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# Identification of ornithine decarboxylase as a trait gene for growth in replicated mouse lines divergently selected for lean body mass

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**Summary**

Studies of lines of mice selected for body mass have shown that there is a significant genetic component affecting this trait although the nature of the genes involved remains to be elucidated. Using replicate lines of mice, our studies have shown that two different variants of the mouse ornithine decarboxylase (ODCase) gene have been selected in replicate lines of mice selected for high and low lean body mass respectively. One variant is associated with an increased peak of ODCase activity in embryos (10–13 days of gestation) in all high mass lines and with a restriction fragment length polymorphism of the expressed gene. The increased ODCase activity coincides with increased ODCase mRNA levels in the high mass selected lines. These results provide evidence implicating ornithine decarboxylase as a major factor in cell growth, and as a candidate 'trait gene'.

## 1. Introduction

Both farm and laboratory animals can be selected on a variety of different criteria resulting in increased growth rate. However, very little is known about the biochemical alterations that correlate with the altered growth rate or the products of these trait genes on which selection is being imposed. The identity of the trait genes selected is important with respect to manipulating body mass of domestic livestock. Replicate selected mouse lines have already been proven as a model system by the studies of Falconer (1973) and Sharp *et al.* (1984). These studies showed that mice could be selected for high or low body mass, with heritabilities ranging between 0·15 and 0·5 (McCarthy, 1982) thus demonstrating a considerable genetic component in the determination of growth.

Previous research directed at identification of the biochemical correlates for increased growth has proved inconclusive (see Bulfield, 1980, for review). One of the main problems in investigations of this type is differentiating between genetic drift and changes resulting from the selection process. This problem has been avoided by the use of replicate selected lines (Falconer, 1973; Sharp *et al.* 1984; Bishop & Hill, 1985) which are selected from the same base population. Any differences between the selected and nonselected lines which are correlated with the selection process will be found at high frequency or fixed in each selected line whereas a difference which is not correlated with the selection would be distributed at random between the lines. This has been validated by Garnett & Falconer (1975) who showed that the $Hbb^s$ allele was fixed in lines selected for high body mass; a similar study showed an association between body mass and the *H-2* locus (Simpson *et al.* 1982). Since these studies were published, further selected lines have been produced by selecting for high or low lean body mass from a common population at the age of 10 weeks (Bishop & Hill, 1985; Sharp *et al.* 1984).

The role of polyamine metabolism and, in particular, the enzyme ornithine decarboxylase (ODCase EC 4.1.1.17)) has not been examined in relation to the selection for lean body mass in these lines of mice, although a number of studies have implicated polyamine metabolism as playing a central role in cellular growth and proliferation as outlined below. Increased levels of ornithine decarboxylase, the initial enzyme in the pathway for synthesis of polyamines (Tabor & Tabor, 1984), and polyamines themselves have been implicated in rapid cell growth and differentiation. ODCase activity is increased in response to stimulation of cells by mitogens and growth factors although the role of these changes in determining growth is not fully understood. It has been shown by using difluoromethyl ornithine (DFMO, a highly specific

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irreversible inhibitor of ODCase), that ODCase activity is essential for normal development of the mouse embryo (Fozard *et al.* 1980) and normal weight gain in young rats (Seiler *et al.* 1978). There is also evidence that DNA, RNA and protein synthesis are affected by the level of ODCase (Pegg & McCann, 1982; Tabor & Tabor, 1984). Changes in the regulation of ODCase have been implicated in tumour formation (Alonen-Hongisto *et al.* 1987) where differences in the methylation state of the ODCase gene have been found between normal and tumour cells. Furthermore it has recently been shown that broiler chickens (selected for increased muscle mass) have a 20-fold increase in the specific activity of muscle ODCase compared to nonselected lines (Bulfield *et al.* 1988).

The association between levels of ODCase activity and growth provides the starting point for the investigation in which we report studies on ornithine decarboxylase in mouse lines selected for high and low lean body mass; the results suggest that ODCase is a growth trait-gene.

