of the Dp/+; pc^{ch}/pc^{ch}; *s*/*s* animals clearly demonstrate a clonal inheritance based on the cell lineage relationships established from the study of chimaeric mice. Similarly, there is little doubt that the small pigmented sectors observed in Dp+/+T16H; *c*/*c* mice represent discrete clonal populations. The demonstration of a determinative event analogous to that found in *Drosophila* creates greater difficulties, however.

For the *Drosophila* position effect variegation it is thought that the determinative event marks the occasion when some molecular action takes place to bring about subsequent gene suppression. Thus, Baker (1971) suggests that one might at this time look for the appearance of new (suppressor) molecules possibly attached to the sites of affected genes. This interpretation does not fit well with the observations made in the mouse, for these indicate a variety of late-occurring events. Considering first the age effect: here we observe what can only be the reactivation of the rearranged loci in cells in which they had formerly been inactive. Not only is this reactivation evident from the onset of pigment production but the order of reactivation of the loci studied is related to their distances from the breakpoints(s) (cf. the spreading effect phenomenon). Thus, timing is involved; first the more distant *p* locus is reactivated, then the *c* locus.

If we now consider the small pigmented sectors observed in Dp+/+T16H; *c*/*c* animals, we find once again a situation which can most easily be interpreted in terms of reactivation. In this case the *c* locus appears to have been reactivated in some cells during the melanoblast migratory phase. Since the cells are multiplying at this stage, small pigment-producing clones result. By contrast, in Dp+/+T16H, pc^{ch}/pc^{ch} mice, large c^{ch} areas representing whole bands may be found with small + areas probably also being present. Similar observations were also made in Dp/+ pc^{ch}/pc^{ch}; *s*/*s* animals; the c^{ch} areas generally comprised whole bands but some bands showed colour combinations suggestive of a late reversal of inactivation. It may be concluded that c^{ch} clones are generally established before the time of pigment site colonization; they derive from early-occurring events.

It may be noted that all the late-occurring events mentioned above indicate that

a reactivation process is taking place. This leads to a key question: Do the earlier-occurring events give rise to the different clones through a process of gene suppression as deduced in *Drosophila*, or could it be that these early events, like the later ones, stem from reactivational changes? It would seem reasonable to suppose that only one mechanism is responsible for the variegation no matter whether determined early or late and, from the consideration of the later events, this would appear to be the reactivation of loci that had been inactivated at some early stage of development.

Evidence supporting this conclusion comes from the study of the retinal variegation of the eye. Like the coat, eye pigmentation results from the action of melanocytes but these are non-migratory, retain their pigment granules *in situ* and function for only a short time in development. Deol & Whitten (1972) have made a comparative study of the *c*-variegation in the eye of Dp heterozygotes and chimaeric mice and found that the patches were smaller and more numerous in the heterozygotes and that, in addition, there was an excess of + areas. On the basis of the known development of the eye they concluded that the process leading to the production of pigmented and unpigmented melanocytes in the heterozygotes, taken to be *X*-inactivation, occurred on the 7th day or later. This is later than the time at which *X*-inactivation is generally thought to occur. It must be noted, however, that Deol and Whitten were not necessarily assessing the time of *X*-inactivation but, rather, the timing of events at the rearranged *c* locus. Their observations might better be taken as evidence that the rearranged *c* locus, like the *p* locus, can be reactivated early, i.e. up to the time when pigment is laid down (12 days; Grünberg, 1943). This is the period when many of the events discussed in relation to the coat variegation are taking place.

We may now summarize the reactivation model of the mechanism which brings about mouse position effect variegation. (1) *X*-chromosome inactivation leads to the inactivation of adjoining autosomal loci and this can most easily be interpreted in terms of a structural effect (Cook, 1973) caused by the proximity of the tightly coiled, condensed, heteropyknotic *X*-chromosomal regions preventing the potentiality for transcription or translation. It would be expected that the extent of this effect along the autosome would be limited but whether or not it is fixed or liable to show some degree of variability is probably of little importance so far as the final phentoype is concerned. (2) The effect of the proximity of the heterochromatic *X* upon the adjoining autosomal regions tends to be reduced with time and/or the number of cell generations subsequent to inactivation. The rate at which this happens cannot be uniform from cell to cell but certainly it must be progressive in the sense that affected loci the farthest removed from the breakpoint will be set free of the inactivating influence first. (3) If reactivation occurs early and in multiplying cells, then the change will be inherited clonally. These clones will be stable so far as the reactivated gene is concerned but the progressive reactivation of other loci will result in the production of sub-clones. (4) With sufficient time a high proportion of cells will have their affected loci reactivated and if this occurs in cells still capable of expressing gene activity, e.g. the melanocytes of the hair

follicle, this will be detectable. By contrast, reactivation of the *c* locus in retinal melanocytes of the adult will not be detectable since pigment is only laid down at one point in development.

