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DIETARY REGULATION OF GENE EXPRESSION: Enzymes Involved in Carbohydrate and Lipid Metabolism

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CONTENTS

Introduction[1]

Alterations in diet have striking effects on the expression of a number of genes in vertebrates. In this review, I emphasize regulation of genes involved in the hepatic synthesis of fatty acids, cholesterol, and glucose. These genes have been studied extensively and illustrate hormone- and metabolite-mediated mechanisms in the regulation of gene expression by diet. In addition, each gene is regulated by several hormones, a common property of genes regulated by diet.

[1]Abbreviations used are: L-PK, liver-type pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; ME, malic enzyme; FAS, fatty acid synthase; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CAT, chloramphenicol acetyltransferase; T3, triiodothyronine; CHO, chinese hamster ovary.

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Insulin, glucagon, and triiodothyronine (T3) are involved in mediating the effects of diet on metabolic pathways of the liver. The level of insulin in blood is elevated in well-fed animals, especially when the diet is rich in carbohydrate. Insulin promotes the oxidation of glucose, the storage of glucose calories as glycogen and triglyceride, and the synthesis of protein. It also inhibits the mobilization of protein and fat and the synthesis of glucose. Secretion of glucagon is stimulated by starvation and inhibited by feeding diets high in carbohydrate. Glucagon's metabolic effects are generally opposite of those for insulin, although mechanisms for achieving the effects are often different. Circulating T3 is increased by feeding and decreased by starvation. T3 stimulates the accumulation of several enzymes, especially those involved in fat synthesis. Simple sugars, especially fructose (which stimulates accumulation of lipogenic enzymes) and cholesterol (which is a feedback inhibitor of enzymes involved in cholesterol biosynthesis) are the metabolites that I discuss here.

Points of Regulation in the Pathway of Gene Expression

In the context of this article, the product of the pathway of gene expression is an enzyme that functions in, and may control flux through, a traditional metabolic pathway. Both synthesis and degradation of that enzyme will influence its cellular level. In most cases, experimental analysis has established enzyme synthesis as the regulated process. The rate of synthesis of a specific enzyme is almost entirely determined by the concentration of its mRNA because the factors required for synthesis of proteins do not, in general, distinguish among different mRNAs. In a few situations, the efficiency of translation of an mRNA may be regulated, or mRNA may be sequestered in nontranslatable messenger ribonucleoprotein particles (translational control). In the examples that I describe, however, diet or hormones or metabolites usually cause coordinated changes in the rate of enzyme synthesis and abundance of enzyme mRNA, which indicates control at a pretranslational step.

Pretranslational control can be exerted at several different points in the pathway for production of mature mRNA (Figure 1). Initiation of transcription is the first step in that pathway. Next comes elongation of the nascent transcript, followed by termination downstream from the eventual 3'-end of the transcript. The sequence AAUAAA, located upstream of the termination site in the RNA, directs cleavage and polyadenylation to form the 3'-end of the transcript, usually about 20 bases 3' to the AAUAAA sequence. The 5'-end of the transcript is capped with a 7-methyl guanylate residue in an unusual 5'-5' triphosphate linkage. The primary transcript of a typical eukaryotic gene contains intron sequences that do not appear in the mature mRNA because they are removed in one or more splicing reactions.

The mature mRNA is transported from the nucleus to the cytoplasm. In addition, the rate of degradation of a splicing intermediate may influence the rate of the productive splicing reaction, adding another potential site for regulation of mRNA production. Finally, an extranuclear process, degradation, also influences the concentration of a mRNA in the cytoplasm.

Initiation of transcription has been the step most commonly observed to control the rate of production of specific mRNAs. Degradation rates of mature and intermediate transcripts also have been implicated in the regulation of mRNA concentration. Capping and polyadenylation probably make the transcript resistant to exonucleolytic attack, but have thus far not been implicated in regulation of gene expression. It is uncertain whether the rare occurrence of regulation at other potential sites is attributable to lack of control at these steps or to the difficulty of measuring their rates.

