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A Cloned cDNA for Duck Malic Enzyme Detects Abnormally Large Malic Enzyme mRNAs in a Strain of Mice (Mod-1^n) That Does Not Express Malic Enzyme Protein†

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ABSTRACT: Sensitive immunochemical assays were used to measure the mass and rate of synthesis of malic enzyme protein in wild-type and Mod-1^n mutant mice fed a high carbohydrate/low fat diet supplemented with thyroid hormone. Malic enzyme activity in the fed, wild-type mice was 100-fold higher than in starved, wild-type mice. Neither activity, mass, nor synthesis of malic enzyme could be detected in fed, mutant mice. However, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase responded to these dietary manipulations with normal or supranormal increases in activities, respectively, in mutant mice. A cDNA clone containing an almost complete copy of the mRNA for malic enzyme from duck liver was used to analyze poly(A+) RNA from C57BL/6J-DBA/2J hybrid mice that had been fasted and refed a high carbohydrate/low fat diet supplemented with thyroid hormone. The ^{32}P-cDNA probe hybridized to two RNAs of 2250 and 2950 nucleotides. The same two RNAs were detected in RNA from starved mice except at much lower concentrations. A similar analysis of RNA from Mod-1^n mice fed the high carbohydrate–thyroid diet also revealed two hybridizing RNAs but each was 700–800 nucleotides longer than its counterpart in wild-type mice. The abundance of malic enzyme mRNA in the fed, mutant mice was about the same as that in fed, wild-type mice. The mutant malic enzyme mRNAs also were present in RNA from starved mice but at much lower concentrations. These results suggest that the mutation responsible for the Mod-1^n phenotype is in the structural gene for malic enzyme. Undetectable synthesis of enzyme in the presence of a normal abundance of mRNA suggests either a protein product with an exceptionally short half-life or an altered mRNA that cannot be translated in liver.

Malic enzyme (EC 1.1.1.40) catalyzes the NADP-dependent oxidative decarboxylation of malate to pyruvate and CO_2 (Frenkel, 1975). Malic enzyme and other enzymes involved in lipogenesis are regulated coordinately by diet and hormones in the livers of both birds and mammals. Starvation causes a decrease in the rate of synthesis of malic enzyme and the other enzymes of lipogenesis, while refeeding, especially with diets low in fat and high in carbohydrate, stimulates the synthesis of these enzymes (Silpananta & Goodridge, 1971). Thyroid hormone stimulates the synthesis of malic enzyme both in vivo and in cells in culture, while glucagon or cAMP decreases the rate of enzyme synthesis (Goodridge & Adelman, 1976; Li et al., 1975).

Cytoplasmic malic enzyme has been mapped to the Mod-1 locus of chromosome 9 of the mouse (Henderson, 1966). The enzymatic activity catalyzed by malic enzyme and the subcellular distribution and physiological regulation of that activity are virtually identical in mammals and birds, despite the fact that the ancestors of these animal classes diverged over 200 000 000 years ago (Dickerson, 1971). The conservation of these functional and regulatory properties through eons of evolution indicates that malic enzyme has an important function(s) in homeothermic vertebrates. Surprisingly, however, mice homozygous for a mutant allele of cytoplasmic malic enzyme, Mod-1^n, are viable, despite the complete lack of cytoplasmic malic enzyme activity in their liver and kidney (Johnson et al., 1981).

The study of Mod-1^n mutant mice was undertaken to determine the mechanism by which this mutation produced a "null" phenotype and to determine the functional consequences of the lack of cytoplasmic malic enzyme activity. In addition, we planned to use RNA from Mod-1^n mice to help select malic enzyme specific cDNA clones from a cDNA library. The last objective was based on the hypothesis that the mRNA population of Mod-1^n mice would lack malic enzyme specific sequences but would be otherwise identical with that of wild-type mice. It could thus be used as an absolute "minus" in a "plus/minus" selection. We were surprised to find that although Mod-1^n mice neither accumulate nor synthesize hepatic malic enzyme, livers from Mod-1^n mice do contain malic enzyme specific mRNAs. These mRNAs are 700–800 nucleotides longer than those detected in RNA from wild-type mice.

