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Pre-translational control of hepatic malic enzyme expression during the development of the rat

David J. MANN* and Ernest BAILEY†
Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K.

The expression of hepatic cytosolic malic enzyme in the developing rat has been studied by molecular-biological techniques. Malic enzyme mRNA was barely detectable throughout the neonatal period, but increased to significant levels immediately before weaning. Northern-blot analysis demonstrated that the two major malic enzyme mRNA species displayed non-co-ordinate control during development, with the 2.0 kb form accumulating to a greater extent than the 3.1 kb form. A novel 1.6 kb mRNA species was found to predominate in foetal samples. Tri-iodothyronine treatment of neonatal rats caused premature induction of all three malic enzyme mRNA species. Dietary studies also showed precocious induction of the mRNA with diets high in carbohydrate, but not with those high in fat.

INTRODUCTION

Cytosolic malic enzyme (EC 1.1.1.40) is believed to play a key role in the generation of NADPH for lipogenesis. After birth, the activity of the rat hepatic enzyme correlates closely with the rate of lipid biosynthesis [1,2]. Previous studies have shown a dramatic increase in hepatic malic enzyme activity at the suckling–weaning transition [3]. Considerable dietary and hormonal changes occur at this developmental stage, including replacement of the high-fat maternal milk diet by solid high-carbohydrate food, an elevation of the circulating levels of tri-iodothyronine (T_3) [4] and the relieving of the insulin resistance which is apparent in the neonatal animal [5]. Variations in these parameters have been shown to alter hepatic malic enzyme expression in adult rats [6–8], and therefore could be perceived to influence the elevation of the activity of this enzyme at the time of weaning.

Previous studies on the expression of hepatic cytosolic malic enzyme during the development of the rat have focused on enzyme activity [1,9] and immunoreactive protein [2]. In the present study we have attempted to analyse the contribution of pre-translational events to the observed changes in malic enzyme protein levels during the early developmental period. Using molecular-hybridization techniques, we demonstrate that the increase in malic enzyme expression occurring at weaning is preceded by a similar specific increase in its mRNA. In addition, the malic enzyme mRNA species detected by Northern blotting exhibit non-co-ordinate control around about the time of weaning. Analysis of the effects of diet and hormones demonstrates that these parameters are likely to influence the expression of this enzyme during this developmental period.

EXPERIMENTAL

Animals

The rats used were of an inbred albino Wistar strain, maintained at 22 °C on a 12 h-light/12 h-dark rotation in the University of Sheffield animal house. The normal gestation period of the rats was 22 days. Foetal age was calculated from the day of mating. Day 0 was taken as being the first 24 h after birth. Newborns were given unlimited access to their dams. Litter sizes were standardized to 8–12 pups each. T_3 was administered as a single dose intraperitoneally at 7 days of age in 0.9% (w/v) NaCl at a dose (2 μg/g body wt.) calculated to maintain greater than 95% receptor saturation for 24 h [10]. Treated animals were found to show similar weight gains to their saline-injected siblings (results not shown). Normally, animals were fed *ad libitum* on a CRM diet (Argo Animal Foods, Sheffield, U.K.) in which 70% of the available energy is starch. In addition, some animals were weaned on to either a solid high-fat-content diet [11], in which the proportions of carbohydrate, protein and fat were identical with those of rat milk (13:37:50, by wt.), or a high-carbohydrate-content diet [6], which contained 70% (w/w) sucrose. Weaning was normally performed at 21 days *post partum*, by separation of parent and offspring. Early weaning was performed at 16 days after birth. Animals were killed by cervical dislocation, and the liver was rapidly removed and either flash-frozen in liquid N_2 or placed on ice for immediate use. All animals were taken between 09:00 and 10:00 h to avoid possible diurnal variation.

Enzyme activity measurements

Malic enzyme [12] and lactate dehydrogenase [13] were assayed in liver supernatants prepared in 300 mM-Tris/HCl/2 mM-EDTA/1 mM-dithiothreitol, pH 7.6. One unit of enzyme activity was defined as the reduction of 1 nmol of $NADP^+$ (or NAD^+)/min at 30 °C. DNA was quantified in crude homogenates by the method of Labarca & Paigen [14] with salmon sperm DNA as an internal control.

