

Estimation of the primordial pool size of the mouse liver using a histochemically demonstrable X-linked enzyme in the adult female mouse

KATHRYN A. WAREHAM AND E. D. WILLIAMS

Department of Pathology, University of Wales College of Medicine, Heath Park, Cardiff, CF4 4XN, UK

SUMMARY

We have used a histochemical technique to study cellular mosaicism in the livers of female mice heterozygous for an abnormal form of the X-linked enzyme ornithine carbamoyltransferase. The proportion of cells expressing the normal enzyme was found to be consistent within a liver lobe, but discordant between lobes, with as few as 4 % or as many as 91 % of cells being enzyme positive. Systematic observation of variation of the proportion of positive cells in different lobes of 12 mice was used to calculate the primordial pool size for each lobe and for the liver as a whole. Although confidence limits for this small study are wide, the data suggest that the liver pool is about 15 cells, and that it enlarges to at least 50 cells before subdividing into six lobe pools ranging in size from a minimum of 4 to a maximum of at least 14 cells.

INTRODUCTION

The use of genetic mosaics in the study of development is not a new concept. Allophenic (tetraparental) mice have been studied biochemically and histochemically in order to estimate the number of patches or clones of cells in an organ or the cell number from which it is derived, as well as estimating the number of cells at the time of X-chromosome inactivation or inner cell mass determination (Mintz, 1967; Nesbitt & Gartler, 1971; Wegmann, 1970; West, 1976), although, as has been pointed out, care must be taken in assuming that the data derived from tetraparental mice apply equally to normal animals (Nesbitt & Gartler, 1971; West, Bücher, Linke & Dünnwald, 1984). X-linkage mosaics have been subjected to similar studies using biochemical and cytogenetic techniques (McMahon, Fosten & Monk, 1983; Nesbitt, 1971; Rabes *et al.* 1982).

We report here, for the first time, the use of a histochemically demonstrable X-linked enzyme to determine the primordial pool size of the mouse liver and its constituent lobes from adult tissue.

Key words: liver, primordial pool size, X-linkage, ornithine carbamoyltransferase, histochemistry, mouse.

MATERIALS AND METHODS

(A) *Tissues*

The mice used in the study were heterozygous for the X-linked sparse fur (*spf*) gene. The *spf* phenotype is associated with a mutation of the structural gene for the urea cycle enzyme ornithine carbamoyltransferase (OCT). OCT is found predominantly in the liver (Reichard, 1957) but is also to be found in smaller quantities in the small gut and colon (Qureshi, Letarte & Oullett, 1985). The mutation results in an abnormal form of OCT which functions poorly at pH 7.4 (DeMars, LeVan, Trend & Russell, 1976). This change in pH optimum is used as the basis for the histochemical demonstration of the mosaic cell population in *spf/+* female heterozygotes (Wareham, Howell, Williams & Williams, 1983).

The mouse liver is usually regarded as being divided into four major lobes. The largest are the left lateral (L) and bifurcated median (M), the next is the right and the smallest is the caudate (Danforth & Centre, 1953; West, 1976). The latter two lobes are also bifurcated and in the *spf* colony exist as morphologically distinct entities. The ventral portion of the right lobe (Rv) is ovoid while the dorsal portion (Rd) is more angular. The dorsal (Cd) and ventral (Cv) caudates are both leaf shaped and almost entirely separated. In view of these obvious characteristics it was decided to treat the liver as being composed of six discrete lobes and as such one tissue block was taken from each. Twelve mice aged between 4 and 6 months old were used in the study. The mice were killed by ether inhalation and the livers removed; in six of the mice the caudates were not sampled due to handling difficulties associated with their small size.

(B) *Histochemistry*

The six tissue blocks (1 cm × 1 cm × 0.3 cm) from each mouse were fixed, washed and frozen as described by Wareham *et al.* (1983). 5 µm cryostat sections were stained for the presence of OCT using a modification of the Mizutani technique (Mizutani, 1967; Wareham *et al.* 1983).