Experimental Procedures

Animal Care. Wild-type mice were obtained from Jackson Laboratory or raised in the Animal Resource Center of Case Western Reserve University. Breeding pairs of Mod-1^n mice were obtained from Dr. Susan E. Lewis, Research Triangle Institute, Research Triangle Park, NC, and were raised in our Animal Resource Center. Mice were fed standard chow diet, high carbohydrate/low fat diet, or high carbohydrate/low fat diet supplemented with 1% desiccated thyroid powder (Nutritional Biochemicals, Cleveland, OH), as indicated. In some experiments, mice were fed Kraft natural brick cheese (composition by weight: fat, 28%; protein, 28%; carbohydrate, 0%; water, 44%).

Purification of Malic Enzyme and Preparation of Antiserum. Male Sprague-Dawley rats were fed for 7 days with the high carbohydrate/low fat diet supplemented with desiccated thyroid powder. After decapitation, their livers were removed, forced through a stainless steel mesh into 3 volumes of 0.25

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M sucrose, 1 mM dithiothreitol, and 10 mM Tris, pH 7.5, and homogenized in a Dounce tissue grinder (Kontes Glass Co.). A postmitochondrial extract was prepared from this homogenate by centrifugation at 12000*g* for 20 min. The extract was adjusted to pH 5.2 with 1 M acetic acid and the precipitate removed by centrifugation. The supernatant solution was adjusted to 60% of saturation with solid ammonium sulfate and the resulting precipitate removed by centrifugation. Malic enzyme was precipitated from the supernatant solution by adding ammonium sulfate to 75% of saturation. The precipitate was dissolved in 30 mM Tris-HCl, pH 7.5, and dialyzed against the same buffer to remove ammonium sulfate. The dialysate was then applied to a column of DEAE-Sephacel (Pharmacia) and the column washed extensively with 30 mM Tris-HCl, pH 7.5. Malic enzyme was eluted from the column with 30 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.5. Fractions containing malic enzyme activity were applied directly to a column of 2′,5′-ADP–Sepharose (Pharmacia). After the column was washed extensively with 50 mM Tris-HCl, malic enzyme was eluted with 0.5 mM NADP and 50 mM Tris-HCl, pH 7.5. Purified malic enzyme was homogeneous as judged by electrophoresis through SDS[1]–polyacrylamide slab gels (Laemmli, 1970).

Partially purified mouse malic enzyme was prepared essentially as described above, except that the pH 5.2 supernatant solution was applied directly to a 2′,5′-ADP–Sepharose column. Fractions containing enzyme activity were pooled and frozen.

Antiserum to rat malic enzyme was raised in a female goat by subcutaneous injection of 5 mg of purified protein in Freund's complete adjuvant, followed by booster injections of 2, 2, and 1 mg in Freund's incomplete adjuvant. The goat was bled three times: prior to immunization (preimmune serum), after the second booster injection (anti-malic enzyme antiserum 1), and after the last booster injection (anti-malic enzyme antiserum 2). Antiserum 1 had immunologic properties different from those of the last bleeding; it was not capable of direct immunoprecipitation of rat or mouse malic enzyme but had a high titer for the indirect precipitation of malic enzyme when used in conjunction with Pansorbin (*Staphylococcus aureus* Cowan I cells, Calbiochem). Anti-malic enzyme antiserum 2, however, is capable of direct immunoprecipitation of rat and mouse malic enzyme.

Malic Enzyme Immunotitration. Increasing amounts of partially purified mouse malic enzyme (0–60 milliunits) were incubated with 2 μL of anti-malic enzyme antiserum 1 or preimmune serum, plus or minus either the equivalent of 10 milliunits of malic enzyme inactivated with *N*-ethylmaleimide or 100 μL of a 50% homogenate of Mod-1^n liver, in a total volume of 200 μL of 88 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 5 mg/mL bovine serum albumin (Pentex fraction V, fatty acid poor, Miles), pH 7.5. After 1 h at 37 °C, 10 μL of Pansorbin was added, and incubation was continued for 1 h at 0 °C. Each reaction was diluted to 1 mL with the same buffer, centrifuged to remove the Pansorbin–antigen–antibody complex, and assayed for malic enzyme activity (Wise & Ball, 1964).