RNA preparation

The method employed was a modification of that described by MacDonald *et al.* [15]. The tissue was roughly ground under liquid N_2 and then quickly homogenized in 10 ml of 4 M-guanidinium thiocyanate/100 mM-Tris/HCl/1% (v/v) β-mercaptoethanol (pH 7.5)/g of sample for 45 s at full speed with a Silverson Tissumiser at room temperature. The homogenate was made 4% (w/v) in sodium lauryl sarcosinate, mixed thoroughly and centrifuged briefly to remove insoluble debris.

Potassium acetate (pH 5.5) and acetic acid were added to 100 mM and 80 mM respectively and the RNA was precipitated by addition of 0.75 vol. of ethanol. After 2 h at −20 °C the nucleic acid was pelleted at 10000 *g* for 10 min. The pellet was resuspended in 5 ml of 7.5 M-guanidinium chloride/10 mM-dithiothreitol, pH 7.0. Then 0.05 vol. of 2 M-potassium acetate,

Abbreviations used: T_3, tri-iodothyronine; poly(A)$^+$, polyadenylated RNA.

* Present address: Department of Biochemistry, University of Dundee, Dundee DD1 4HN, U.K.
† To whom correspondence should be sent.

pH 5.5, was added, and 0.5 vol. of ethanol was then slowly mixed into the solution. After a further 2 h at −20 °C and centrifugation as above, the RNA precipitate was dispersed in 70% (v/v) ethanol and re-pelleted by brief centrifugation. The pellet was resuspended in 20 mM-EDTA, pH 7.0, and vortex-mixed for 4 min in the presence of 2 vol. of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). After centrifugation, the upper aqueous phase was removed to a sterile tube, and the organic phase and interface were re-extracted with a further 1 vol. of EDTA solution exactly as before. The aqueous phases were pooled and the RNA was precipitated by addition of 0.1 vol. of 3 M-sodium acetate, pH 5.2, and 2.5 vol. of ethanol. The RNA was stored in this form until required, when it was again pelleted by a 10 min centrifugation as described above and resuspended in an appropriate amount of solvent.

Polyadenylated [poly(A)$^+$] RNA was isolated from total RNA by the method of Aviv & Leder [16]. Rat liver polysomes were isolated exactly as described by El-Dorry [17] and poly(A)$^+$ RNA was subsequently selected [18].

RNA quantification

Assays were routinely performed as follows: approx. 0.2 μg of poly(A)$^+$ RNA was diluted to 249 μl with hybridization solution (10 mM-Tris/HCl/300 mM-NaCl/10 mM-$MgCl_2$, pH 7.5). Then 2.5 μg of [^{3}H]poly(U) (0.37 kBq; Du Pont, Stevenage, Herts., U.K.) was added, and the solutions were mixed and incubated at 30 °C for 20 min. RNAase A, freshly boiled for 5 min and quenched on ice, was then added to 10 μg final concn. and the reaction mixture was incubated for a further 15 min at 30 °C. The undegraded [^{3}H]poly(U) was then radioassayed after collection on glass-fibre filters (Whatman, Maidstone, Kent, U.K.) by precipitation with 2.5% (w/v) trichloroacetic acid [19].

Samples were assayed in triplicate and the mean value was used to determine their relative poly(A)$^+$ content. With each set of assays, two controls were routinely performed, one involving assay with no added poly(A)$^+$ RNA to indicate the completion of the RNAase A digestion, and a second with no added poly(A)$^+$ RNA or RNAase A to indicate the degree of endogenous degradation of the [^{3}H]poly(U).

Hybridization analysis

Equal concentrations of poly(A)$^+$ RNA in 50% (v/v) formamide/2.2 M-formaldehyde/20 mM-Mops/5 mM-sodium acetate/1 mM-EDTA, pH 6.5, were denatured at 65 °C for 15 min. The samples were then spotted on to the nylon membrane with a Bio-Rad Dot Blot apparatus. The samples were allowed to drain from the wells under gravity over a period of about 2 h, and each well was then washed under vacuum with 1 M-ammonium acetate. After air-drying, the RNA was covalently attached to the nylon membrane by u.v. irradiation [20]. A range of concentrations was established for each sample to enable accurate quantification of the resultant autoradiographs.

