Differential transcription and expression of ornithine decarboxylase in embryos of replicated mouse lines divergently selected for lean body mass

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Embryos of replicated mouse lines divergently selected for high or low lean body mass have been shown to differ approximately twofold in the activity of ornithine decarboxylase during embryogenesis and that this difference has been shown to be associated with a restriction enzyme polymorphism in the structural gene [Gray and Tait (1993) *Genet. Res.* 62, 31–37]. In the

present paper we report that the differences in enzyme activity are due to changes in transcription and show that the enzyme produced has the same specific activity in all selected lines, precluding any significant structural change in the enzyme being associated with the differences in the structural gene.

INTRODUCTION

A number of studies have indicated that polyamine metabolism plays a central role in cellular growth and proliferation although the precise mechanism is not understood. Increased levels of ornithine decarboxylase (ODCase; EC 4.1.1.17), the initial enzyme in the pathway of polyamine synthesis [1], 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 difluoromethylornithine (DFMO), a highly specific irreversible inhibitor of ODCase, that ODCase activity is essential for normal development of the mouse embryo [2] and normal weight gain in young rats [3]. There is also evidence that DNA, RNA and protein synthesis are affected by the level of ODCase [1,4]. Studies on tumour cell lines have shown that changes in the regulation of ODCase occur in tumour formation [5] and that these are associated with differences in the methylation state of the ODCase gene.

A further area of research in which the levels of ODCase activity have been implicated as determinants of growth and cell mass is the study of enzyme levels in lines of mice and chickens for lean body mass and breast muscle respectively. Comparisons of ODCase specific activity in breast muscle between lines of chickens (broilers) selected for increased muscle mass and non-selected lines (layers) have shown a 20-fold difference [6].

Studies of lines of mice selected for lean body mass [7,8] have shown that there is a significant genetic component affecting this trait, although the nature of all the genes involved remains to be elucidated. Using replicated lines of mice differing in lean body mass, we have shown that there is an increased peak in the specific activity of ODCase activity between days 10 and 13 of gestation in the mice selected for high lean body mass [9]. This increased level of ODCase activity is correlated with increased steady-state levels of ODCase mRNA, implying that the increased activity is due to increased levels of the enzyme. Restriction-

fragment-length-polymorphism analysis of the expressed ODCase gene has shown that a specific polymorphism has been selected in all the high-mass lines. This implies that selection for variation in ODCase may be a component contributing to high lean mass [9]. These results provide evidence that ODCase is a major factor in cell growth, and a candidate trait-gene for lean body mass.

A priori the differences in the levels of ODCase activity could be due to variants of the enzyme with different specific activities or different levels of enzyme protein. To investigate this, an antibody-based assay system has been developed, and we present data showing that the level of ODCase enzyme protein is increased in the high-mass lines correlating with the increase in enzyme activity and mRNA levels. In order to examine the basis of the increased levels of ODCase mRNA, the level of transcription of the ODCase gene has been determined by nuclear run-on assays of nuclei from embryos of both high- and low-line mice. The results obtained suggest that the observed differences in ODCase mRNA are primarily due to differences in transcription rates although a role for mRNA stability cannot be completely excluded.

MATERIALS AND METHODS

Experimental animals

The mouse lines used in this study were kindly provided by Professor W. G. Hill from Edinburgh University [7], as described previously [9].

ODCase assay

ODCase activity was measured essentially by the method described for histidine decarboxylase [10,11] with 5 mM L-[1-¹⁴C]ornithine (specific radioactivity 333 nCi/μmol) in place of

Abbreviations used: ODCase, ornithine decarboxylase (EC 4.1.1.17); DFMO, DL-α-difluoromethylornithine; GPH, general protein selected high line; GPL, general protein selected low line; DTT, dithiothreitol; GST, glutathione S-transferase.

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histidine and the addition of 1 mM dithiothreitol (DTT), 1 mM benzamidine and 1% (v/v) aprotinin to the homogenization buffer. The protein content of homogenates was estimated by the dye-binding method of Bradford with BSA as standard [12].

