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## Nutritional and hormonal regulation of genes for lipogenic enzymes

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The *de novo* synthesis of long-chain fatty acids, a process that occurs primarily in liver in many species, is high in animals fed on diets with a high content of carbohydrate, and low in starved animals. The activities of the 'lipogenic' set of hepatic enzymes follow the same pattern. Two members of this lipogenic set are cytosolic malic enzyme (*EC* 1.1.1.40), which furnishes most of the NADPH for fatty acid synthesis in avian species, and fatty acid synthase (*EC* 2.3.1.85), a multifunctional polypeptide that uses seven molecules malonyl-CoA, one molecule acetyl-CoA and fourteen molecules NADPH, to synthesize one molecule saturated $C_{16}$ fatty acid. Malic enzyme and fatty acid synthase are expressed at high levels in chicks and ducklings fed on a normal mash diet and at low levels in starved birds (Goodridge, 1968, 1973; Goodridge *et al.* 1984). When we first began these studies many years ago, our long-term objectives were (1) to identify humoral factors responsible for communicating the state of alimentation of a whole animal to its liver and (2) to define at the molecular level each step in the intracellular signalling pathway between binding of a humoral factor to its specific hepatic receptor and altered activity of these enzymes. We undertook a series of parallel investigations on malic enzyme and fatty acid synthase. Here mainly our work with malic enzyme will be discussed.

### IDENTIFICATION OF HUMORAL FACTORS

Insulin and glucagon play important roles in the metabolic transition from the fed to the starved state. Insulin levels are high in animals fed on high-carbohydrate diets and low in starved animals. Glucagon levels are the opposite, low in fed animals and high in starved ones. The levels of expression of the lipogenic enzymes in diabetic animals resemble those in the starved state; diabetic animals have low levels of insulin and high levels of glucagon. In isolated liver tissue, insulin stimulates and glucagon inhibits incorporation

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of precursors into long-chain fatty acids. Finally, the increases in lipogenic enzyme activities that accompany the transition from the starved to the fed state are blocked by the simultaneous administration of glucagon. Most of these points were well established by other groups before or as we began these studies (Volpe & Vagelos, 1976; Wakil *et al.* 1983; Goodridge, 1985).

The responsiveness of malic enzyme to thyroid state in rats had been previously established (Tepperman & Tepperman, 1964). Measurements of triiodothyronine in fed and starved animals indicated that triiodothyronine also might play a role in mediating the responses of the lipogenic enzymes to diet. Triiodothyronine levels are decreased with starvation and increased when starved animals are fed, with kinetics consistent with a role in regulation of enzyme levels (for review, see Goodridge *et al.* 1986).

Our major contribution to the identification of humoral agents was the development of a cell culture system for hepatocytes in which hormones stimulated or inhibited the activities of the lipogenic enzymes to extents comparable to the effects of starvation or refeeding (Goodridge *et al.* 1974; Goodridge & Adelman, 1976). Isolated hepatocytes were prepared from the livers of chick embryos incubated for 17–19 d and incubated in a chemically-defined medium for up to 1 week. In the absence of added hormones, malic enzyme activity remained about the same as that in freshly-prepared hepatocytes. Triiodothyronine alone caused about a twenty-fivefold increase in activity. Insulin alone had only a small effect on activity but amplified the effect of triiodothyronine by another threefold. Glucagon virtually completely blocked the effects of triiodothyronine plus insulin (Goodridge & Adelman, 1976). Thus, the starved and fed states could be mimicked in culture with appropriate hormone additions. These experiments provided the first evidence that thyroid hormone, insulin and glucagon played direct roles in the long-term regulation of the activities of the lipogenic enzymes and supported the hypothesis that they were important in mediating nutritional regulation of these enzymes in vivo.

Recent work from our laboratory suggests that insulin-like growth factor 1 (IGF-1) may be added to this list. Like insulin, IGF-1 has little effect by itself but markedly amplifies the response of malic enzyme to triiodothyronine (Goodridge *et al.* 1989*a*). IGF-1 levels in the blood are elevated in the fed state and decreased in the starved state. In intact animals, it is probable that insulin, IGF-1, triiodothyronine and glucagon are involved in communicating the state of alimentation to the liver. Nevertheless, definitive evidence that these hormones and growth factor are the only or even primary humoral agents that mediate nutritional effects is lacking; other agents, as yet unidentified, also may play important roles.

## INTRACELLULAR SIGNALLING PATHWAYS THAT REGULATE MALIC ENZYME ACTIVITY

Intracellular signalling pathways are often branching; binding of a hormone can lead to several different end-effects. Our strategy was to trace each signalling pathway backwards from its distal end, altered enzyme activity, to its proximal end, binding of hormone to its appropriate cellular receptor, thus assuring ourselves that the signalling pathways we were analysing were those involved in regulation of malic enzyme. Nutritional studies in vivo were executed in parallel with experiments in hepatocytes in culture, providing a test of the relevance of mechanisms observed in culture.