Northern blots were performed by the formaldehyde method of Lehrach *et al.* [21] with samples containing equal amounts of poly(A)$^+$ RNA (up to 10 μg). Processing and transfer were performed as described by Maniatis *et al.* [22]. In order to assess accurately the molecular masses of species detected by Northern analysis, RNA standards (0.24–9.49 kb ladder; GIBCO, Uxbridge, Middx., U.K.) were included, processed and transferred to the nylon membrane along with the samples. After cross-linking by u.v. irradiation, the membrane-bound RNA was stained with Methylene Blue [22]. The distances of migration of the RNA markers and the residual rRNA in the poly(A)$^+$ samples were noted and a calibration plot was made [22]. This staining procedure was found to have no effect on the subsequent hybridization of the membrane.

Prehybridization and hybridization were performed at 55 °C, by using 1 ml of the following solution per 5 cm^2 of membrane: 50% (v/v) deionized formamide/5 × SSPE [1 × SSPE is 150 mM-NaCl/10 mM-NaH_2PO_4/1 mM-EDTA (pH 7.4)]/0.5% (w/v) polyvinylpyrrolidone/0.5% (w/v) BSA/0.5% (w/v) Ficoll 400/heat-denatured sonicated salmon sperm DNA (500 μg/ml)/poly(A) (50 μg/ml)/*Escherichia coli* tRNA (50 μg/ml). Polyethylene glycol 6000 (10%, w/v) was also included to accelerate the rate of hybridization [23].

The malic enzyme cDNA (pME6, ref. [24]) and that for albumin (pRSA13, ref. [25]) were kindly given by Dr. V. M. Nikodem, National Institutes of Health, Bethesda, MD, U.S.A. and Dr. J. Bonner, California Institute of Technology, Pasadena, CA, U.S.A. respectively. Recombinant plasmid DNA was restriction-digested to liberate the cDNA insert, which was then purified after electrophoresis from a 1% (w/v) low-melting-temperature agarose gel [22]. This insert was labelled by nick translation with [α-^{32}P]dATP (Amersham International, Amersham, Bucks., U.K.) [26]. Typically, incorporations of (1–2) × 10^8 c.p.m./μg of DNA were achieved. Alkali-denatured [26] cDNA probe was included in the hybridization solution at approx. 5 × 10^5 c.p.m./ml. Hybridization was performed for 12–16 h. After this time, blots were washed with two changes of 5 × SSPE for 20 min each at 65 °C, followed by two changes of 0.1 × SPPE/0.1% (w/v) SDS at 65 °C for 20 min each. Autoradiography was then performed at −70 °C with intensification. Probes were removed by placing in 1% (v/v) glycerol at 85 °C for 4 min, the efficiency of probe removal being assessed by autoradiography.

An Helena Laboratories Quick Scan R and D densitometer was used to quantify autoradiographs, after ensuring that the exposure was within the linear response range of the film.

RESULTS

Dot-blot analysis

Poly(A)$^+$ RNA isolated from the livers of rats of ages ranging from 20 days gestation to over 100 days *post partum* was spotted on to a nylon membrane and hybridized with the nick-translated cDNA coding for malic enzyme. As an internal standard, albumin mRNA levels were subsequently assessed. The results of these experiments are presented in Figs. 1 and 2. Expression of malic enzyme mRNA in rat liver is very low until 16 days after birth, but subsequently increases rapidly until about 35 days *post partum*, thereafter declining to adult levels. This developmental profile is quantitatively consistent with data presented previously [2], which demonstrated the appearance of malic enzyme activity and immunoreactive protein at the suckling–weaning transition. The expression of malic enzyme mRNA slightly precedes that of activity/protein levels, indicating that the induction of expression occurring at this time is due primarily to pre-translational events.