## 2. Materials and methods

### (i) *Experimental animals*

The mouse lines used in this study were kindly provided by Professor W. G. Hill from the Department of Genetics Edinburgh University (Bishop & Hill, 1985). The mouse lines used in these experiments were produced by selecting, within litter, for high (GPH) or low (GPL) lean body mass from a common population at the age of 10 weeks (Bishop & Hill, 1985; Sharp *et al.* 1984). The weight difference at 10 weeks between the high and low lines is approximately 2-fold (GPH6, 40 g and GPL6, 20 g at generation 20) (McKnight & Goddard, 1989). The work presented in this paper used three independently selected replicate lines of high body mass mice (GPH1-3) and three independently selected low lines (GPL1-3) at generation 20, as well as the lines GPH6 and GPL6, obtained by interbreeding the three replicate lines of each group. The GP6 lines were used at generation 5, i.e. 6 generations after intercrossing the replicates from generation 20, Embryos were produced by mating 1 male with 4 female mice; 16 h later all females with coital plugs were removed and placed in separate cages. Detection of the coital plug was taken as day zero of pregnancy. Embryos were then removed from the pregnant mice at the required times and stored in liquid nitrogen for later analysis.

### (ii) *Ornithine decarboxylase assay*

Ornithine decarboxylase activity was measured essentially by the method described for histidine decarboxylase (Bulfield & Nahum, 1978; Martin *et al.* 1984) with 5 mM-L-[1-$^{14}$C]ornithine (specific activity 333 nCi $\mu$mol$^{-1}$) in place of histidine and 1 mM dithiothreitol, 1 mM benzamidine, 1 % (v/v) aprotinin present in the homogenization buffer. The protein content of homogenates was estimated by the dye-binding method of Bradford (1976), using bovine serum albumin as standard.

### (iii) *Isolation of DNA and RNA*

DNA was isolated from adult mouse livers by the modified method of Maniatis *et al.* (1982), in which the tissue is first powdered in liquid nitrogen before extracting the DNA. RNA was isolated from embryos stored frozen in liquid nitrogen by the method of Chirgwin *et al.*, 1979. Poly(A$^+$) RNA was isolated from total RNA on oligo(dT) cellulose (Boehringer) according to the manufacturer's instructions.

### (iv) *Preparative and analytical agarose gel electrophoresis*

Agarose gel electrophoresis of restriction endonuclease digests was carried out in Tris borate buffer on 1% agarose gels for enzymes with four-base recognition sequences or 0·6% agarose gels for enzymes with six-base recognition sequences.

DNA was isolated from preparative gels by cutting the required zone from the gel followed by electro-elution in a Bio-Rad electro-eluter according to the manufacturer's instructions.

### (v) *RNA dot blots and quantification of mRNA*

Dot blot assays were carried out by denaturing the RNA in 50% formamide (v/v) with 10% formaldehyde (v/v) at 65 °C for 15 min and spotting aliquots onto Amersham Hybond-N nylon membranes on a vacumn dot blotter. RNA dots were fixed by UV irradiation for 7 min on a transilluminator. Quantitation of ODCase mRNA in total RNA dots was carried out as follows: two sets of dots were made, one set (10–12 $\mu$g) was hybridized with a labelled ODCase cDNA probe in 50 mM-PIPES [piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)] pH 6·8, 0·1 M-NaCl, 1 mM-EDTA, 5% SDS. Hybridization was carried out for 16 h at 65 °C. Filters were washed twice for 15 min in SSC (15 mM sodium citrate, pH 7·0, 0·15 M-NaCl) with 5% SDS at 65 °C and then exposed to X-ray film. The second set of dots from the same sample preparation (1–5 $\mu$g) were hybridised with an excess of oligo(dT) (labelled to low specific activity) in order to saturate all oligo(dT)-binding sites [poly(A) tails of mRNA] on the filter (Harley, 1987). Hybridization was carried out as described above except that the hybridization temperature was reduced to 20 °C. The filters were washed four times in 2 × SSC at 20 °C and then exposed to film for 2 h. Once the autoradiographs of the two filters were developed and checked for background the individual dots were cut from the