In Vivo Synthesis of Malic Enzyme. Wild-type (C57BL/6J-DBA/2J hybrids) and Mod-1^n mice were fed Kraft natural brick cheese for 5 days, starved for 2 days, then injected with 15 μg of L-triiodothyronine/100 g body wt (Towle et al., 1980), and allowed continuous access to the high carbohydrate/low fat diet for 12 h. Each mouse was then injected intraperitoneally with 1 mCi of [^{3}H]leucine and killed 1 h later by decapitation. The livers were removed, homogenized in 3 volumes of 0.25 M sucrose, 10 mM Tris-HCl, and 1 mM dithiothreitol, pH 7.4, and centrifuged at 100000*g* for 1 h. The supernatant solutions were assayed for malic enzyme activity. Incorporation of [^{3}H]leucine into soluble protein was determined by precipitation with trichloroacetic acid (Goodridge & Adelman, 1976). Samples of liver extract (wild-type mice) containing 268 milliunits of malic enzyme activity were incubated with 75 μL of anti-malic enzyme antiserum 2 for 1 h at 37 °C, followed by 1 h on ice. Samples of liver extract from Mod-1^n mice, equivalent in volume to that from the wild-type mice, were adjusted to 268 milliunits of malic enzyme activity with partially purified, unlabeled malic enzyme and treated the same way as the extracts from wild-type mice. The antigen–antibody complexes were collected by centrifugation through a cushion of 0.5 M sucrose and 1% Nonidet P-40 (Shell Oil). Immunoprecipitates were washed twice, dissolved in 50 μL of a dissociation mixture containing 80 mM Tris-HCl, 4% SDS, 5% 2-mercaptoethanol, 12% sucrose, and 0.002% bromphenol blue, and heated to 100 °C for 5 min. The dissociated immunoprecipitates were subjected to electrophoresis through 10% polyacrylamide–SDS tube gels (Laemmli, 1970). After electrophoresis, the gels were fixed in 12.5% trichloroacetic acid, stained with Coomassie Blue, destained, and sliced. The slices were dissolved in 0.6 mL of 30% hydrogen peroxide/perchloric acid (2:1 v/v) and assayed for radioactivity by liquid scintillation spectrometry.

Cloning of Duck Malic Enzyme cDNA. RNA was extracted from the livers of white Peking ducks by a modification of the guanidine thiocyanate procedure (Chirgwin et al., 1979; Mori et al., 1979). A cDNA library was prepared from duck poly(A+) RNA essentially as described previously (Morris et al., 1982). A random array of colonies was prepared on nitrocellulose filters, replicated (Hanahan & Meselson, 1980), and screened with a ^{32}P-labeled, single-stranded DNA probe synthesized from a goose cDNA cloned into M13mp8 (Winberry et al., 1983). One of the eight positive colonies, pDME1, contained an insert of 2000 base pairs. The insert liberated from pDME1 by *Pst*I digestion was subcloned into the *Pst*I site of M13mp8 to generate M13DME1m, a subclone of the mRNA-sense strand.

Northern Gel Analysis of Mouse RNA. Total RNA was extracted from mouse liver by homogenization in 5 M guanidine thiocyanate, 50 mM lithium citrate, 0.1% lithium dodecyl sulfate, and 0.1 M mercaptoethanol (Derman et al., 1981) and then processed as described by Mori et al. (1979). Poly(A+) RNA was prepared by chromatography on oligo(dT)–cellulose in citrate buffer (Maniatis et al., 1982). RNA samples were treated with formaldehyde and subjected to electrophoresis in 0.9% agarose gels (Lehrach et al., 1977). The separated RNAs were transferred to nitrocellulose and baked for 2 h at 80 °C in vacuo (Thomas, 1980). Nitrocellulose filters containing RNA were prehybridized at 37 °C in 50% deionized formamide, 20 mM PIPES (pH 6.4), 0.8 M NaCl, 2 mM EDTA, 200 μg/mL denatured salmon sperm DNA, and 0.5% SDS. After 24 h, ^{32}P-cDNA synthesized from the M13DME1m template was added directly to the prehybridization buffer (Winberry et al., 1983). After 48 h of hybridization, filters were washed 3 times in 300 mL of 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, and 0.5% SDS at 37 °C, followed by three washes in 0.30 M NaCl, 0.030 M sodium citrate, and 0.5% SDS, also at 37 °C. The filters were then air-dried and subjected to autoradiography

[1] Abbreviations: SDS, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Table I: Malic Enzyme, Glucose-6-phosphate Dehydrogenase, and 6-Phosphogluconate Dehydrogenase Activities in Liver Extracts from Wild-Type or Mutant Mice[a]

enzyme	fasted		refed		refed plus thyroid	
	wild-type	mutant	wild-type	mutant	wild-type	mutant
malic enzyme	1.7 ± 0.3	<0.02	76 ± 12	<0.02	160 ± 22	<0.02
glucose-6-phosphate dehydrogenase	6.0 ± 0.6	5.5 ± 0.6	79 ± 4	91 ± 19[b]	73 ± 12	48 ± 18
6-phosphogluconate dehydrogenase	16 ± 1	13 ± 1	50 ± 3	80 ± 5[b]	57 ± 5	39 ± 10