The results presented for malic enzyme contrast markedly with those determined for albumin. Dot-blots which had been hybridized with the malic enzyme cDNA were re-probed with the nick-translated albumin insert after ensuring that the previous signal had been completely removed. The level of albumin mRNA increased slightly during the first few days after birth and subsequently maintained a relatively stable level by about week 3 of life (Fig. 2). These data are in agreement with previous findings [27,28] and indicate the specific nature of the malic enzyme induction.

In addition to the dot-blots constructed by using poly(A)$^+$ RNA isolated from total liver RNA, polysomal poly(A)$^+$ RNA was isolated from animals at different developmental ages and the concentrations of malic enzyme mRNA and albumin mRNA were assessed. The malic enzyme polysomal poly(A)$^+$ RNA

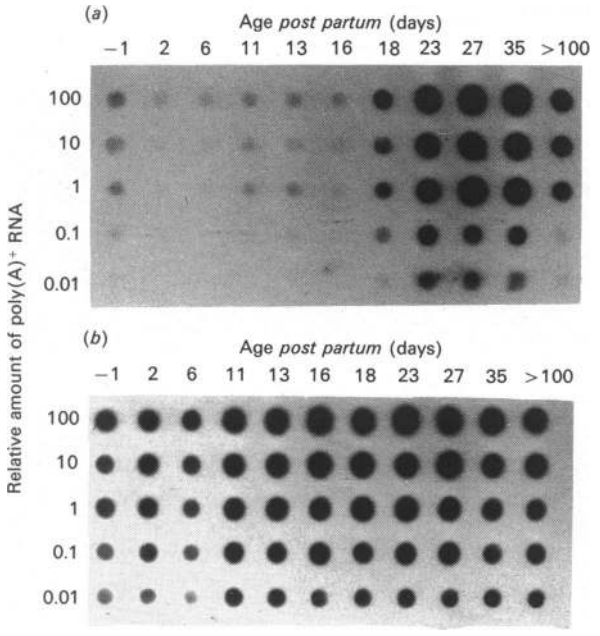

Fig. 1. Dot-blot analysis of the time course of developmental changes in mRNA levels of malic enzyme (*a*) and albumin (*b*)

Samples comprising serial dilutions of quantified poly(A)$^+$ RNA, isolated from 3–10 pooled rat livers from animals of the indicated ages *post partum*, were hybridized with the appropriate nick-translated probe. The blots shown are representative examples of 4 separate experiments.

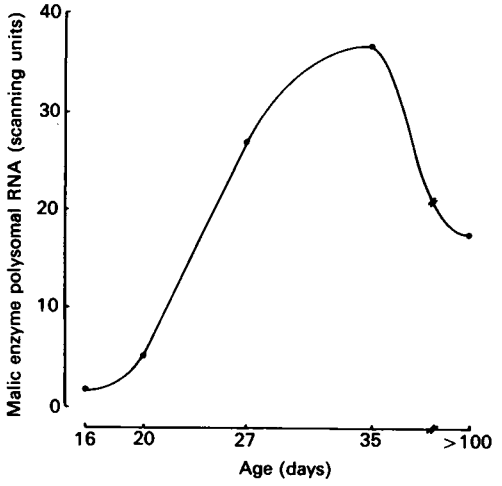

Fig. 3. Dot-blot analysis of the changes in malic enzyme mRNA levels in polysomal poly(A)$^+$ RNA immediately before and after weaning

The data points represent the means of two separate experiments performed with duplicate samples and normalized against adult values. Variation between samples was less than 10% of the normalized value in each case.

showed increases which exactly paralleled those determined for poly(A)$^+$ RNA isolated from total RNA (Fig. 3). Albumin polysomal poly(A)$^+$ RNA exhibited little change in abundance over the period studied (results not shown).

Very small amounts of malic enzyme mRNA could be detected throughout the neonatal period over and above non-specific background, as judged by hybridization to *Escherichia coli* tRNA. Similar observations have been reported during the developmental expression of rat liver tryptophan 2,3-dioxygenase [28] and phosphoenolpyruvate carboxykinase [29]. The production of such mRNA during the neonatal period may reflect a lack of regulatory stringency in gene expression of these normally housekeeping proteins. In addition, a slightly larger signal (4 times that detected at day 13) was registered with poly(A)$^+$ RNA isolated from foetal liver at 21 days gestation.