(C) *Scoring technique*

The incubated sections were scored for OCT-staining cells using a point count system. A grid of 100 points was superimposed on the projected image of the section, the actual area being sampled was 6.25 mm² per field. Each point was scored as either positive (stained hepatocyte), negative (unstained hepatocyte) or non-hepatocellular (vascular tissue, non-parenchymal cells and section defects). The proportions of the positive and negative areas were calculated and regarded as equivalent to the proportions of the positive and negative cells, the two cell types having previously been shown to be of similar size.

(D) *Sampling regime*

The results of a pilot study to investigate variation in staining within a section (eight fields scored separately in each of 12 sections) showed that there was no significant variation in the distribution of staining ($P > 0.1$). A second pilot study investigated variation between different levels from a lobe. Six levels from both the median and dorsal caudate lobe were studied, no significant variation was found within a lobe ($P > 0.1$ in each case).

In view of these findings four fields were scored from each of two sections taken from widely separated levels from each block. The estimated proportion of positive cells from the two levels showed a highly significant correlation ($r = 0.90$, $P < 0.001$).

RESULTS

(A) *Distribution of positive cells*

All the lobes studied displayed a mosaic staining pattern. However, the extent of staining varied not only between animals but also between different lobes of the same individual. Fig. 1 shows fields from the L and Rv lobes of animal 10.

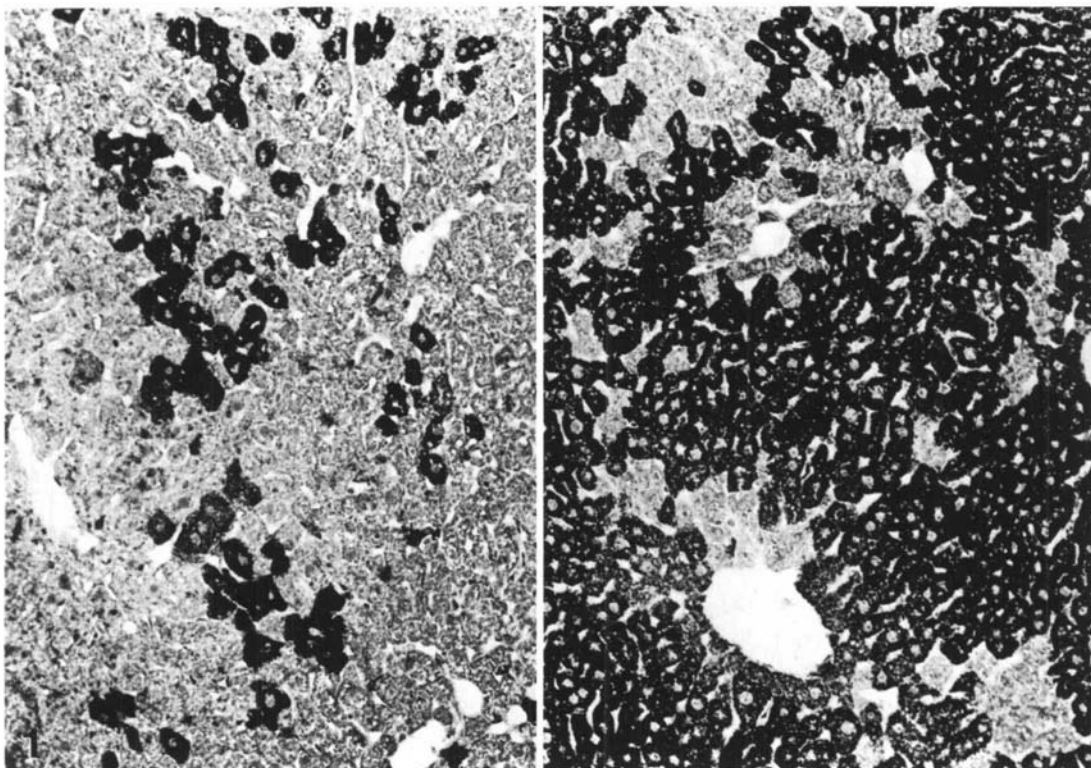


Fig. 1. Composite photomicrograph of two lobes from the same heterozygous *spf* female mouse ($XX \times X^{spf}Y$) stained for the presence of OCT showing marked intra-animal variation lobar in staining (left – left lateral lobe; right – right ventral lobe). Sections were incubated for 15 min at room temperature in a metal-salt precipitation medium containing ornithine, carbamyl phosphate and lead nitrate. The reaction product was visualized by exposure of the sections to H_2S gas dissolved in water giving a final reaction product of PbS (brown stain) at the site of normal OCT activity. $\times 750$.