Isolation of nuclei

Nuclei were isolated from embryos (frozen in liquid nitrogen) by the method of Northemann [13]. Frozen embryos were homogenized in buffer (0.3 M sucrose, 5 mM DTT, 5 mM MgCl₂, 10 mM Tris/HCl, pH 7.5, 0.1% Triton X-405) with 10 strokes in a glass homogenizer with a loose Teflon pestle. The homogenate was then filtered through four layers of cheesecloth and 2 ml of buffer made up of 2.3 M sucrose, 5 mM MgCl₂, 5 mM DTT and 10 mM Tris/HCl, pH 7.5, added per ml of homogenate. After mixing, the homogenate was layered over a 2.5 ml cushion of 2.3 M sucrose, 2 mM MgCl₂ and 10 mM Tris/HCl, pH 7.5 and the nuclei were pelleted by centrifugation at 95000 *g* at (25000 rev./min) for 1 h at 4 °C in an SW40.1 rotor. The pelleted nuclei were then resuspended in 0.5 ml of 50 mM Hepes buffer, pH 7.5, containing 50% glycerol, 5 mM MgCl₂ and 0.1 mM EDTA (nuclei buffer) and frozen in liquid nitrogen until required. Numbers of nuclei were estimated by staining with ethidium bromide/Acridine Orange and counting in a haemocytometer using a top-illumination u.v. microscope.

Cell-free transcription assay

Cell-free transcription assays were carried out using a modification of the method described by Northemann [13]. Aliquots of the frozen nuclei were thawed, briefly centrifuged, and resuspended in nuclei buffer at a concentration of 0.5×10^9 – 1×10^9 nuclei/ml. The assay mixtures contained 20 μ l of resuspended nuclei (1×10^7 – 2×10^7) to which 170 μ l of reaction mixture was added consisting of 50 mM Hepes, pH 7.5, 25% glycerol, 50 mM NaCl, 2.5 mM MgCl₂, 5 mM DTT, 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 9 mM phosphocreatine, 40 μ g/ml creatine kinase (Boehringer), 0.05 mM EDTA and 1 unit/ μ l RNAase inhibitor (Stratagene). The assays were started by the addition, with mixing, of 10 μ l of [α -³²P]UTP (10 mCi/ml; 3000 Ci/mmol) and incubated at 25 °C for 35 min.

The assays were terminated by the addition of 400 μ l of RNazol B RNA-isolation solvent (Cinna/Biotec Laboratories), and nuclear RNA was isolated according to the manufacturer's instructions. Dextran sulphate and glycogen (both at 5 μ g/ml) were added to the nuclear extract, and nuclear RNA was precipitated by the addition of an equal volume of propan-2-ol. The RNA pellet was then washed three times in 70% ethanol and redissolved in hybridization solution consisting of 20 mM Pipes, pH 6.4, 50% formamide, 0.8 M NaCl, 2 mM EDTA, 0.2% SDS, 0.01% Ficoll DL 400, 0.02% polyvinylpyrrolidone, 200 μ g/ml tRNA and 100 μ g/ml polyadenylic acid.

The nuclear RNA was then denatured at 70 °C for 10 min and an aliquot (2 μ l) taken for liquid-scintillation counting before being added to the hybridization mixtures. Each labelled nuclear extract was hybridized at 42 °C for 48 h to 5 μ g of a full-length genomic clone of ODCase spotted on to Hybond-N hybridization membrane. The filters were washed three times for 15 min in $1 \times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate)/0.01% SDS at a temperature of 65 °C and exposed to a Molecular Dynamics phosphor imager screen for 48 h. Counts bound were determined using the integration function of the phosphor imager and results expressed as total phosphor imager counts bound by target sequence per 5000 c.p.m. added to the hybridization mixture. This is defined as a transcription unit. Transcription rates were

estimated in duplicate for each time point and the experiment was repeated three times on independently isolated batches of nuclei.

Construction of ODCase fusion protein

An ODCase fusion protein was generated using the pGEX-2T vector [14] which produces a glutathione S-transferase (GST) fusion protein cleavable at the fusion point by thrombin. The full-length ODCase cDNA was provided by L. Sanders (Department of Molecular Biology and Genetics, Johns Hopkins University, Baltimore, MD, U.S.A.) as a subclone of pmODC-1 [15] in the SP65 vector. There were no suitable restriction sites in the ODCase cDNA which would allow cloning in-frame into the pGEX-2T vector, therefore the cDNA was modified by PCR using mismatch oligonucleotides to generate new restriction sites allowing cloning into the *Bam*HI–*Eco*RI restriction sites of the vector. The 5'-oligonucleotide had the sequence TTC CCT GTA AGC ACC*TCG AGA ACC ATG AGC AGC TT. The protein ATG start codon is marked in bold and the *Xho*I site (underlined) created by substituting C for A in the original sequence (*). The 3'-oligonucleotide sequence, CTT CCA GGC AAG AGC TAC AAG AAT TC*C ATC TAG AG, shows the *Eco*RI site created (underlined) and the substitution C and G (*) resulting in a C-to-G substitution in the original cDNA sequence. The plasmid was digested with *Bam*HI and *Eco*RI followed by a partial filling in of the cleaved *Bam*HI site with dG and dA. The PCR product formed from the original cDNA template was digested with *Eco*RI and *Xho*I followed by a partial filling in of the cleaved *Xho*I site with dC and dT. Ligation of the modified vector and PCR product results in the in-frame insertion of the full ODCase coding sequence while maintaining the thrombin-cleavage-recognition sequence. On cleavage of the fusion protein by thrombin the ODCase produced has a four-amino acid 5' extension of Gly-Ser-Arg-Thr. The production and purification of the fusion protein is described in the Results section.