Size distribution of the malic enzyme mRNA

In order to determine the size of hepatic malic enzyme mRNA during development, Northern-blot analysis was performed, again using the quantified poly(A)$^+$ RNA samples (Fig. 4). Malic enzyme mRNA exists as two major species estimated to be 2.0 and 3.1 kb, similar to previous values in the adult rat [7]. Quantification of the Northern-blot data revealed overall patterns of malic enzyme mRNA expression identical with those determined by dot-blot analysis. However, the ratio of the two major mRNA forms did not remain constant during development (Table 1). At 16 days after birth, the only detectable species was the 3.1 kb form, with the smaller 2.0 kb type first appearing at

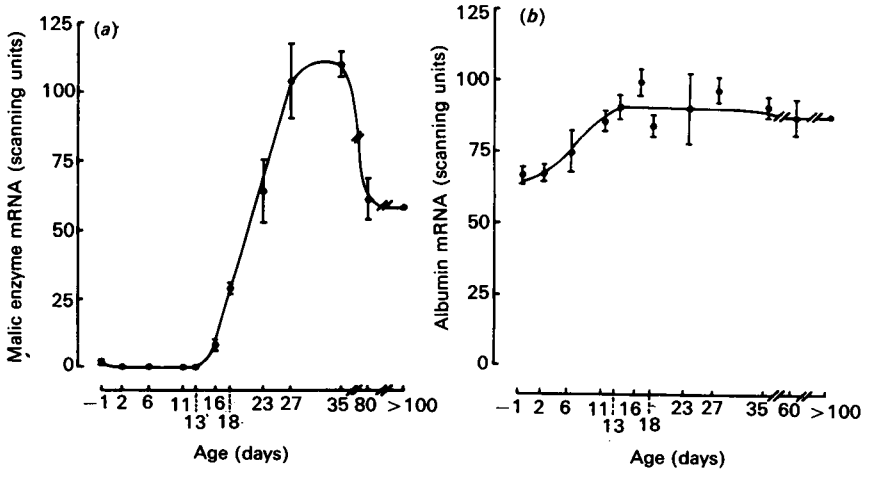

Fig. 2. Quantitative changes in the amounts of mRNA coding for malic enzyme (*a*) and albumin (*b*) during postnatal development

The experimental data were generated by densitometrically scanning blots such as those in Fig. 1. The values indicate relative scanning units normalized against adult values (means ± S.E.M.).

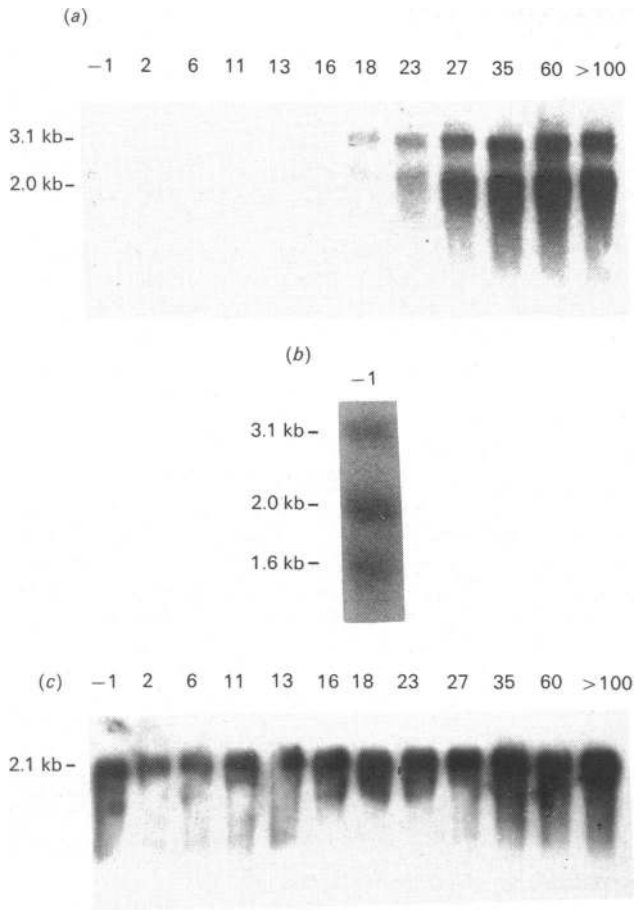

Fig. 4. Northern blot showing the size distribution of rat liver malic enzyme and albumin mRNA during development

Ages *post partum* (days) are indicated. (*a*) Malic enzyme (16 h exposure); (*b*) malic enzyme, foetal-rat liver (2-week exposure); (*c*) albumin (6 h exposure).