[a] Male wild-type (C57BL/6J) or Mod-1^n mice were fed Kraft natural brick cheese for 2 days, starved 2 days, and refed with the high carbohydrate/low fat diet or high carbohydrate/low fat diet supplemented with 1% desiccated thyroid powder for 2 days. Liver extracts were prepared and enzyme activities assayed as previously described (Goodridge, 1968; Rudack et al., 1971). Protein was assayed as described by Lowry et al. (1951) using bovine serum albumin as standard. The results are expressed as nanomoles of NADPH produced per milligram of soluble protein per minute ± SE (n = 3). [b] Each of these groups had four mice.

at −70 °C with Kodak XAR-5 film and Du Pont Lightning Plus intensifying screens.

Results and Discussion

Preliminary Characterization of Mice Carrying the Mod-1^n Mutation. Two electrophoretically distinguishable products of the gene for cytoplasmic malic enzyme have been described in wild-type mouse populations (Henderson, 1966). Mice of the DBA/2J and C57BL/6J strains are homozygous for the products of the Mod-1^a and Mod-1^b genes, respectively. The Mod-1^n allele was discovered in an F_1 mouse derived from a cross between a mutagenized DBA/2J male and a normal C57BL/6J female. Malic enzyme activity in this F_1 mouse migrated as a single band and to the same position as that of the male parent. This F_1 mouse lacked not only Mod-1^b enzyme activity but also the ability of the Mod-1^b product to form mixed tetramers with the Mod-1^a product. This result suggested the presence of a null allele (Johnson et al., 1981).

Cytoplasmic malic enzyme activity was undetectable in liver extracts from Mod-1^n mice fed normal diets (Lee et al., 1980) or from those fed a high carbohydrate/low fat diet supplemented with thyroid hormone (Table I). In wild-type mice, this dietary manipulation increased malic enzyme activity by almost 100-fold. Under these dietary conditions, the mutant mice have less than 0.5% of the hepatic malic enzyme activity of wild-type mice.

The dehydrogenases of the pentose phosphate pathway are potential alternative sources of NADPH. When mice were fed the normal chow diet, the activities of both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in Mod-1^n mice were indistinguishable from those of wild-type mice (Lee et al., 1980; our unpublished results). When starved mice are refed a high carbohydrate/low fat diet, lipogenesis and utilization of NADPH are increased severalfold (Allman & Gibson, 1965). The activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were stimulated in both wild-type and mutant mice (Table I). Even under these conditions, there was no difference in glucose-6-phosphate dehydrogenase activity between the wild-type and mutant mice. The activity of 6-phosphogluconate dehydrogenase, however, was 60% ($p < 0.05$) higher in liver extracts from the mutant. The further addition of thyroid powder to the high carbohydrate/low fat diet had no effect on the pentose phosphate pathway enzyme activities in the wild-type mice and abolished the compensation of 6-phosphogluconate dehydrogenase activity observed in the mutant. The changes in dehydrogenase enzyme activities caused by diet and thyroid hormone in these experiments are similar to those previously reported for the rat (Miksicek & Towle, 1982; Tepperman & Tepperman, 1964). Our results suggest that Mod-1^n mice have all of the components required for the induction of these "lipogenic" enzymes, which is caused by refeeding high carbohydrate diets. Furthermore, one of the pentose phosphate dehydrogenases may increase in activity in the Mod-1^n mouse, compensating for the lack of cytoplasmic malic enzyme activity. Such a result implies that the rate of utilization of NADPH may regulate the activity of 6-phosphogluconate dehydrogenase.