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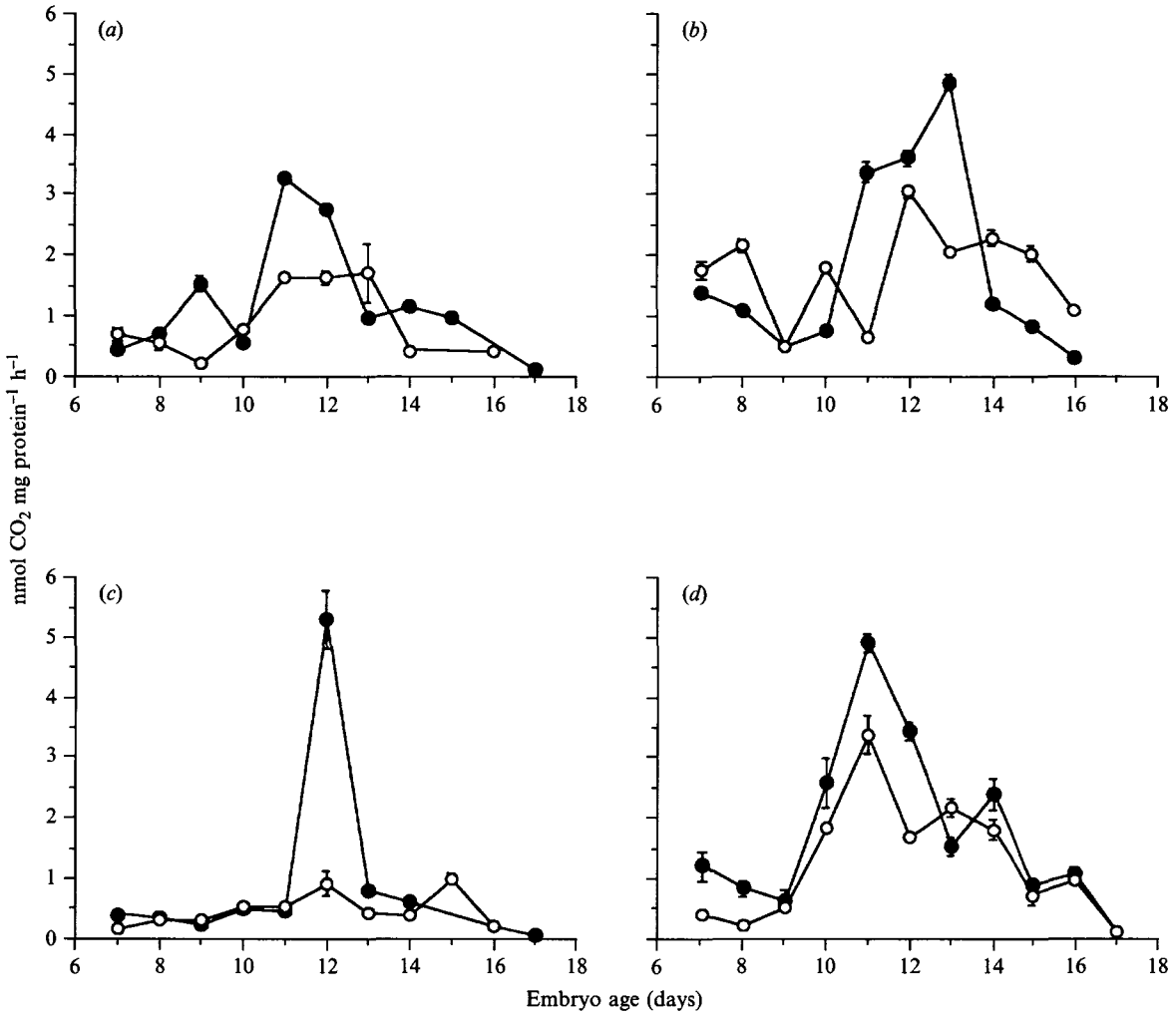


Fig. 1. Variation in ODCase specific activity during embryogenesis. ●, GPH lines; ○, GPL lines. The panels show the mean specific activity and standard deviations, from three experiments, in the replicate mouse lines. (*a*) GPH1 and GPL1, (*b*) GPH2 and GPL2, (*c*) GPH3 and GPL3, (*d*) GPH6 and GPL6.

filters and the amount of radioactive probe bound estimated by liquid scintillation counting. The amount of ODCase mRNA detected by the ODCase cDNA could then be expressed relative to the amount of total mRNA [as given by the amount of oligo(dT) bound].