The reactivation model has several advantages over the suppression concept as the mechanism leading to position effect variegation. First, the actual inactivation process can be visualized as the not unexpected consequence of placing a gene close to structurally different chromatin (Cook, 1973). This dispenses with the need of postulating suppressor molecules which bind non-specifically with genes in the proximity of the breakpoint (Baker, 1971). Secondly, the extent of inactivation of the autosomal loci need not be specific whereas precise programmes of suppression are integral to the suppression model. Thirdly, the relative stability of clones is readily understandable in terms of the continuing proximity of heterochromatin but requires the faithful replication of suppressor molecules operating to fixed but different distances along the autosomal regions in different clones. Finally, basic to the reactivation model is the probability of eventual gene activity (or potential for activity), evidence for which is here presented.

Position effect variegation is a general phenomenon recognized in a number of organisms other than *Drosophila* and the mouse but it is from the *Drosophila* data that the suppression model for the phenomenon has been derived (Baker, 1971). It is therefore pertinent to ask whether the reactivation model here proposed is totally at variance with these data. Most probably it is not. In the first place, in contrast to the mouse none of the cell types that can be investigated for position effect variegation in *Drosophila* continue dividing beyond a fixed point in development and gene activity is probably similarly limited. Thus, the probability of detecting late-occurring or reactivational events is very small. Secondly, evidence taken to demonstrate the stability of the different clones in *Drosophila* organ cultures (Hadorn *et al.* 1970) is only satisfactory for the wild-type clones, which, by the reactivation model, should be stable anyway. The stability of the clones with the recessive phenotype was not demonstrated with certainty for these did not survive beyond an early stage of the experiment.

It may be concluded that there is at least a possibility that *Drosophila* position effect variegation results from the reactivation of previously inactivated loci as has been postulated for the mouse and indeed it seems possible that the many systems which modify *Drosophila* position effect variegation (parental source effects, homozygosity *v.* heterozygosity for the rearrangement in the mother, etc.) may be more readily explainable in terms of the reactivation model. For example, such effects may derive from temporary acquired differences in the degree of coiling of the heterchromatic regions.

The data presented also provide information on systems other than position effect variegation. Thus one observation is pertinent to the mechanism of action of *Xce* (Cattanach & Williams, 1972). It was found that the high-variegation group of animals, which are heterozygous for *Xce* (Dp $Xce^a/+Xce^b$), had twice as many c^{ch} bands as the low-variegation group, which is homozygous for the Xce^b allele. As already noted, this observation is consistent with the fact that the *Xce* hetero-

zygosity leads to an excess of cells with the X^T inactive and hence liable to have a *c* locus inactive but the *p* locus active. However, if this is the reason, then it must be concluded that the excess of X^T-inactive cells must exist at the time when events producing the c^{ch} clones first occur. Since these events must occur early in development and within a few days of *X*-inactivation, there would seem to be little time in which cell selection could operate to produce the non-random *X*-chromosome activity observed in the adult. Furthermore, since the non-randomness is not great, were cell selection the responsible mechanism then it would seem necessary to postulate that this only operates during the early stages of development. The data would thus seem to be more consistent with the alternative mechanism proposed for *Xce* action (Cattanach & Williams, 1972), namely that the non-randomness stems from a bias to the randomness of the *X*-inactivation process itself.

The data presented also substantially confirm and extend the conclusion of Mintz (1967, 1970) and to some extent also those of Schaible (1969) that there are a number of paired sites on the head and body which single melanoblasts colonize and produce clones of cells which migrate laterally down each side of the body. As deduced by Mintz (1967, 1970) three bands can be seen on the head (and neck) and it is shown that their regular boundaries can be identified. In addition, there is a twin head-spot. Four bands can readily be detected in the thoracic and lumbar regions of the body and probably three more exist in the rump area. The uncertainty about the precise number here stems from the extensive amounts of cell mingling typical in this area. Possibly, clearer patterns in rump area might be obtainable with the use of the spotting gene, *Rump white*, which preferentially affects this region.

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