Antibody production and purification

Antibody to ODCase was produced by immunizing a New Zealand White rabbit with 250 μ g of fusion protein using Alum (Pierce Imject) as an adjuvant according to the manufacturer's instructions, and then boosting with 150 μ g and 100 μ g of fusion protein at 4 weeks and 8 weeks respectively. Small samples of blood were taken to estimate antibody titre over this time period. The rabbit was exsanguinated 8 days after a further two boosts (4 weeks apart) using 250 μ g of purified recombinant ODCase.

Antibody was partially purified using octanoic acid fractionation as follows: 1 vol. of serum was mixed with 2 vol. of 60 mM sodium acetate, pH 4, and stirred gently for 5 min. The solution was then acidified by the dropwise addition of 75 μ l of *n*-octanoic acid/ml of serum (starting volume) and stirred at room temperature for 1 h. Precipitated material was removed by centrifugation at 100000 *g* for 1 h. The supernatant was then collected and dialysed against 10 vol. of Tris/saline or PBS and stored as frozen aliquots until required.

Antibody estimation of ODCase protein

Direct estimation of ODCase protein was carried out as follows: embryo homogenate (180 μ l) prepared as for the ODCase enzyme assay (see above) was mixed with labelling solution (20 μ l) containing 5 μ Ci of DL- α -[3,4-³H]DFMO (27.12 Ci/mmol; Dupont), 10 mM DTT and 10 mM pyridoxal 5-phosphate and incubated at 37 °C for 2 h. The labelled homogenate was then

incubated overnight at 4 °C with 15 μ l of octanoic acid-fractionated antibody. Protein A–Sepharose (Sigma; 50 μ l of a 50% suspension) was added to the incubation mixture and gently mixed for 1 h. The Protein A–Sepharose was then collected and washed five times by centrifugation, twice in 1 ml of NET buffer (50 mM Tris/HCl, pH 7, 0.15 NaCl, 5 mM EDTA, 0.5% Triton X-100), once in 1 ml of NET high salt (as NET but 0.6 M NaCl) and a further twice in NET buffer. The washed Sepharose was then resuspended in scintillant for liquid-scintillation counting.

RESULTS

Transcription rates of ODCase in high- and low-mass mouse lines

Transcription rates of ODCase were estimated by nuclear run-on assays in embryos of the replicated selected mouse lines on the days of gestation corresponding to the peaks of enzyme activity shown in Figure 1 [9]. The results of the nuclear run-on experiments are presented in Table 1 for duplicate determinations on three independent batches of nuclei from the six lines of mice. The results, expressed as transcription units as defined in the Materials and methods section, show that the rates of transcription in the high-line embryos (GPH1,2,3) are greater than in the low-line embryos (GPL1,2,3). The mean $\pm$ S.D. for each mouse line is presented in the last column of Table 1. The specific activity of ODCase for embryo extracts taken from each of the three independent high lines (GPH1,2,3) and three independent

low lines (GPL1,2,3) (see Figure 1) are also shown for comparison. A paired Students *t* test (assuming unequal variance) of the mean transcription rates of each high line and low line show that they are significantly different at $P = 0.03$. A pooled correlation between ODCase activity and transcription was estimated as follows. First the mean values for the high selected and low selected lines were subtracted from the values for the individual replicate lines, and the resulting six-line deviations were correlated. The calculated correlation value was 0.994, which shows that enzyme activity is highly correlated with transcription. All transcription assays were corrected for non-specific binding which was estimated by digesting the input nuclear RNA produced with DNAase-free RNAase before hybridization and also hybridization to vector sequence without the ODCase gene.