(vi) *Southern transfer and hybridization*

Agarose gels of restriction digests were transferred to Amersham Hybond-N nylon membranes by the capillary method of Southern, 1975. DNA was fixed to the filters by UV irradiation. Hybridization of radiolabelled probe to Southern transfers was carried out in 7% sodium dodecylsulphate (SDS) in 0·5 M (with respect to sodium) sodium phosphate buffer pH 7·2 at a temperature of 65 °C for 16 h. Pre-hybridization was carried out in the same buffer at the hybridization temperature for 30 min. After hybridization the filters were washed three times in hybridization solution at 65 °C. The filters were then exposed overnight to Amersham Hyperfilm-MP at −70 °C with intensifying screens.

(vii) *Radioactive probes*

cDNA probes were labelled to high specific activity with [$\alpha$-$^{32}$P]dCTP using a random primer kit (Boehringer) as per the manufacturers instructions. The probes used were all purified inserts or fragments of inserts purified free of vector sequence by low melting point agarose electrophoresis. Using the hybridization protocols described above it was found that removal of unincorporated label was not required for low backgrounds. The full-length ODCase cDNA was provided by L. Sanders (Dept of Molecular Biology and Genetics, Johns Hopkins University) as a subclone of pmODC-1 (Kahana & Nathans, 1985) in the SP65 vector. Sub-fragments of the ODCase cDNA were produced by digestion with the appropriate restriction enzymes using sites identified in the published sequence. The fragments were purified by two rounds of electrophoresis on low melting point agarose gels. Low specific activity oligo(dT) was prepared by labelling with [$\gamma$-$^{32}$P]ATP using T4 kinase as described by Harley, 1987.



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## 3. Results

The specific activity of ODCase in homogenates of whole embryos from all the replicate lines from day 7 to 17 of gestation was determined (Fig. 1), detection of the coital plug being taken as day zero. The data show that ODCase activity is developmentally regulated with a peak of activity occurring between days 10 and 13. Each time-point represents the mean and standard deviation of 6 assays comprising triplicate determinations on each of two homogenates of 2–6 embryos, depending on age (6 at day 7, to 2 at day 17), obtained from two duplicate matings for each time point. Comparison of the developmental time courses of ODCase specific activity between pairs of high and low line mice (Fig 1) shows that the difference in peak activity varies between 1·3 and 6·4-fold, the mean difference being 2·5 ± 1·2-fold (mean and standard deviation from two sets of embryos for each line and three samples for each time point within a set). The fact that this difference is observed in all the independently selected high lines suggests that it is correlated with selection for high lean weight.

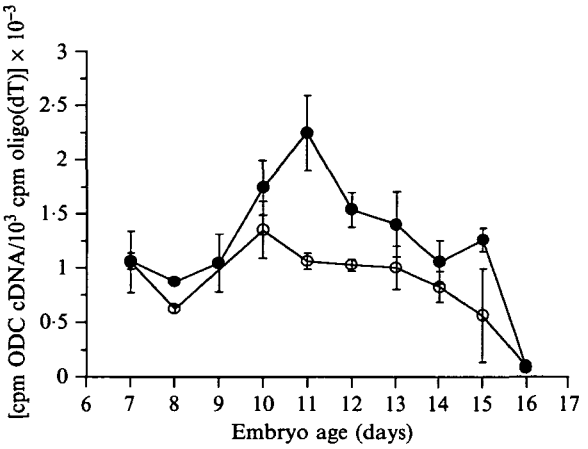


Fig. 2. Variation in ODCase mRNA levels with day of embryogenesis. The results shown are the mean and standard deviations for three determinations of ODCase mRNA levels in GPH6 (●) and GPL6 (○) embryos. The mRNA levels are expressed as ratio of ODC mRNA probe per $10^3$ counts of oligo(dT) bound (see text for details).