Analytical Methods

Analysis of the mechanisms involved in regulation of enzyme activity has been facilitated greatly by the availability of antisera to purified enzymes. Antibodies bind to antigens with high affinity and specificity, thus providing a

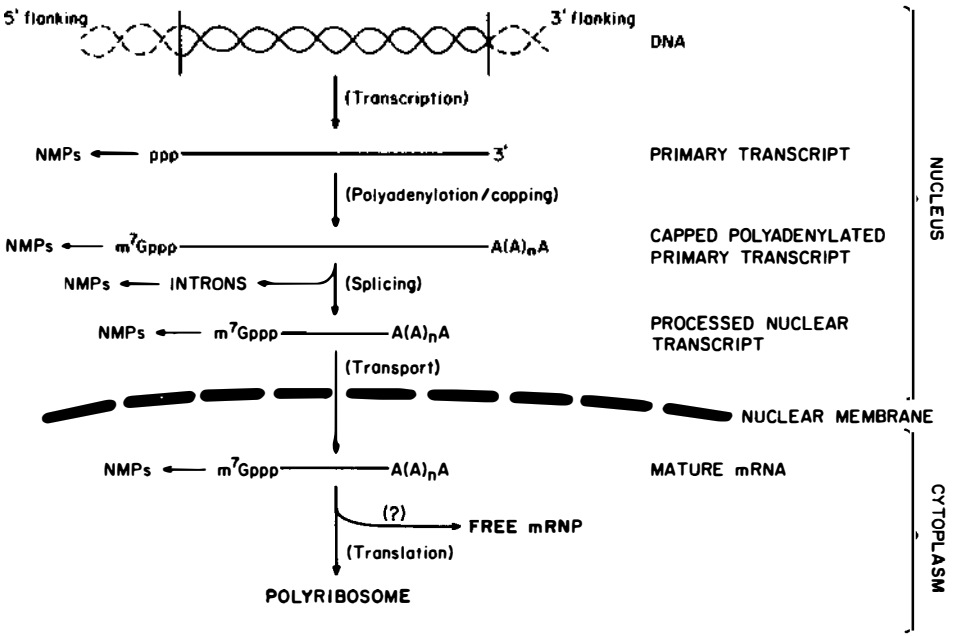

Figure 1 The pathway for gene expression. A generalized scheme for the transcription, processing, transport, and degradation of intermediate and final products in the synthesis of mRNA. NMP = nucleoside monophosphate; m^7G = 7-methyl guanosyl residue; mRNP = messenger ribonucleoprotein particle.

critical reagent for measurement of mass, synthesis rate, and degradation rate of individual enzymes. Complementary DNAs (cDNA) bind to mRNA with even higher affinity and specificity. Physical techniques for the purification of specific mRNAs are not as effective as those for proteins, so that purification of cDNAs was generally not feasible until molecular cloning techniques were developed. It is now theoretically possible to isolate cloned cDNA for any protein, provided that there is a valid assay or a partial amino acid sequence.

Concentrations of specific mRNAs can be estimated in two ways. First, extracts of RNA are translated in a cell-free system, such as the rabbit reticulocyte lysate. The amount of specific protein formed is a measure of the translational activity of the mRNA in question. Even though the components are more complex, this is analogous to determining the concentration of an enzyme by measuring its enzymatic activity. The second method assesses mass of the specific mRNA by virtue of its hybridization to a specific, labeled cDNA. Under suitable conditions, the amount of radioactive cDNA that binds to an RNA preparation is proportional to the concentration of complementary mRNA in that preparation. There are several variations of this assay, all generally analogous to determining enzyme concentration with one of several currently used immunological assays. In the studies reviewed below, one or more of these methods were used to measure concentration of specific mRNAs.

Cloned cDNAs (or genomic DNAs) also are used to measure the synthesis of specific mRNAs. In this case, a labeled ribonucleotide precursor is incubated with cells or nuclei. Cloned DNA attached to a solid support is used as an affinity matrix to rapidly purify the specific mRNA from other labeled RNAs. The analogy here would be to the use of antiserum for rapid purification of an enzyme from a crude extract of metabolically labeled proteins. A commonly used version of this procedure is the "run-on" assay for assessing transcription rate (77). Nuclei are isolated from animals or cells that have been subjected to an experimental treatment. The isolated nuclei are then incubated in vitro with ^{32}P-UTP under conditions that allow extension of preexisting RNA chains but not significant reinitiation. On average, each RNA polymerase molecule engaged in transcription at the time of preparation of the nuclei will extend its chain by about 100 nucleotides before detaching from the DNA. All labeled mRNA molecules are trimmed to the size of the probe by treating the mRNA/cDNA duplex with nuclease, thus removing single-stranded RNA. The assay is thus a measure of the number of RNA polymerase molecules engaged in transcription at the time the tissue or cells were harvested. If neither elongation nor termination of transcription are rate-limiting, the number of polymerase molecules bound per unit length of a specific gene should be proportional to the rate of initiation of transcription of that gene in the intact cell. Most of the transcription studies discussed in this review employed the "run-on" assay.