Production of fusion protein and validation of antibody assay

The fusion protein was isolated from cultures of XL-1 Blue bacteria transformed with the fusion construct described in the Materials and methods section by the methods of Smith and Johnson [14]. Figure 2 shows an SDS/PAGE analysis of the affinity-purified fusion protein (track F) and a time course of the cleavage of the protein by thrombin. Incubation of the fusion protein with thrombin [14] results in cleavage of the fusion protein producing free GST (authentic sample shown in track G) and ODCase. The time course in tracks 1, 2, 4, 8 and 16 (representing the digestion time in minutes) shows that digestion

Figure 1 Variation in ODCase specific activity during embryogenesis

●, General protein selected high line (GPH); ○, general protein selected low line (GPL). Results are means $\pm$ S.D. from three experiments in the replicate mouse lines. (a) GPH1 and GPL1; (b) GPH2 and GPL2; (c) GPH3 and GPL3 [9].

Table 1 Transcription of ODCase in high- and low-line mouse embryos

Nuclei were prepared from the same batch of embryos as used for determination of ODCase activity in Figure 1 and the transcription activity of ODCase was determined in duplicate in three independent experiments as described in the Materials and methods section. Means $\pm$ S.D. are presented in the last column.

Mouse line	Embryo age (days)	ODCase activity (nmol/h per mg of protein)	Transcription units						
			1	1	2	2	3	3	Mean
GPH1	11	3.25	58.3	30.4	30.0	40.3	30.1	47.7	39.5 $\pm$ 11.7
GPH2	13	4.77	75.2	62.3	32.0	46.5	60.3	39.1	52.6 $\pm$ 16.2
GPH3	12	5.25	80.4	75.1	44.5	40.7	57.7	58.3	59.5 $\pm$ 15.9
GPL1	11	1.60	32.2	32.3	24.7	24.0	27.2	31.1	28.6 $\pm$ 3.8
GPL2	13	2.05	27.1	27.9	27.5	31.3	48.2	29.3	31.9 $\pm$ 8.1
GPL3	12	0.90	16.8	17.1	22.2	18.2	21.5	31.4	21.2 $\pm$ 5.5

Figure 2 SDS/PAGE analysis of thrombin cleavage of ODCase-GST fusion protein

Lane G, GST alone; lane F, glutathione-affinity-purified fusion protein; lanes 1–16 thrombin (0.4 unit/ml) digestion of fusion protein (lane numbers represent the digestion time in minutes); lanes M, molecular-mass markers.

is complete after 16 min and yields the 50 kDa ODCase polypeptide and 24 kDa GST. After thrombin cleavage, the recombinant ODCase was purified by a further round of chromato-

Figure 3 Antibody binding of [³H]DFMO-labelled recombinant ODCase

Recombinant ODCase (0.3 μ g) was labelled and immunoprecipitated with various dilutions of antibody as described in the Materials and methods section.

graphy on a glutathione-affinity column, and used to validate the conditions for the DFMO/antibody assay of ODCase protein.

In order to check that the antibody added to the assays was sufficient to bind the ODCase present, 0.3 μ g of recombinant protein was incubated with ³H-labelled DFMO and incubated with antibody as described in the Materials and methods section except that the antiserum was diluted or concentrated before addition. The addition of undiluted antiserum as described above is sufficient to bind 90 % of the labelled protein (Figure 3) as 0.3 μ g of recombinant ODCase binds 6.7×10^4 c.p.m. [³H]DFMO. Further increases in antibody concentration do not increase the amount of labelled ODCase precipitated.

The assays of ODCase activity and DFMO-binding-antibody precipitation were carried out with increasing amounts of recombinant ODCase as described in the Materials and methods section. Comparison of the two assays (Figure 4) shows that the antibody binds DFMO-labelled protein in proportion to the measured enzyme activity until the amount of ODCase exceeds the binding capacity of the antibody present at about 0.4 μ g of recombinant ODCase.

These results show that the DFMO/antibody assay for protein is a true reflection of the amount of ODCase protein present and that the range of protein levels over which this relationship is linear more than covers the levels of ODCase protein likely to be encountered in homogenates.

ODCase protein levels

The amount of ODCase protein in the embryos from day 10 to 13 of gestation in all high and low selected mouse lines was estimated by binding the irreversible inhibitor [³H]DFMO to the enzyme and immunoprecipitating the labelled protein using the antibody raised against recombinant ODCase as described in the Materials and methods section. The amount of enzyme protein, expressed as d.p.m./mg of total protein, was then compared with the enzyme activity as determined by the ODCase assay described above for each of three pairs of high- and low-line mice. The results of both assays are presented in Table 2. Comparisons of the relationship between enzyme activity and amount of protein for the high and low selected lines were made by regression analysis. If the specific activity of the ODCase protein is the same in both lines, a plot of ODCase activity against amount of protein, as given by DFMO binding, would be expected to be a straight line passing through the origin. Different slopes for the high and low selected lines were compared with a common slope for both using an *F* test. Before this step the consistency of the three replicates for each selected line was assessed in a similar manner. For the individual high lines, an *F* test for equal slopes gives $F_{2,9} = 2.26$ and for the low lines $F_{2,9} = 2.09$ which are not

Table 2 Comparison of ODCase activity and amount of ODCase protein in high- and low-line mouse embryos

Assays were carried out as described in the Materials and methods section. All data points are means of triplicate determinations from two independent experiments.