Immunologically Cross-Reactive Malic Enzyme Is Undetectable in Liver from Mod-1^n Mice. When kidney or liver extracts made from Mod-1^n mice fed normal chow diets were subjected to Ouchterlony double-diffusion analysis, no malic enzyme protein was detected (Lee et al., 1980). The level of Mod-1^n gene product might have been too low to be detected by the relatively insensitive Ouchterlony procedure, or alternatively, the product might bind to the antibody but not be precipitated by it. We therefore measured the level of cross-reactive material in extracts prepared from mice fed the high carbohydrate/low fat diet supplemented with thyroid powder and, in addition, used a more sensitive immunochemical assay. In this assay, a limiting amount of the nonprecipitating antiserum 1 was added to increasing amounts of partially purified, mouse malic enzyme. The resulting antigen–antibody complexes were precipitated with Pansorbin. The residual malic enzyme activity in the supernatant solution was then assayed. Addition of an enzymatically inactive competitor for the antibody should compete with active enzyme for the limiting number of antibody molecules. This should decrease the amount of active enzyme that is precipitated and result in more enzyme activity remaining in the supernatant solution. This assay is only suitable for the detection of immunologically cross-reactive material that has an undetectable enzyme activity. To test the assay, wild-type malic enzyme was inactivated with *N*-ethylmaleimide. Enzyme inactivated in this manner should retain most of its immunoreactivity. Addition of 10 milliunits of *N*-ethylmaleimide-inactivated malic enzyme to the reaction caused a 6- or 7-milliunit shift in the titration curve (Figure 1). The inactivation process may have caused a small change in immunoreactivity of the enzyme. Enzyme inactivated by limited digestion with trypsin gave the same result (data not shown). However, the addition of 0.1 mL of a 50% liver homogenate prepared from Mod-1^n mice fed the high carbohydrate/low fat diet supplemented with thyroid hormone caused no shift in the curve. Therefore, 0.1 mL of this extract contained less cross-reactive material than 10 milliunits of malic enzyme. An equivalent amount of liver homogenate prepared from wild-type mice would contain about 2000 milliunits of malic enzyme. Thus, liver extract prepared from Mod-1^n mice contained less than 0.5% of the amount of antigenically reactive malic enzyme found in wild-type mice.

Synthesis of Malic Enzyme Is Undetectable in Liver of Mod-1^n Mice. The lack of antigenically detectable malic enzyme protein in Mod-1^n mouse liver could be due either to

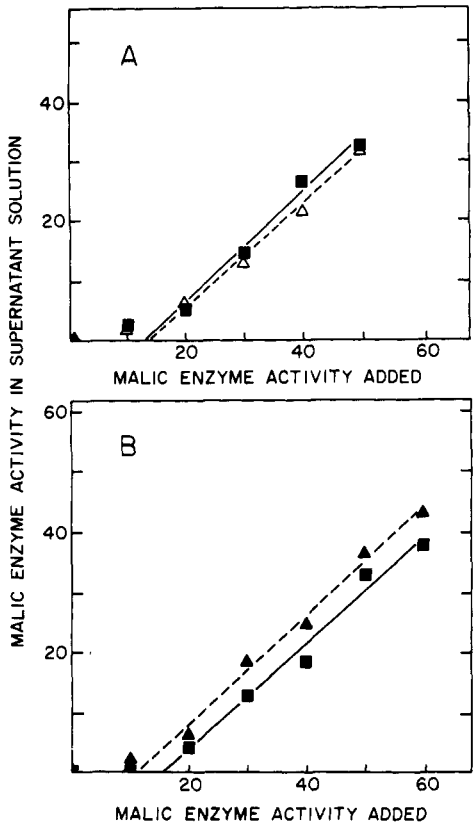

FIGURE 1: Immunoreactive malic enzyme is undetectable in liver extracts from Mod-1^n mice. Panel A: Anti-malic enzyme antiserum (2 μL) was incubated with the indicated quantities (in milliunits) of malic enzyme for 1 h at 37 °C, in the presence (△) or absence (■) of 100 μL of liver extract from a null mouse in a total volume of 200 μL. Pansorbin was then added to each reaction. After an additional 1 h of incubation at 4 °C, antigen–antibody complexes bound to Pansorbin were removed by centrifugation. Malic enzyme activity was assayed in the supernatant solution. Identical sets of tubes were assayed with preimmune goat serum instead of the antiserum. In these tubes, malic enzyme activity in the supernatant solution was equivalent to the amount added. The results are expressed as milliunits of enzyme activity added or recovered. Panel B: Identical with the experiment in panel A except that 10 milliunits of *N*-ethylmaleimide-inactivated malic enzyme (▲) was added instead of liver extract from a null mouse.