Cloned cDNAs also can be used as affinity matrices in the measurement of synthesis of specific mRNAs in intact cells labeled with ^{3}H-uridine. Degradation of specific mRNA can be measured by labeling the cells, followed by incubation in the absence of label.

The methods described above, or variations thereof, are used in experiments designed to identify the regulated step in the pathway for expression of a specific gene. In two of the examples discussed here, transcription has been identified as the regulated step, leading to the next level of analysis, identification of *cis*-acting[2] elements in the DNA. All expressed genes have promoter elements. In eukaryotes they usually consist of "TATA" and "CCAAT" sequence elements positioned about 20 and 100 bp, respectively, upstream of the start-site for transcription. These sequences are involved in the binding of RNA polymerase and are responsible for accurate initiation of transcription. Sequences that regulate the rate of transcription also are found in the 5'-flanking DNA of many genes. DNA-mediated gene transfer and site-specific mutagenesis are techniques being used to test the functional significance of sequences thought to be involved in the control of transcription. Briefly, suspected regulatory regions of genes are joined to the structural sequence of a heterologous "reporter" gene and inserted into a hormone- or metabolite-sensitive cell. If the test sequence confers hormone sensitivity on a normally insensitive reporter gene, then the test sequence must contain a *cis*-acting hormone regulatory element. Deletion, insertion, and point mutations are then introduced into the test sequence to determine the exact base sequence responsible for hormone sensitivity. The uses and limitations of these techniques have been reviewed recently (56, 127). Similar approaches will be useful for identifying *cis*-acting sequences involved in control at any point in the pathway of gene expression.

L-Type Pyruvate Kinase

L-type pyruvate kinase (EC 2.7.1.40) (L-PK[1]) catalyzes the conversion of phosphoenolpyruvate to pyruvate. Along with phosphoenolpyruvate carboxykinase (PEPCK), discussed below, L-PK plays an important role in directing pyruvate toward glucose synthesis, or toward oxidation or incorporation into long-chain fatty acids. The concentration of L-PK is decreased by starvation and increased by refeeding, especially when the diet is high in carbohydrate (59, 110). The diabetic state also causes a large decrease in L-PK, which is restored completely with insulin and partly by feeding a diet high in fructose (44, 102, 121). These changes in concentration of L-PK protein are due to changes in the rate of synthesis of the enzyme (12). Thyroid hormone also stimulates accumulation of L-PK in thyroidectomized animals (9).

[2]In this context, *cis* refers to sequences in the same DNA molecule as the regulated gene; *trans* refers to diffusible factors.

Complementary DNA for L-PK mRNA has been cloned by two groups (90, 104) and used to determine the size of L-PK mRNA and its abundance in liver. Feeding a carbohydrate-rich diet to starved rats causes a 40- to 100-fold increase in the level of L-PK mRNA in liver (13, 90, 120). Three L-PK mRNAs (3.2, 2.2, and 2.0 kb) are expressed in liver and vary in an equivalent manner during dietary manipulations. The three mRNAs have the same 5'-end and coding sequence, differing only by the length of the 3'-noncoding region (72).

Regulation of the synthesis of L-PK by hormones and metabolites also is predominantly pretranslational. Thus, accumulation of L-PK is stimulated by insulin and is inhibited by the withdrawal of insulin (diabetes) or the administration of glucagon or cAMP (84, 90, 116). Thyroid hormone also increases the abundance of L-PK mRNA (84). Fructose and certain other simple sugars stimulate increased accumulation of L-PK, even in diabetic animals (102). These sugars share a common property in that they enter metabolic pathways of liver via enzymes other than hexokinase or glucokinase. The insulin-independent effect of fructose is exerted at a pretranslational step because the abundance of L-PK mRNA also is increased in diabetic animals fed fructose (48, 88).

Starvation, feeding a carbohydrate-rich diet, insulin, and cyclic AMP (glucagon) exert their effects on the concentration of L-PK mRNA by regulating gene transcription (89, 115). In contrast, feeding fructose to diabetic animals does not stimulate transcription of the gene (89). The effect of fructose is blocked by glucagon, which suggests that glucagon may regulate accumulation of L-PK mRNA at both transcriptional and post-transcriptional steps. The accumulation of L-PK mRNA induced by thyroid hormone also has a major post-transcriptional component (115). Thus, regulation of the level of L-PK mRNA is not exclusively transcriptional. Furthermore, metabolite and multihormonal control of gene expression may be achieved by regulating different steps with different hormones or metabolites.