Changes in the activity of an enzyme can be due to regulation of the number of enzyme molecules per cell or to regulation of the catalytic efficiency of a constant number of enzyme molecules per cell. We used a specific polyclonal antibody raised against purified chicken malic enzyme to show that both nutritional and hormonal regulation of malic enzyme were due to control of enzyme concentration (Silpananta & Goodridge, 1971; Goodridge & Adelman, 1976). The concentration of an enzyme can be controlled by regulating the rates of either its synthesis or its degradation. The antibody was used as an affinity reagent to purify malic enzyme from crude extracts in a specific, rapid, and quantitative manner. In pulse-labelling experiments, nutritional manipulation in vivo or hormonal treatment in culture had equivalent effects on the concentration and relative rate of synthesis of hepatic malic enzyme. In pulse-chase experiments, we were unable to detect a selective effect of nutritional state or hormones on the degradation rate constant for malic enzyme (Silpananta & Goodridge, 1971; Goodridge & Adelman, 1976). We concluded that the activity of malic enzyme was regulated by controlling its rate of synthesis.

Regulation of the rate of synthesis of an enzyme can be achieved by controlling the number of specific mRNA molecules per cell or by controlling the efficiency with which a constant number of molecules of mRNA are translated into protein. Discriminating between these two alternatives is analogous to determining whether enzyme activity is regulated by controlling concentration or catalytic efficiency. In this case, the reagent analogous to a specific antibody is a complementary DNA (cDNA). We isolated a cloned malic enzyme cDNA using recombinant DNA techniques (Winberry *et al.* 1983). The amount of malic enzyme mRNA in different populations of RNA was determined by 'Northern' analysis. Total RNA was extracted from liver or cells in culture under conditions that preserve the integrity of all mRNA. The RNAs were separated by size on denaturing agarose gels and blot-transferred to a solid matrix, usually nitrocellulose or a nylon-based membrane. The transfer process produces on the membrane, a mirror image of RNAs on the gel. Under appropriate conditions, the transfer process is essentially quantitative. After fixing the RNA to the membranes, it was hybridized to $^{32}$P-labelled malic enzyme cDNA. After hybridization, the membrane was washed to remove non-specifically bound label and then exposed to X-ray film.

Within the limits of resolution of agarose gels, a single malic enzyme mRNA species of about 2·1 kilobases (kb) is expressed in hepatocytes in vivo and in culture irrespective of nutritional or hormonal state. The amount of the mRNA, however, was quite different under those different conditions. Liver RNA from starved chicks had very little malic enzyme mRNA; its abundance was increased thirty-five- to one hundredfold by feeding (Winberry *et al.* 1983). In culture, very little malic enzyme mRNA was detectable in hepatocytes incubated without hormones or with insulin alone. Triiodothyronine alone caused a large increase in this mRNA and that increase was further amplified by addition of insulin. The combination of insulin and triiodothyronine resulted in a fifty- to 150-fold increase in the abundance of malic enzyme mRNA relative to hepatocytes without hormone or with insulin alone (Winberry *et al.* 1983). Addition of glucagon almost completely inhibited the stimulation caused by insulin plus triiodothyronine (Winberry *et al.* 1983; Back *et al.* 1986). From these experiments, we conclude that diet and hormones regulate the synthesis of malic enzyme by controlling the abundance of malic enzyme mRNA, i.e., at a pretranslational step.

The second messenger for most of the hepatic actions of glucagon is cyclic AMP. Thus,

it is probable that cyclic AMP also mediates the action of glucagon on expression of malic enzyme. Forskolin, a drug that raises the intracellular concentration of cyclic AMP, and dibutyryl cyclic AMP, an analogue of cyclic AMP that enters cells more readily than the natural compound, also inhibited the triiodothyronine-induced accumulation of malic enzyme mRNA, providing additional evidence that cyclic AMP is the intracellular second messenger for glucagon in this system (Goodridge *et al.* 1989*b*).

Regulation of the abundance of an mRNA is more complex than regulation of the concentration of an enzyme because the process analogous to enzyme synthesis, rate of production of the mature cytoplasmic mRNA, can be controlled at several different steps. Thus, initiation, elongation and termination of transcription, processing of the primary transcript, stability of nuclear precursor transcripts and transport of mRNA from nucleus to cytoplasm are all potential regulatory points in the production of mature cytoplasmic mRNA. In addition, the level of cytoplasmic mRNA will be influenced by the rate of its degradation. The rates of several of these processes have been estimated for malic enzyme mRNA under different nutritional and hormonal conditions.