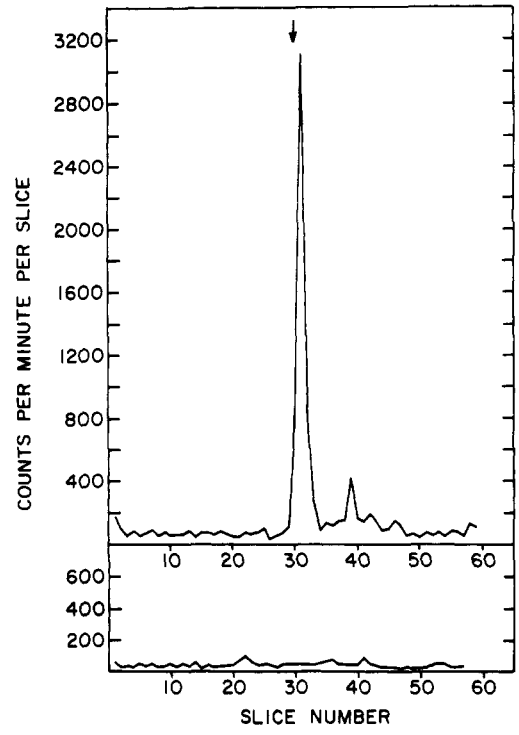

FIGURE 2: SDS–polyacrylamide gel electrophoresis of malic enzyme immunoprecipitates from wild-type (upper tracing) and Mod-1^n (lower tracing) mice. Wild-type (F_1 hybrids, C57BL/6J × DBA/2J) or Mod-1^n mice were fed Kraft natural brick cheese for 5 days, starved for 2 days, and refed with the high carbohydrate/low fat diet for 12 h. They were injected intraperitoneally with L-triiodothyronine (15 μg/100 g body wt) at the same time that refeeding was started. The treated mice were injected intraperitoneally with 1 mCi of [^{3}H]leucine and killed 1 h later. Malic enzyme was immunoprecipitated from an amount of liver extract containing 268 milliunits of enzyme activity or an equal amount of extract from Mod-1^n mice. Direct immunoprecipitation with antiserum 2 and electrophoretic analysis of the immunoprecipitate were carried out as described under Experimental Procedures. The position of authentic mouse malic enzyme is indicated by the arrow.

a lack of synthesis or to very rapid degradation of the Mod-1^n protein product. Relative synthesis of malic enzyme, therefore, was measured in the livers of wild-type and Mod-1^n mice. The mass of malic enzyme in the liver was reduced by feeding Kraft natural brick cheese, a food high in fat and protein but low in carbohydrate. After 1 week on the cheese diet, malic enzyme activity was reduced further by starvation for an additional 2 days. The mice were then injected intraperitoneally with thyroid hormone and refed with the high carbohydrate/low fat diet for 12 h. This regimen was designed to maximally stimulate synthesis of malic enzyme while keeping the mass of malic enzyme low, thus allowing immunoprecipitation of enzyme from a large quantity of liver tissue. Enzyme precipitated from extracts of liver from wild-type mice contained about 5000 cpm, from which we calculated a relative rate of synthesis of about 0.4% of that for total soluble protein (Figure 2). Radioactive malic enzyme was undetectable in the liver extracts from Mod-1^n mice.

Mod-1^n Mice Express Malic Enzyme mRNAs That Are Longer Than Those of Wild-Type Mice. Our first cloned malic enzyme cDNA was from domestic geese. It represents about half the total length of mature mRNA for malic enzyme and includes some of the coding sequence. It would not hybridize to mouse RNA, even under conditions of relatively low stringency (data not shown). The cloned goose cDNA was used to isolate a nearly full-length malic enzyme cDNA clone from a duck liver library. Surprisingly, the duck malic enzyme cDNA hybridized to RNA species of 2250 and 2950 bases in a Northern analysis of poly(A+) RNA from fed, thyroid hormone treated, wild-type mice (Figure 3). The same two RNAs were detected in RNA from starved, wild-type mice but at much lower concentrations. RNA from the fed, thyroid hormone treated, mutant mice also gave two strong hybridization signals, whereas that from the starved, mutant mice gave weak but discrete signals. (In Figure 3, hybridization signals are not visible for the RNA samples from starved mice. They were visible on the original film.) However, the malic enzyme mRNAs from Mod-1^n mice were 700–800 nucleotides larger than the corresponding mRNAs from wild-type mice. After this work was completed, we learned that another laboratory also had observed two abnormally large mRNAs for malic enzyme in the liver of Mod-1^n mice (Suk et al., 1984).