Table 1. Change in the ratio of the abundance of the 2.0 kb and 3.1 kb forms of malic enzyme mRNA during development calculated from scanning-densitometer quantification of the Northern blot shown in Fig. 4

Age (days)	Ratio of 2.0:3.1 kb mRNA species
16	0.05:1
18	0.95:1
23	3.19:1
27	3.27:1
35	4.14:1
> 100	3.10:1

day 18. After weaning at day 21, both species increased rapidly in abundance. By 23 days of age, the 2.0 kb form predominated and the larger species had attained a level of expression which was maintained throughout the remainder of the developmental period into adulthood. The smaller form increased dramatically, until at 35 days after birth it was over 4-fold more abundant than the other species. In the adult rat, the final ratio of small to large malic enzyme mRNA species was found to be approx. 3:1, similar to that reported previously [7]. Thus, during the early life of the rat, an increase in the 2.0 kb species relative to the 3.1 kb form was observed.

Prolonged exposure of these developmental Northern blots revealed the presence of three malic enzyme mRNA species in foetal-rat liver (Fig. 4). The additional mRNA band was approx. 1.6 kb in length and comprised about 60% of the total malic enzyme mRNA in foetal-liver samples as judged by scanning densitometry. This mRNA was retained by oligo(dT)–cellulose columns, indicating the presence of a poly(A) tract. The malic enzyme mRNA coding sequence of the rat is 1716 nucleotides long [30], so that this novel species cannot fully encode native malic enzyme. Adult-rat liver appears not to express this mRNA form (D. J. Mann & E. Bailey, unpublished work; [7]).

When the same blot used to generate the data for the malic enzyme mRNA size distribution was probed specifically for albumin mRNA, a single band was observed at each age (Fig. 4). This mRNA shows an increase identical with that shown by the corresponding dot-blots. The albumin mRNA appears at a position equivalent to 2.1 kb, in close agreement with other estimates [27].

Table 2. Changes in rat liver malic enzyme activity and mRNA following dietary manipulation

Animals were weaned at 21 or 16 (early weaning) days *post partum* and fed on the indicated diet for 7 days (Control, high-starch; Fat, high-fat; Sucrose, high-sucrose). Activity data are the means ± S.E.M. ($n-1$ of 5–6 separate experiments. mRNA results are scanning-densitometer quantifications (means ± S.E.M.; $n-1$) of 3 separate dot-blots, the values given being relative scanning units normalized to the control value.

Treatment	Malic enzyme activity (units/μg of DNA)	Malic enzyme mRNA (relative scanning units)
Control	23.7 ± 2.3	30.0
Fat	6.5 ± 1.0	16.0 ± 3.0
Sucrose	41.9 ± 3.2	38.6 ± 4.1
Early weaning, control	49.6 ± 2.9	51.9 ± 7.8
Early weaning, fat	10.0 ± 1.1	10.2 ± 1.8
Early weaning, sucrose	16.1 ± 0.3	9.8 ± 0.7

Dietary effects

In order to investigate the effects of the dietary change at the suckling–weaning transition on the developmental expression of hepatic malic enzyme, rats were weaned at 16 or 21 days *post partum* on to one of three diets: a control diet which was high in starch, a high-fat diet or a high-sucrose diet. After 7 days, animals on all diets showed similar weight gains as compared with their respective controls, except for those weaned early on to the sucrose diet. These failed to grow normally, owing to their inability to digest sucrose [31]. The enzyme activity and mRNA abundance data are summarized in Table 2. The levels of malic enzyme mRNA and activity corresponded closely to one another. It is evident that animals weaned on to the high-fat diet or weaned early on to the sucrose diet showed lower levels of malic enzyme expression than did control weaned animals, whereas rats weaned at 21 days of age on to the high-sucrose diet showed elevated amounts of malic enzyme mRNA and activity. Northern-blot analysis showed similar patterns of change of