The mean percentage of stained cells for each of the lobes sampled and the overall liver means giving equal weighting to the measurements for each lobe are shown in Table 1. The range of staining was between 4 % (Rv of animal 1) and 91 % (Rd of animal 3) with an overall mean of 44.5 % which is not significantly different from the expected 50 % predicted by the Lyon hypothesis (Lyon, 1961). Although there was significant variation between the lobes of an animal (Table 1) the range of staining for a given lobe was great (19–91 % for Rd and 4–86 % for Rv).

(B) Estimation of primordial pool size

The estimation of the primordial pool sizes of the liver and its lobes was made using an equation derived from the binomial distribution:

$$N = \frac{p q}{v}$$

where N is the pool size, p is the proportion of positive cells, $q = 1 - p$ (the proportion of negative cells) and v is the variance of p (Fialkow, 1973; Nesbitt & Gartler, 1971).

(1) *Liver pool size*

Three approaches were used to calculate the primordial pool size of the mouse liver (a) from the raw data summarized in Table 1, (b) after weighting the data to account for the different sizes of each lobe and (c) using an estimate for v derived from the analysis of mosaic variation (Appendix).

All three approaches give a pool size of 14–15 cells with 95 % confidence limits of 5–30 cells.

(2) *Lobar pool sizes*

In view of the range of staining seen between the morphologically associated lobes, particularly Rd and Rv (e.g. animals 1, 4, 7, 10) and the overall poor correlation coefficients (Table 2) it was decided to make estimates for the lobar primordial pool for all six lobes separately (Table 3). Despite the small sample size, wide confidence limits and the fact that the observations have not been corrected for the variation between the means for the whole liver, it is clear from the data that the lobes are derived from a larger pool than that determining the liver as a whole.

DISCUSSION

A number of interesting observations has been made even though the model used in this study is very simplistic with a number of gross assumptions, e.g. random growth and sampling from a large random pool so that the effects of

Table 1. *Mean percentage of OCT-positive cells found in the liver lobes of 12 heterozygous spf female mice*

Animal	Liver lobe						Liver mean (un-weighted)	Variation between lobes (P)
	Left lateral	Median	Right ventral	Right dorsal	Caudate ventral	Caudate dorsal		
1	42 ± 9	45 ± 15	4 ± 3	36 ± 8			32 ± 19	<0.001
2	26 ± 14	42 ± 16	48 ± 17	61 ± 10			44 ± 15	<0.001
3	64 ± 11	67 ± 6	86 ± 12	91 ± 8			77 ± 13	<0.001
4	24 ± 8	33 ± 10	38 ± 12	19 ± 9			29 ± 9	<0.001
5	39 ± 10	29 ± 10	57 ± 11				42 ± 12	<0.001
6	35 ± 14	65 ± 13	67 ± 8	41 ± 7			52 ± 16	<0.001
7	10 ± 4	19 ± 10	20 ± 13	61 ± 15	45 ± 15	54 ± 11	35 ± 21	<0.001
8	33 ± 10	26 ± 15	67 ± 13	52 ± 19		43 ± 20	44 ± 16	<0.001
9	25 ± 11		40 ± 12	44 ± 17	45 ± 17	58 ± 14	42 ± 12	<0.05
10	38 ± 17	58 ± 9	84 ± 8	57 ± 14	70 ± 13	51 ± 9	60 ± 16	<0.001
11	47 ± 10	55 ± 20	35 ± 16	45 ± 22	19 ± 14	25 ± 7	38 ± 14	<0.001
12	46 ± 12	57 ± 14	40 ± 13	26 ± 12	36 ± 11	30 ± 13	39 ± 11	<0.001

Table 2. *Product-moment correlation coefficients for the liver lobes of heterozygous spf female mice (Spearman's Ranked correlation coefficients gave similar values.)*

Median	Left lateral 0.74** (11)	Median			
Right ventral	0.42 (12)	0.42 (11)	Right ventral		
Right dorsal	0.29 (11)	0.17 (10)	0.52 (11)	Right dorsal	
Caudate ventral	-0.28 (5)	-0.02 (4)	0.72 (5)	0.48 (5)	Caudate ventral
Caudate dorsal	-0.79 (6)	-0.65 (5)	0.13 (6)	0.59 (6)	0.71 (5)

() no. of pairs of data.