We used two methods to estimate rate-constants for degradation of malic enzyme mRNA. First, we calculated the degradation rate-constant from the kinetics with which the abundance of malic enzyme mRNA approached steady-state in the livers of ducklings that were starved or ducklings that were refed after a period of starvation (Berlin & Schimke, 1965). The half-life of malic enzyme mRNA was 3 to 5 h in refed birds and about 1 h in starved animals (Goldman *et al.* 1985; half-life equals the natural logarithm of 2 divided by the degradation rate-constant). The three- to fivefold change in half-life is not sufficient to account for the about one hundredfold change in level of malic enzyme mRNA. In hepatocytes in culture, we measured the rate of loss of malic enzyme mRNA after inhibiting total transcription with actinomycin D or α-amanitin. The half-life of malic enzyme mRNA was about 1·5 h in hepatocytes treated with insulin, triiodothyronine and glucagon and 8–11 h in those treated with insulin and triiodothyronine (Back *et al.* 1986). This six- to eightfold increase in degradation rate is not sufficient to account for the 94–98% inhibition of abundance of malic enzyme mRNA caused by glucagon.

Two other techniques have been employed to estimate the rates of nuclear processes. In the transcription run-on assay, nuclei are isolated from organs or cells under conditions that 'freeze' the transcription complex on the DNA with an active RNA polymerase II (*EC* 2.7.7.6) and nascent RNA still attached. These complexes are elongated in vitro in the presence of $^{32}$P[UTP] and other components required for transcription elongation. In this in vitro system, there is no re-initiation of transcription, and the engaged RNA polymerases extend existing nascent chains by essentially the same amounts regardless of the gene or the position on the gene (McKnight & Palmiter, 1979). The newly-synthesized RNAs are isolated and hybridized to specific DNA fixed to hybridization membranes. The rate of incorporation of $^{32}$P[UTP] into RNA specifically-bound to DNA represents average polymerase density over the part of the gene represented by the particular cDNA or genomic DNA probe used. If, as is generally assumed for eukaryotic genes, initiation is the limiting step in transcription, then polymerase density will be directly proportional to the rate of transcription initiation.

In nuclei from the livers of starved chicks, transcription of the malic enzyme gene is barely detectable. Refeeding causes a thirtyfold or greater increase in transcription rate

with the maximum rate of transcription being achieved about 3 h after refeeding is started. Starvation of fed animals causes an equally rapid decrease in the transcription of the malic enzyme gene (Ma *et al.* 1990). In nuclei isolated from hepatocytes incubated in culture with insulin alone, transcription of the malic enzyme gene was similar to that in liver nuclei from starved birds, barely detectable. Triiodothyronine caused about a fortyfold or greater increase in transcription and glucagon almost completely blocked that increase (Salati *et al.* 1991). The triiodothyronine-induced increase in transcription of the malic enzyme gene was evident as soon as 1 h after adding the hormone. Inhibition by cyclic AMP was detected within 30 min after adding the drug.

Earlier experiments implicated post-transcriptional mechanisms in the regulation of malic enzyme mRNA by diet, triiodothyronine and glucagon (cyclic AMP) (Goldman *et al.* 1985; Back *et al.* 1986). The experiments just described indicate that primary regulation of the abundance of malic enzyme mRNA is via the rate of transcription, both in vivo by nutritional state and in hepatocytes in culture by triiodothyronine and glucagon. What is the basis of this discrepancy? The present results were obtained with genomic DNA probes. In the earlier experiments, an artifact caused by sequences added to cDNA during the cloning steps greatly inflated the rate of transcription of the malic enzyme gene in nuclei from starved liver or hepatocytes incubated with insulin alone or with insulin, triiodothyronine and glucagon. These and other confounding problems with the transcription assay make it useful to obtain confirming evidence using a different method. If transcription is the regulated step, the concentrations of nuclear intermediates in the synthesis and processing of a mRNA should be low in the non-induced state and high in the induced state. If processing, transport to the cytoplasm, or stability of the cytoplasmic mRNA are regulated, then the concentrations of some or all of these intermediates should be similar in the induced and non-induced states. We measured the abundance of nuclear precursors for malic enzyme mRNA by extracting RNA from isolated nuclei and then using labelled intronic DNA as hybridization probes in 'Northern' analyses. Nuclear precursors for malic enzyme mRNA were virtually undetectable in the RNA from livers of starved chicks and abundant in RNA from the livers of fed birds (Ma *et al.* 1990), adding additional confidence to our conclusion that nutritional regulation of the expression of malic enzyme is primarily transcriptional.