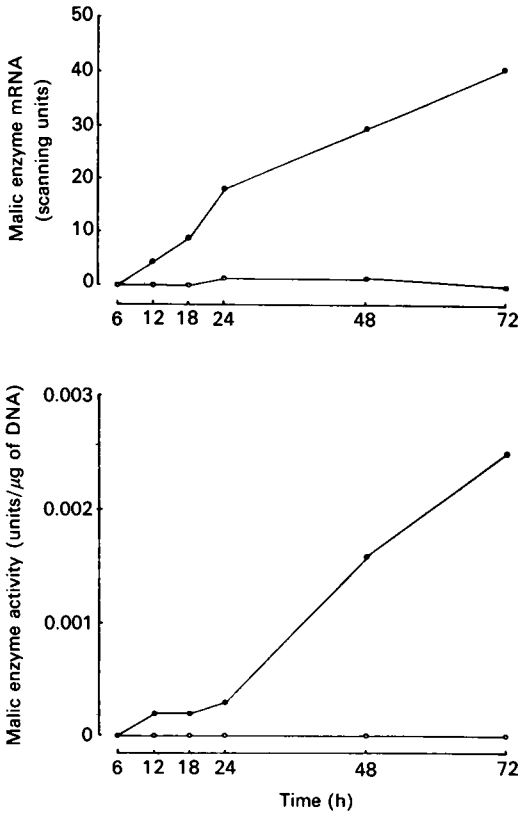

Fig. 5. Changes in malic enzyme activity and mRNA after treatment with T_3 (●) compared with saline controls (○) treated at zero time

Enzyme-activity results represent the means of 5–6 separate experiments. mRNA data are mean scanning-densitometer quantifications of 3 separate dot blots, the values indicated being relative scanning units normalized to values for 72 h + T_3.

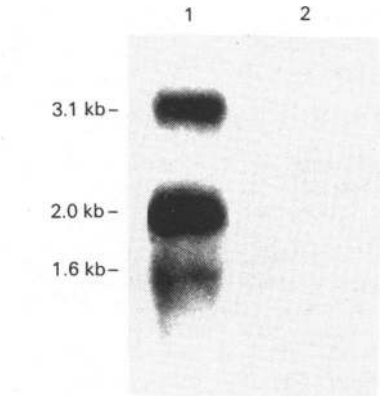

Fig. 6. Northern blot showing the size distribution of hepatic malic enzyme mRNA after 2 days treatment of neonatal rats with either T_3 (1) or saline (2)

malic enzyme mRNA to those determined by dot-blot studies (results not shown).

Neither the levels of rat hepatic albumin mRNA nor lactate dehydrogenase activity were altered by these dietary manipulations (results not shown).

Effects of T_3

T_3 has been shown to induce malic enzyme activity and immunoreactive protein in neonatal rats [2]. The results presented here show that this increase is secondary to an elevation in the level of the malic enzyme mRNA species (Fig. 5). Control animals displayed barely detectable amounts of malic enzyme mRNA and activity. These results also demonstrate that malic enzyme expression is only slowly responsive to T_3, in agreement with results obtained previously in adult rats [32].

Northern-blot analysis revealed that the two major malic enzyme mRNA species were induced to the same extents by T_3 treatment (Fig. 6). However, at the earlier time points, the novel 1.6 kb malic enzyme mRNA species was detected, although it was not present after 3 days treatment or in similarly treated adult rats (D. J. Mann & E. Bailey, unpublished work; [7]), and therefore appeared to be selectively and transiently expressed.

Rat hepatic albumin mRNA levels and lactate dehydrogenase activity were unaffected by these treatments, demonstrating a specific effect on malic enzyme expression (results not shown).

DISCUSSION

The results presented here demonstrate that the increases in malic enzyme activity associated with normal or premature weaning or the administration of T_3 are secondary to increases in the abundance of the specific mRNAs coding for this enzyme.