Malic enzyme mRNAs of two different lengths also have been found in the Sprague-Dawley rat (Magnuson & Nikodem, 1983) although both were significantly larger than those of the wild-type mouse. Thyroid hormone also stimulated accumulation of both malic enzyme mRNAs in the rat (Magnuson & Nikodem, 1983). In the Mod-1^n mouse, both pentose phosphate pathway dehydrogenases and both malic enzyme mRNAs show wild-type patterns of regulation by the combination of low fat/high carbohydrate diet and thyroid hormone. Thus, the Mod-1^n mutation is not in a gene for a

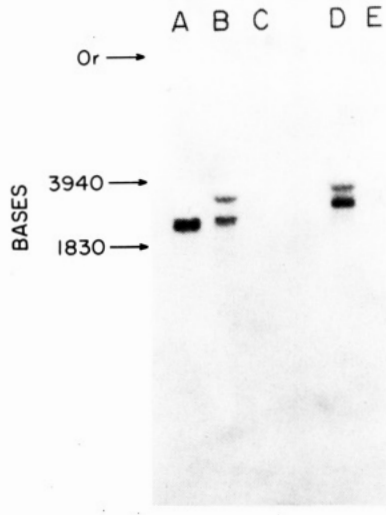

FIGURE 3: Determination of size and abundance of malic enzyme mRNA by Northern analysis. Total (duck, 0.3 μg per lane) or poly(A+) (mouse, 7 μg per lane) RNA samples were subjected to electrophoresis under denaturing conditions as described under Experimental Procedures. The RNA was transferred to nitrocellulose and hybridized with ^{32}P-cDNA synthesized from M13DME1m. The markers were duck 18S and 27S ribosomal RNA (Spohr et al., 1976) and were located by staining the gel with ethidium bromide before transfer. Or refers to the origin. The RNAs were prepared as described under Experimental Procedures. Sources of RNAs were as follows: total RNA from the liver of a fed duckling (lane A); poly(A+) RNA from livers of wild-type mice fed high carbohydrate/low fat diet supplemented with thyroid hormone (lane B) or starved (lane C); poly(A+) RNA from the liver of Mod-1^n mice fed high carbohydrate/low fat diet supplemented with thyroid hormone (lane D) or starved (lane E).

component of the pathway by which the level of malic enzyme is regulated; it must be in the structural gene for cytoplasmic malic enzyme. In addition, since both malic enzyme mRNAs were longer in the Mod-1^n mutant, both must have been transcribed from the same gene.

Since in vivo synthesis of malic enzyme was undetectable in Mod-1^n mice, it was surprising to find malic enzyme RNAs in their livers. There are three possible explanations for these results. The first possibility is that the protein product of the Mod-1^n gene may be so aberrant that it is not recognized by antisera against malic enzyme. This possibility seems unlikely because another laboratory has found that RNA extracted from the livers of mutant mice directed the synthesis, in vitro, of an abnormally large peptide that was precipitated by anti-malic enzyme antiserum (Suk et al., 1984). This raises the trivial possibility that our failure to detect synthesis of malic enzyme was due to the inability of our antisera to recognize a slightly altered malic enzyme molecule but minimizes the probability that a grossly aberrant protein is expressed. It also seems unlikely that our antisera are significantly different from those raised by other laboratories. Our antisera are specific for mouse malic enzyme, quantitatively precipitating only malic enzyme from extracts of liver or 3T3-L1 cells (Figures 1 and 2 and Goodridge et al., 1984). Furthermore, although the sensitivity of their assays was not as great as ours, antisera raised against mouse malic enzyme by other laboratories also has been unable to detect malic enzyme protein in Mod-1^n mice (Lee et al., 1980). The second possibility is that the mutant may produce an enzyme which is sufficiently like the normal enzyme to be immunologically reactive but which is degraded too quickly to be detected by measurement of synthesis in vivo. The third possibility is that protein product was not made in vivo, due to a mutation that rendered the mRNA nontranslatable in liver despite its translatability in a cell-free system derived from rabbit reticulocyte lysates. For example, the mutant mRNA may have missing or altered recognition sites for translational initiation factors that are not required when translating RNA in reticulocyte lysates. At present, our results cannot discriminate between rapid degradation and lack of synthesis.

A mutant RNA longer than wild type could be due to initiation of transcription at an incorrect site, incomplete processing of the primary transcript, insertion in the correct reading frame, or termination of transcription at an incorrect site. The molecular nature of the Mod-1^n mutation is the subject of continuing investigation.