In order to examine whether this increased enzyme activity was due to increased levels of gene product, levels of ODCase mRNA from the mouse lines GPH6 and GPL6 (high and low lean mass lines) were quantified by dot blot analysis; all loadings of RNA were normalized for mRNA content by hybridization with oligo deoxythymidine [oligo(dT)] as described in Materials and methods. The results presented are mean and standard deviation of six determinations as described for the ODCase assays. A clear significant difference in steady-state ODCase mRNA is shown between embryos from the high and low lines (Fig. 2). It can be seen from the data (Figs 1, 2) that ODCase

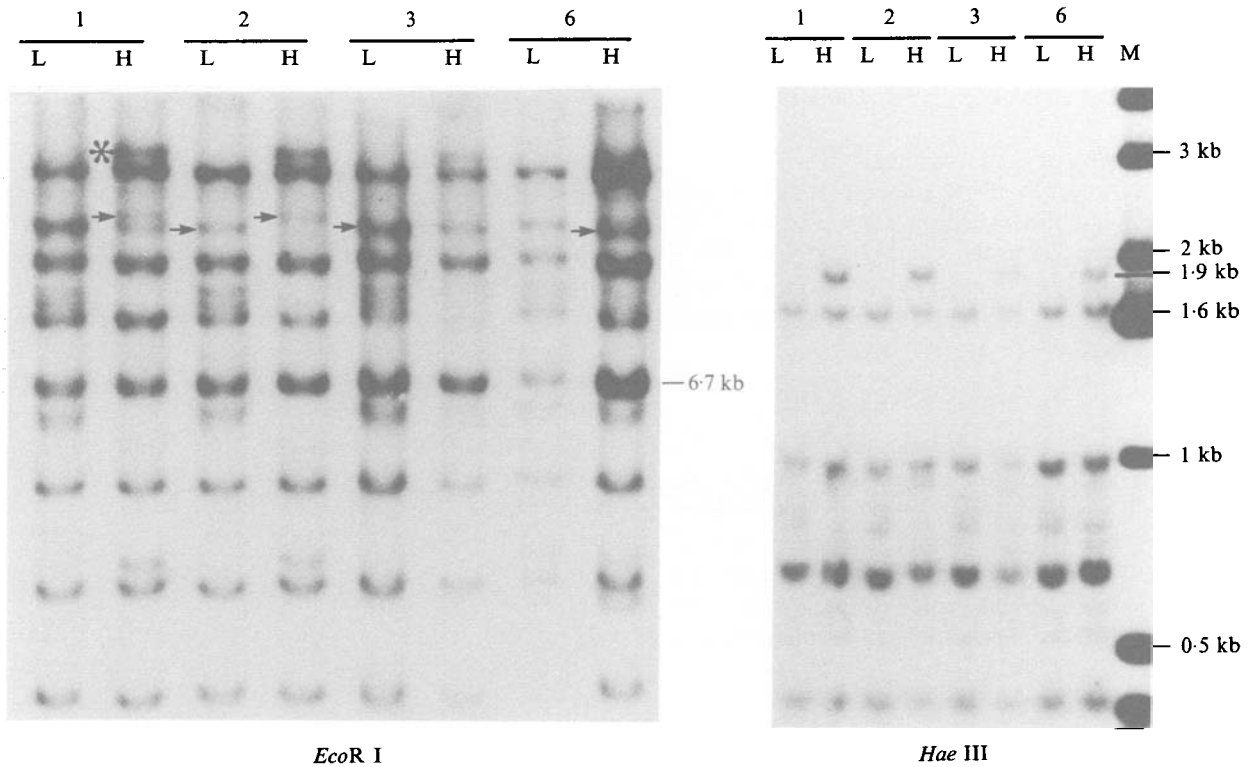


Fig. 3. Southern blots of genomic DNA from all replicate mouse lines digested with *Eco*R I and *Hae* III and probed with the full-length mouse ODCase cDNA. The *Hae* III blot shows a 1·9 kbp polymorphism restricted to all the GPH lines, while the *Eco*R I digest shows one high-molecular-weight band (*) restricted to the high lines and other polymorphic bands (→) distributed randomly between the lines.