## IDENTIFICATION OF *CIS*-ACTING SEQUENCE ELEMENTS

Malic enzyme is one of a small set of genes, the transcription of which is stimulated by feeding and inhibited by starvation. Similarly, it is one of small but different sets of genes stimulated by triiodothyronine or inhibited by glucagon. Each gene regulated by a specific physiological event must be marked in some way to identify it as a specific target. Primary structure differentiates one gene from another. Thus, sequence elements must identify a gene for regulation by a particular hormone or agent. In eukaryotes, short *cis*-acting (in the same molecule as the regulated gene) DNA sequence elements are usually found in the 5′-flanking region of the regulated gene. For a particular agent or hormone, each gene in the set of regulated genes contains *cis*-acting regulatory elements that are similar in sequence. A common method for identifying *cis*-acting elements is a functional assay in which putative regulatory DNA elements are attached to a reporter gene. Expression of the chimeric gene is tested after transfection of the DNA into hormone-sensitive cells (Wynshaw-Boris *et al.* 1986). Fragments of 5′-flanking sequence

that confer hormone sensitivity on expression of the reporter gene contain the relevant regulatory sequence. Deletions, insertions and point mutations are then used to determine the specific nucleotide sequence required for the response.

We prepared a chimeric gene containing 5 kb 5′-flanking DNA of the malic enzyme gene ligated to the bacterial gene, chloramphenicol acetyltransferase (*EC* 2.3.1.28; CAT). After transient transfection into chick-embryo hepatocytes in culture, CAT expression was stimulated about fifteenfold by triiodothyronine and that rate was inhibited about 65% by cyclic AMP (D. A. Fantozzi, S. A. Klautky and A. G. Goodridge, unpublished results). Thus, 5′-flanking DNA of the malic enzyme gene contains triiodothyronine- and cyclic AMP-responsive elements. The nucleotide sequence of the first 400 bp upstream from the start site reveals several half-sites for putative triiodothyronine-response elements but no sites for cyclic AMP-response elements. Additional work is under way to identify more precisely the involved DNA sequences.

To continue our 'reverse' progress along the signalling pathway from altered enzyme activity to hormone receptor, we must next identify the elements of that signalling pathway that interact with the specific *cis*-acting sequence elements on the malic enzyme gene. These elements are *trans*-acting (i.e., diffusible) factors that bind to *cis*-acting-hormone-response elements and regulate transcription. DNase (*EC* 3.1.21.1) footprint analysis, in vitro transcription and DNA–protein-binding assays are being used in this process. Published literature for other genes (Evans, 1988) and our preliminary findings suggest that the primary *trans*-acting factor in the response of the malic enzyme gene to triiodothyronine will be the triiodothyronine receptor, a known DNA-binding protein. If so, the signalling pathway by which triiodothyronine regulates malic enzyme activity will be complete. Cyclic AMP probably regulates transcription of the malic enzyme gene by activating the catalytic subunit of the cyclic AMP-dependent protein kinase (*EC* 2.7.1.37). How the kinase regulates transcription is unknown but probably involves phosphorylation of *trans*-acting factors that bind to the 5′-flanking region of the malic enzyme gene. Understanding how cyclic AMP inhibits transcription and how the two hormone-signalling pathways interact to regulate transcription of the malic enzyme gene are our near-term objectives.

The actions of triiodothyronine and cyclic AMP probably will not provide a complete explanation of how changes in the state of alimentation regulate transcription of the malic enzyme gene. Other agents also regulate transcription of this gene, and interactions of triiodothyronine, cyclic AMP and these 'other' agents must bring about the integrated transcriptional response to changes in nutritional state. As discussed earlier, insulin and IGF-1 amplify the stimulation by triiodothyronine. In addition, corticosterone is without effect by itself or with triiodothyronine if enzyme activity is measured within the first 3 d that chick-embryo hepatocytes are in culture. After 3 d in culture, the ability of malic enzyme to respond to triiodothyronine decreases rapidly and by about 1 week, there is essentially no response. If corticosterone is included in the culture medium, however, it has no effect by itself, but maintains the responsiveness of malic enzyme to triiodothyronine for more than 2 weeks. Finally, short- and medium-chain fatty acids inhibit the triiodothyronine-stimulated rate of transcription of the malic enzyme gene (C. Roncero and A. G. Goodridge, unpublished results). Additional tissue-specific *trans*-acting factors are probably responsible for tissue-specific expression and regulation of this gene.

In summary, feeding increases and starvation decreases the activity of hepatic malic enzyme. In hepatocytes in culture, insulin and triiodothyronine increase and glucagon, via cyclic AMP, decreases activity of this enzyme. In all cases, regulation is primarily at transcription initiation. Hormone-response elements for triiodothyronine and cyclic AMP are located in the 5 kb 5′-flanking DNA. The long-term challenge is to understand how diet controls the intracellular activities of the many different effectors of this gene, and how the actions of those effectors and tissue-specificity factors are integrated to bring about the regulated expression of malic enzyme.

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