** $P < 0.01$.Table 3. *Estimates of the primordial pool sizes for the individual lobes of the mouse liver*

Lobe	No. of cells in pool	95 % confidence limits
Left lateral	12	4-24
Median	9	3-19
Right ventral	4	1-8
Right dorsal	7	2-13
Caudate ventral	7	1-20
Caudate dorsal	14	2-35
Total lobe pool	53	

sampling without replacement can be ignored. Despite this the model is as useful for a single tissue as the more complex models developed for multiple tissue samples (Nesbitt, 1971; Stone, 1983).

Potential pitfalls of this approach have been considered by McLaren (1972) and Lewis, Summerbell & Wolpert (1972) who drew attention to the effects that clonal growth and prior sampling events may have on any estimates for pool size based on the binomial theorem.

The figures calculated for the liver primordial pool size agree well with those of Rabes *et al.* (1982). Using an X-linked electrophoretic variant of phosphoglycerate kinase they calculated the liver to be derived from about 13 cells. They also agree with Wegmann's figure (Wegmann, 1970) using allophenic mice mosaic for β -glucuronidase (17 cells). Wegmann actually refers to this figure as the number of 'clones' in the sample but Mintz (1971) and Rabes *et al.* (1982) have interpreted the data as giving an estimate of pool size rather than the number of clones. Rabes *et al.* (1982) suggested that the sample size determines what is being calculated when using the equation employed here and also by Wegmann: a large sample gives rise to an estimate of pool size while a small sample estimates patch or clone size within the tissue at the time of sampling. This interpretation of the equation places Wegmann's data as an estimate of primordial pool size and explains the vast discrepancy between his number of clones in the liver (340) and number of coherent clones calculated by West (1976), using similar allophenic mice, to be several million. Furthermore, the estimate of a patch size of about eight hepatocytes calculated by Rabes *et al.* (1982) using a small sample agrees with West's estimate (1976).

The three independent estimates of the primordial pool size of the mouse liver discussed here are much lower than those provided from intertissue correlation in both neonate (Nesbitt, 1971) and embryonic mice (McMahon *et al.* 1983). However, the large pools indicated by these latter studies are not compatible with the extreme variation in the proportions of cells expressing the two phenotypes that we have observed.

The variation in the extent of the mosaicism between lobes, especially between Rd and Rv, highlights the importance of sampling from all areas; the lack of correlation found also suggests that the right ventral and dorsal lobes may not be as closely associated as deduced from their morphology. It is suggested that all six lobes should be regarded as having independent origins within the common primordial liver pool. As we have previously commented, the figures for each lobe's pool size are based upon a simple calculation which has not been corrected for the bias introduced by the relatively small size and variation in composition of the whole liver pool. This means that our estimates for the lobe pool sizes are likely to be low. The sum of the estimates for the lobe pools is about 50 cells, compared with 14–15 cells for the whole liver pool, suggesting that at least two to three cell divisions occur before lobar differentiation.

We thank the Cancer Research Campaign for funding and Dr R. Newcombe for statistical advice.