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Monoclonal Antibodies against Distinct Determinants of Histone H5 Bind to Chromatin†

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ABSTRACT: A series of monoclonal antibodies specific for distinguishable epitopes in chromosomal protein histone H5 were obtained from mice immunized with either free H5 or H5·RNA complexes. The antibodies elicited by H5 could be distinguished from those elicited by H5·RNA by their binding to native or acid-denatured H5, by their interaction with the globular region of H5, and by their cross-reactivity with H1°. The specificity of the antibodies was assessed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting experiments. The antibodies could distinguish between H5 and the closely related histones H1 and H1°. The binding of some of the antibodies to the antigens was dependent on the type of assay used, suggesting nonrandom binding of the antigen to the solid supports used in ELISA and immunoblotting. Competitive ELISA experiments indicate that 8 of the 11 antibodies characterized bind to distinct epitopes. Three monoclonal antibodies bind to epitopes which are in close spatial proximity, causing mutual steric hindrance. The monoclonal antibodies bind to nuclei of fixed cells and to isolated chromatin, indicating that the epitopes are present both in the purified protein and in chromatin-complexed H5. These monoclonal antibodies can be used to study the organization of distinct regions of histones H5 and H1° in chromatin and chromosomes.

The lysine-rich histone group differs from the core histones in its tissue and species specificity and in the manner in which it is organized in chromatin. While the core histones are highly conserved during evolution (Elgin & Weintraub, 1975), the lysine-rich histones display a remarkable species and tissue specificity (Bustin & Cole, 1968). The core histones are organized into an octamer around which the nucleosomal DNA is wound, while the lysine-rich histones are not an integral part of the core particle (Kornberg, 1977; McGhee & Felsenfeld, 1980). Several types of experiments suggest that the lysine-rich histones stabilize the structure of the nucleosomes and are involved in the maintenance and control of higher order chromatin structure (Simpson, 1978; Finch & Klug, 1976; Bradburg et al., 1973; Renz et al., 1977). The various functions of the very lysine-rich histones seem to be dependent on specific structural features of the molecule. It has been shown that these histone molecules are composed of three main structural domains (Bustin & Cole, 1970; Chapman et al., 1977) which exert specific effects at various levels of chromatin organization (Allan et al., 1980; Thoma et al., 1983). In most cells, histone H1 is the major very lysine-rich histone species (Elgin & Weintraub, 1975). However, in the nucleated erythrocytes of several species, the H1 molecules are largely replaced by the H5 lysine-rich histone variant (Neelin et al., 1964). Recent studies indicated that most eukaryotic cells have a minor very lysine-rich histone variant, histone H1°, which is closely related to histone H5 (Panyim & Chalkley, 1969; Pehrson & Cole, 1981; Smith et al., 1980). The H1° molecule seems to accumulate in cells which either are terminally differentiated or have a lower rate of cell division (Pehrson & Cole, 1980; Keppel et al., 1977; Chabanas, 1983). The molecular basis of this observation is not understood; however, it is known that H5 imparts greater stability to higher order chromatin structure than does H1 (Thomas & Rees, 1983; Kumar & Walker, 1980). The H1/H5 histones bind to chromatin through a globular domain which seems to locate the molecule at the exit and entrance points of the 165 base pair DNA in the chromatosome (Simpson, 1978). Sequence analysis (Pehrson & Cole, 1981; Smith et al., 1980) and immunological studies have shown that the central globular region of H1 differs from that of H5 and H1° (Mura & Stollar, 1981; Allan et al., 1982). Thus, it is possible that the functional differences between H5/H1° and H1 are related to structural features in the globular region of these proteins.

Immunological approaches have been used for various studies on the specificity of histones and on their role in maintaining the structure and regulating the function of chromatin (Bustin, 1979). So far, most of the serological studies on chromatin were done with polyclonal sera against specific proteins or histone peptide (Bustin, 1979; Absalom & Regenmortel, 1978; Muller et al., 1983; Mathiu et al., 1981). Defined monoclonal antibodies may be useful in studying the organization of specific histone regions in chromatin. Such studies may be particularly applicable to the lysine-rich group since we have shown that in chromatin the antigenic determinants in this histone are relatively exposed (Goldblatt & Bustin, 1975). Defined monoclonal antibodies may provide further insight into the organization of particular regions of the molecules in chromatin and chromosomes.

In the present paper, we describe the preparation and characterization of several monoclonal antibodies against histone H5. The antibodies can be used to distinguish between

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