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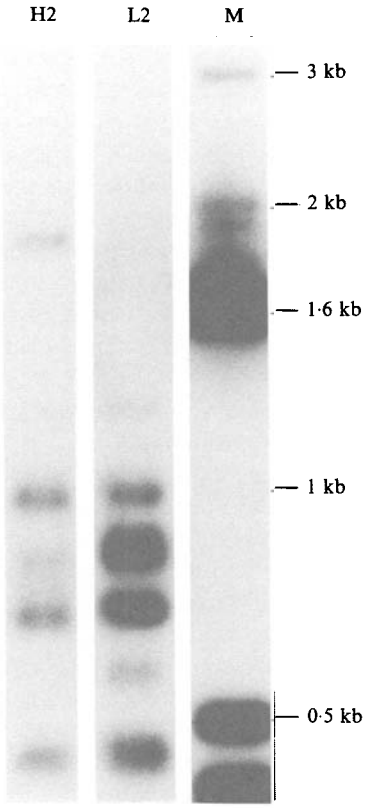


Fig. 4. Southern blot of a *Hae* III restriction digest of the 6·7 kbp *Eco*R I fragment, isolated from genomic digests of GPH2 and GPL2, and probed with a full-length mouse ODCase cDNA. Tracks shown are H2, GPH2 digest, L2, GPL2 digest and M, markers.

mRNA and enzyme activity follow the same approximate developmental time course. Thus the differences in enzyme activity can be explained by increased levels of mRNA for ODCase with resultant increases in the gene product in the embryos from the high body mass lines.

This result led to the analysis of the ODCase gene at the level of the DNA in the replicate lines. Restriction digests (*Eco*R I and *Hae* III) of DNA from all the replicate lines (pooled DNA from 3 non-sibling individuals from each line) were Southern blotted and probed with a full length ODCase cDNA probe as described in Materials and methods. The *Hae* III digest (Fig. 3) clearly shows that a 1·9 kbp restriction fragment length polymorphism (RFLP) is associated with the GPH lines and the alternative allele associated with the GPL lines. An *Eco*R I digest of the same DNA samples (Fig. 3), shows the complexity of the mouse ODCase gene family, which consists of 14 related loci most of which have been mapped to specific chromosomes. Only one functional structural gene, located on chromosome 12, has been identified (Elliot *et al.* 1988) and the remaining loci are considered to be pseudogenes. The structural gene is associated with the 6·7 kbp *Eco*R I fragment (Alonen-Hongisto *et al.* 1987; Brabant *et al.* 1988). The *Eco*R I digest shows a number of RFLPs (arrowed) only one of which (marked *) is present in all the high lines and absent in all of the low lines. The other RFLPs shown are not consistent in their distribution between the high and low lines, suggesting that they are not associated with the selection. The high-molecular-weight RFLP (*), which is an *Eco*R I fragment not associated with the expressed ODCase locus, has been mapped to chromosome 6 (R. W. Elliot, personal communication) and may be a reporter for a closely linked trait-gene which has been selected.

In order to determine whether the 1·9 kbp *Hae* III RFLP was associated with the structural gene, preparative gels of *Eco*R I digests of the lines GPH3 and GPL2 were run and the 6·7 kbp region isolated (Materials and methods) followed by digestion with *Hae* III. Southern blot analysis of these digests using the ODCase cDNA as a probe, (Fig. 4), shows that the 1·9 kbp *Hae* III fragment is presented within the 6·7 kbp *Eco*R I fragment in DNA from the high line but is absent from the low line.