The fundamental change occurring at weaning is an increase in dietary carbohydrate intake. Previous studies have demonstrated the involvement of a carbohydrate-derived signal in the control of malic enzyme expression in adult-rat hepatocytes [33]. The results presented here indicate that carbohydrate feeding is associated with elevated malic enzyme expression. Further, the quality of the carbohydrate is also important, with a sucrose-based diet enhancing the elevation of expression over that of the starch-rich diet in rats weaned at 21 days of age. This may be due to insulin-independent metabolism of fructose [34], derived from the dietary sucrose, thereby enabling enhanced generation of the putative carbohydrate-derived signal. Post-weaning rats only gradually lose the significant insulin resistance typical of the neonatal state [5], so that the generation of the putative signalling molecule from glucose may be initially retarded, as seen with animals weaned early on to the sucrose diet. Weaning on to a high-fat diet did not completely abolish malic enzyme induction, indicating the involvement of factors other than the carbohydrate-derived signal in the control of expression at this time.

Early weaning has no effect on the levels of accumulation of circulating T_3 [35], so that rats prematurely fed a solid diet display adaptations divorced from the developmental increase in this hormone. The fact that the ratio of small to large malic enzyme mRNA species in early-weaned rats was similar to that in normally weaned animals after the same length of time on the specified diet indicates that the change in this ratio at weaning is probably not due to an effect of T_3. However, this change may not be attributed only to the dietary switch, as the levels of other potential regulators, such as insulin [36], are altered by this approach.

The activity of malic enzyme in adult rats has often been used as a measure of thyroidal status, being elevated in hyperthyroid animals and depressed in the hypothyroid state. Malic enzyme expression in neonatal rat liver is competent to respond to T_3. Endogenous serum T_3 increases throughout the neonatal period [4], preceding malic enzyme induction by about 6 days. This temporal discrepancy may be resolved by the intrinsically slow response of malic enzyme expression. Additionally, a threshold level of hormone may be required before induction occurs, as has been described for ovalbumin induction by oestrogen [37].

Malic enzyme mRNA was found in foetal rat liver of 21 days gestation. This level was approx. 4-fold greater than that detected during the neonatal period, with the major mRNA species being

a previously unreported smaller form, approx. 1.6 kb in length. This is likely to encode an anomously low-molecular-mass form of malic enzyme. The mechanism leading to the generation of this message awaits further investigation. The presence of this novel mRNA in foetal liver, but not in non-induced neonatal or adult liver, may provide a useful marker of the state of hepatocyte differentiation.

The size distribution of the malic enzyme mRNA species after weaning exhibits non-co-ordinate control of the two major forms. Recently, Morioka *et al.* [30] have demonstrated by sequencing and S1-nuclease analysis that the molecular basis for the different sizes of these mRNAs is due entirely to the selection of alternative polyadenylation sites, although the mechanism governing this selection is unknown.

Although there are many reports describing multiple mRNA forms coding for a single polypeptide, accounts of non-co-ordinate control of those species, as demonstrated here for malic enzyme, are rare. One of the few documented examples is that of L-type pyruvate kinase. Like malic enzyme, the activity of pyruvate kinase shows a marked induction about the time of weaning [38]. Marie *et al.* [39] have demonstrated that the protein is encoded by three mRNA species which differ only in their 3′ regions owing to variation in the selection of polyadenylation sites. Also, they showed that during postnatal development there is an increase in the production of its smaller mRNA forms relative to the larger. This situation is analogous to that described here for malic enzyme mRNA. In addition, Dozin *et al.* [7] demonstrated that the two forms of the malic enzyme mRNA are found in different ratios in different tissues. Again, Marie *et al.* [39] demonstrated an identical phenomenon for pyruvate kinase mRNA. This correlation between the developmental and tissue-specific expression of malic enzyme and L-type pyruvate kinase provides circumstantial evidence for similar mechanisms of control. Concerted study of these and other proteins with similar patterns of expression, such as glucokinase, fatty acid synthase and acetyl-CoA carboxylase [40], will provide a greater insight into the mechanisms governing metabolic control in eukaryotic development.

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