APPENDIX

*Derivation of v from analysis of mosaic variation**ANOVA table for mosaic variation*

	df	SS	MS	F _s
Between animals	11	8 589.04	780.82	3.41**
Within animals	45	10 319.00	229.31	
	56	18 908.04		

** $P < 0.01$.

$$\text{Variance component } S_A^2 = \frac{MS_{\text{group}} - MS_{\text{within}}}{n_0}$$

$$\text{where } n_0 (\text{average number of lobes}) = \frac{1}{a-1} \left(\sum n - \frac{\sum n^2}{\sum n} \right)$$

$$= 4.73$$

therefore:

$$S_A^2 = 116.6 \times 10^{-4}$$

and

$$\begin{aligned}
 v &= \frac{s^2 + n_0 S_A^2}{n_0} \\
 &= \frac{229.31 \times 10^{-4} + (4.73 \times 116.6 \times 10^{-4})}{4.73} \\
 &= 0.0165.
 \end{aligned}$$

REFERENCES

- DANFORTH, C. H. & CENTER, E. (1953). Development and genetics of a sex-influenced trait in the livers of mice. *Proc. natn. Acad. Sci. U.S.A.* **39**, 811–817.
- DEMARS, R., LEVAN, S. L., TREND, B. L. & RUSSELL, L. B. (1976). Abnormal ornithine carbamoyltransferase in mice having the sparse fur mutation. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1693–1697.
- FIALKOW, P. J. (1973). Primordial cell pool size and linkage relationships of five human cell types. *Ann. hum. Genet.* **37**, 39–48.
- LEWIS, J. H., SUMMERBELL, D. & WOLPERT, L. (1972). Chimaeras and cell lineage in development. *Nature, Lond.* **239**, 276–278.
- LYON, M. F. (1961). Gene action in the X chromosome of the mouse (*Mus musculus*). *Nature, Lond.* **190**, 372–373.
- MCLAREN, A. (1972). Numerology of development. *Nature, Lond.* **239**, 274–276.
- MCMAHON, A., FOSTEN, M. & MONK, M. (1983). X chromosome inactivation mosaicism in the three germ layers and germ line of the mouse embryo. *J. Embryol. exp. Morph.* **274**, 207–220.

- MINTZ, B. (1967). Gene control of mammalian pigmentary differentiation 1. Clonal origin of melanocytes. *Proc. natn. Acad. Sci. U.S.A.* **58**, 344–351.
- MINTZ, B. (1971). Clonal basis of mammalian differentiation. *Soc. exp. Biol. Symp.* **25**, 345–370.
- MIZUTANI, A. (1968). Cytochemical demonstration of ornithine carbamoyltransferase activity in liver mitochondria of rat and mouse. *J. Histochem. Cytochem.* **16**, 172–180.
- NESBITT, M. N. (1971). X chromosome inactivation mosaicism in the mouse. *Devl Biol.* **26**, 252–263.
- NESBITT, M. N. & GARTLER, S. M. (1971). Application of genetic mosaicism to developmental problems. *A. Rev. Genet.* **5**, 143–162.
- QURESHI, I. A., LETARTE, J. & OUELLET, R. (1985). Expression of ornithine transcarbamylase deficiency in the small gut and colon of *spf* mutant mice. *J. Pediat. Gastroenterol. Nut.* **4**, 118–124.
- RABES, H. M., BÜCHER, TH., HARTMANN, A., LINKE, I. & DÜNNWALD, M. (1982). Clonal growth of carcinogen induced enzyme deficient preneoplastic cell populations in mouse liver. *Cancer Res.* **42**, 3220–3227.
- REICHARD, P. (1957). Ornithine carbamoyl transferase from rat liver. *Acta Chem. Scand.* **11**, 523–536.
- STONE, M. (1983). A general statistical model for clone-tissue studies using X-linked data. *Biometrics* **39**, 395–409.
- WAREHAM, K. A., HOWELL, S., WILLIAMS, D. & WILLIAMS, E. D. (1983). Studies of X chromosome inactivation with an improved technique for ornithine carbamoyltransferase. *Histochem. J.* **15**, 363–371.
- WEGMANN, T. G. (1970). Enzyme patterns in tetraparental mouse liver. *Nature, Lond.* **225**, 462–463.
- WEST, J. D. (1976). Patches in the livers of chimaeric mice. *J. Embryol. exp. Morph.* **36**, 151–161.
- WEST, J. D., BÜCHER, T., LINKE, I. M. & DÜNNWALD, M. (1984). Investigation of variability among mouse aggregate chimaeras and X-chromosome inactivation mosaics. *J. Embryol. exp. Morph.* **84**, 307–329.

(Accepted 5 February 1986)