Further analysis of this RFLP was undertaken by isolating sub-fragments of the ODCase cDNA probe covering the 5′ end, a central region and the 3′ end, as illustrated in Fig. 5 (Fragments F1, F2 and F3). A Southern blot of a *Hae* III digest of total genomic DNA from GPH6 and GPL6 probed separately with each of the sub-fragments (1–3, Fig. 5) shows that the 1·9 kbp *Hae* III fragment is not detected by the 3′

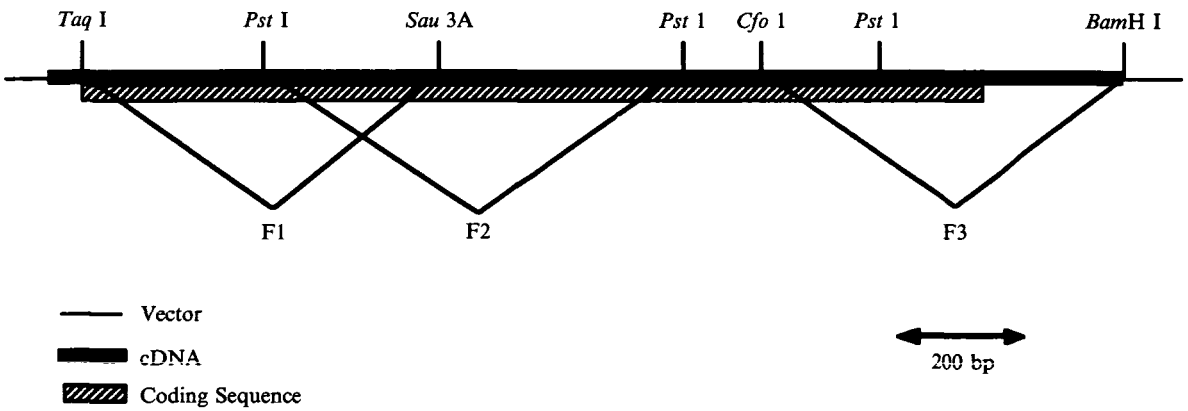


Fig. 5. Restriction map of the mouse ODCase cDNA showing the origins of the sub-fragments F1–F3 isolated and used as probes in Fig. 6.



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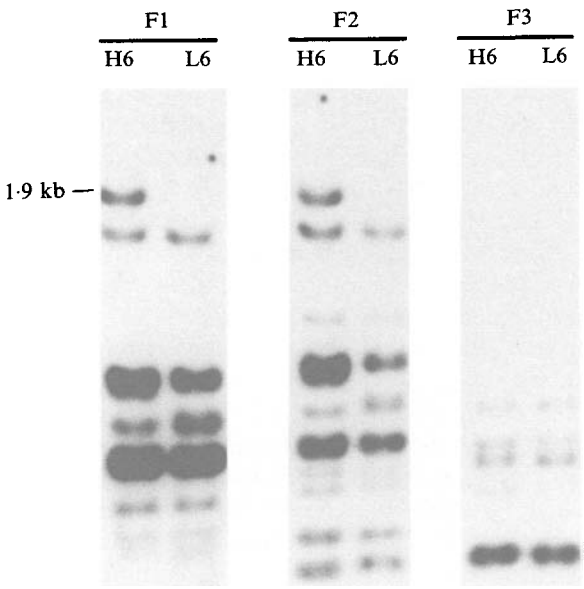


Fig. 6. Southern blot of *Hae* III digested genomic DNA from GPH6 and GPL6 mice probed separately with fragments F1–F3 of the full-length mouse cDNA as shown in Fig. 5.

probe but is detected by both the central region and 5′ probe (Fig. 6). Thus this blot shows that the RFLP is located towards the 5′ end of the gene.

## 4. Discussion

The results presented show that the activity of ODCase is developmentally regulated with a peak of activity occurring between days 10 and 13 of gestation. It may be significant that this time corresponds to the main period of cellular differentiation into organ-specific tissues in the mouse (Hogan *et al.* 1986). The observed peak of ODCase activity is consistently higher in the GPH lines than the GPL lines. The fact that this difference is observed in all the independent replicate lines suggests that this is a result of the selection.