Embryo age (days)	ODCase activity (nmol of CO ₂ /h per mg of protein)		DFMO bound (d.p.m./mg of protein)		ODCase activity (nmol of CO ₂ /h per mg of protein)		DFMO bound (d.p.m./mg of protein)		ODCase activity (nmol of CO ₂ /h per mg of protein)		DFMO bound (d.p.m./mg of protein)	
	GPH1	GPL1	GPH1	GPL1	GPH2	GPL2	GPH2	GPL2	GPH3	GPL3	GPH3	GPL3
10	1.32	1.94	4119	4102	4.53	3.86	6141	5123	1.23	0.34	2365	919
11	1.83	1.90	2891	4396	2.49	2.13	3857	2512	2.76	1.56	5043	3560
12	5.15	1.59	8306	3131	2.76	0.70	4115	1371	2.66	0.69	5140	1261
13	3.24	2.74	5198	4152	2.95	1.24	4969	3079	0.52	2.07	1002	3555

Figure 4 Comparison of ODCase antibody-binding assay and enzyme assay

The plot shows the results of an ODCase assay (■) and antibody-binding assay (□) carried out in parallel using recombinant ODCase as enzyme source. Both assays were carried out as described in the Materials and methods section.

significantly different, $F_{2,9}(0.95) = 4.26$. The plot for all high lines and all low lines (Figure 5) shows that there is little difference in the slopes of the lines; an F test of these data ($F_{1,22} = 0.42$) shows that they are not significantly different at the 5% level [$F_{1,22}(0.95) = 4.30$]. Therefore the specific activity of the ODCase protein must be the same in both the high and low lines.

DISCUSSION

It has been shown that the activity of ODCase is developmentally regulated, with a peak of activity occurring between days 10 and 13 of gestation [2,9]. It may be significant that this time corresponds to the main period of cellular differentiation into organ-specific tissues in the mouse [16]. The peak ODCase activity is consistently higher in the GPH lines than the GPL lines. We have previously shown [9] that the higher activity in the GPH lines of mice is associated with an *HaeIII* restriction polymorphism and that the ODCase mRNA levels are higher in the GPH lines.

A number of explanations for the differences in the activity of the enzyme can be considered; the increased levels of mRNA in GPH lines may result in increased levels of the enzyme protein. The level of enzyme activity could be increased by structural variations between the selected lines leading to increased specific activity or a longer half-life of the enzyme (normal half-life is 10 min [17,18]). Equally the increased levels of ODCase mRNA could be due to a structural change resulting in increased mRNA stability or alternatively alterations in regulatory regions could lead to increased levels of gene transcription.

These questions are answered by the results presented in this paper. The results of the transcription assays (Table 1) clearly show that mice of the GPH line have a higher rate of ODCase transcription than those of the GPL line. From this we can conclude that the increase in transcription is responsible, at least in part, for the higher levels of ODCase mRNA observed in the

Figure 5 Regression-analysis plots of the data presented in Table 2

The antibody-binding results are plotted against ODCase enzyme activity for the high-line mice (○) and low-line mice (●), and the regression lines, H for high-line mice, L for low-line mice and C for all data points, are shown. See the text for details of analysis.

GPH line mice. A component of mRNA stability contributing to the increased ODCase mRNA levels cannot be ruled out by these data although, given the correlation of ODCase enzyme activity with transcription rate, this seems unlikely.

Comparison of the ODCase activity and amount of ODCase protein present (Table 2) demonstrates that the ODCase enzyme has the same specific activity in both the GPH and GPL lines which rules out any structural change that affects activity or stability being responsible for the increased ODCase activity in the GPH lines. If the specific activity had changed it would be expected that there would be more ODCase activity per unit of [^3H]DFMO bound in the GPH lines than the GPL lines, although again a minor component of stability contributing to the observed differences cannot be eliminated. The variation in expression of ODCase shown in Table 2 is a reflection of the changes in ODCase activity during the development of the embryo and the biological variation between selected lines as shown in Figure 1.

From the data we can conclude that the *HaeIII* restriction polymorphism associated with the 5' end of the ODCase structural gene in all the GPH lines [9] is associated with increased transcription of the gene. We can also conclude that there is no change in the specific activity of ODCase between the GPH and GPL lines.

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