A number of reasons for the differences in the specific activity of the enzyme can be suggested; the enzyme in the GPH lines may be a structural variant with higher activity, or the level of the enzyme protein may be increased. The level of enzyme could be raised by increased stability of the protein, resulting in a longer half life (normal half life is 10 min; Russell & Durie, 1987; Canelakis *et al.* 1979), increased half-life of the ODCase mRNA or increased levels of gene transcription.

The proportion of ODCase mRNA was estimated relative to total mRNA [quantified by oligo(dT) binding] and the results show that the differences in ODCase activity between the GPH and GPL lines are mirrored by increased study state levels of ODCase mRNA (Fig. 2). This strongly suggests that the increase in specific activity is due to an increase in the level of ODCase enzyme. The increased level of ODCase mRNA could either be due to increased stability or to increased levels of transcription. This question could be answered by measuring transcription rates in the various lines using nuclear run-on assays.

We have shown that the gene encoding ODCase has a 1·9 kbp *Hae* III restriction polymorphism in all of the high lines, that this RFLP is associated with the expressed ODCase gene, and not with any of the pseudogenes (Fig. 3) and the 1·9 kbp RFLP is located within the 5′ portion of the gene. This latter result is significant in that it indicates that the GPH and GPL variants of the ODCase gene vary at their 5′ ends. This could indicate that there are differences in regulatory sequences which could increase transcription or that there are differences in the 5′ non-coding RNA which could alter the messenger stability.

The polymorphism is unlikely to be due to a gene duplication event since the 1·9 kbp RFLP is not large enough to encode the entire ODCase gene (Coffino & Chen, 1988). Additionally the 3′ end of the cDNA does not hybridize with the 1·9 kbp RFLP and so it cannot encode the full gene. Using the published sequence of the mouse ODCase gene (Coffino & Chen, 1988), oligonucleotides could be synthesized to various regions of the gene and these regions amplified by the polymerase chain reaction (PCR) using DNA from the GPH and GPL lines. Such an analysis would allow localization of the *Hae* III polymorphism which would perhaps give enough information to determine whether or not this RFLP is related to increased transcription or increased stability of the mRNA in the GPH lines.

The high-molecular-weight restriction polymorphism (marked * in Fig. 3) is consistently present in all the high lines and absent from all the low lines. This polymorphism may be a marker for another growth trait gene in a similar fashion to the *Hbb*$^s$ allele fixed in similar lines as reported by Garnett & Falconer (1975). The lack of association between the *Hbb*$^s$ allele and body mass in the control and low mass lines suggested that a gene affecting body mass was closely linked to the *Hbb*$^s$ locus. The other restriction polymorphisms shown (arrows, Fig. 3) are not consistent in their relation to either the low or high mass lines, showing that their distribution is independent of the selection.

The fact that the ODCase specific activity is higher in all the high mass replicate lines and the *Hae* III restriction polymorphism is restricted to the high lines leads to the conclusion that these results are not due to random events independent of the selection. Accurate calculation of the probabilities of these results being obtained by chance in all six independent lines awaits the completion of a computer model of the selection experiment. The probability of obtaining the observed restriction pattern, if the RFLP is distributed randomly, for the GPL lines can be estimated as follows: each lane on the gel received pooled DNA from 3 non-sibling mice, and if any of

these mice contained the GPH RFLP it would have been detected; therefore the probability of all 3 mice being RFLP negative is $(1/2)^3 = 1/8$. This is true for each of the 4 lines giving a final probability of $(1/8)^4 = 1/4096$.

From the results presented we conclude that ODCase is a possible 'trait-gene' and a variant of the gene is associated with the GPH lines which results in higher ODCase activity during embryogenesis. We do not claim that this gene alone is responsible for the observed differences in growth between the GPH and GPL lines. It is more likely that several genes concerned with growth are being selected in the GPH lines. However, the increased ODCase activity may be required to allow the full effect of these other growth genes to be realised, since ODCase activity is essential for DNA replication, RNA synthesis and protein synthesis (Pegg & McCann, 1982; Tabor & Tabor, 1984).

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