Phosphoenolpyruvate Carboxykinase

PEPCK (EC 4.1.1.32) is part of a potentially futile cycle involved in the interconversion of pyruvate and PEP, and plays a central role in the regulation of glucose synthesis. Dietary conditions that stimulate L-PK activity inhibit PEPCK activity and vice versa. Insulin and glucagon have opposite effects on PEPCK, which for both hormones are the converse of their effects on L-PK. The concentration of PEPCK is increased in starved and diabetic animals or animals treated with glucagon or cyclic AMP, and decreased in starved animals refed a diet rich in carbohydrate or in diabetic animals injected with insulin (64). Diet regulates the amount of enzyme protein by controlling enzyme synthesis (46, 111).

The rate of synthesis of PEPCK and the level of its mRNA are high in starved rats. Refeeding a normal chow diet or administering glucose by stomach tube causes rapid decreases in both synthesis and mRNA abundance (8, 57). If rats are treated with cyclic AMP plus glucose, the decrease in enzyme synthesis is blocked (111). Similarly, if starved rats are refed glucose to reduce enzyme synthesis and amount of mRNA, injection of analogues of cyclic AMP causes a rapid increase in the rate of synthesis of PEPCK and the level of its mRNA (8, 49). Glucagon mimics the action of cyclic AMP (5), which suggests that the effects of starvation and refeeding are mediated, in part at least, by changes in the concentration of circulating glucagon.

PEPCK is very high in the liver of diabetic rats. Replacement therapy with insulin causes PEPCK to fall to levels comparable to those in normal animals (64). Insulin, therefore, also plays a role in controlling the level of this enzyme. The concentration of PEPCK mRNA is increased in the liver of diabetic rats and decreased by treatment of such rats with insulin (5). Insulin also inhibits accumulation of PEPCK mRNA in hepatoma cells in culture; this suggests that the effect of insulin in vivo is directly on liver cells and not via changes in the level of some other hormone or factor (1, 45). The correlation between rate of enzyme synthesis and level of enzyme mRNA during dietary and hormonal manipulations indicates that regulation by these agents is pretranslational.

Glucocorticoids and thyroid hormones also lead to increased accumulation of PEPCK in liver. The effect of glucocorticoids in the intact animal is complex because treatment with adrenal steroids increases insulin secretion which, in turn, suppresses PEPCK in liver (43). Stimulatory effects of glucocorticoids are observed in diabetic animals (43), in hepatoma cells in culture (45, 96), and in primary cultures of rat hepatocytes (95). Glucocorticoids amplify the stimulation by cyclic AMP, and this results in either an additive effect, as in Reuber H-35 cells (45), or a synergistic effect, as in rat hepatocytes in culture (50, 95). Thyroid hormone causes increased accumulation of PEPCK in intact rats (82) and in hepatocytes in culture (50). Like glucocorticoids, thyroid hormone amplifies the effects of starvation (82) or cyclic AMP (47, 50). The changes in PEPCK level caused by glucocorticoids and thyroid hormone are due to regulation of the abundance of PEPCK mRNA (47, 50, 69, 96).

Complementary DNA and genomic DNA for PEPCK have been cloned in two laboratories (6, 7, 130, 131) and used to investigate the structure of the mRNA and its gene and the mechanisms by which hormones regulate expression of the gene. PEPCK is a single-copy gene (6, 131) that encodes a single mRNA containing 2624 bases (6). Addition of about 200 adenylate residues at the 3'-end yields an mRNA of about 2.8 kb. The gene for PEPCK spans 6.0 kb and contains 10 exons and 9 introns (6, 131).

Transcription was assessed directly by the "run-on" assay described above. Starvation, cyclic AMP, glucocorticoids, and thyroid hormone stimulate the transcription of the PEPCK gene (63, 69, 96), while glucose administration to intact animals and insulin addition to hepatoma cells inhibit transcription (42, 63, 96). The abundances of nuclear intermediates of PEPCK mRNA also were quantitated, using "Northern" analyses. Increased transcription should result in increased levels of nuclear intermediates with kinetics similar to, but lagging behind, that of transcription. This result was observed for stimulation with cyclic AMP or dexamethasone (11, 63) and inhibition with insulin (42). The magnitude of the changes in transcription is, in general, similar to the magnitude of the changes in steady-state level of mRNA; hence regulation of PEPCK by hormones may be exclusively transcriptional. Figure 2 shows the kinetic relationships between level of mature transcript, level of nuclear transcripts, and transcription rate for cyclic AMP-stimulated accumulation of the PEPCK gene.

Cis-acting sequences that confer hormonal specificity on the regulation of

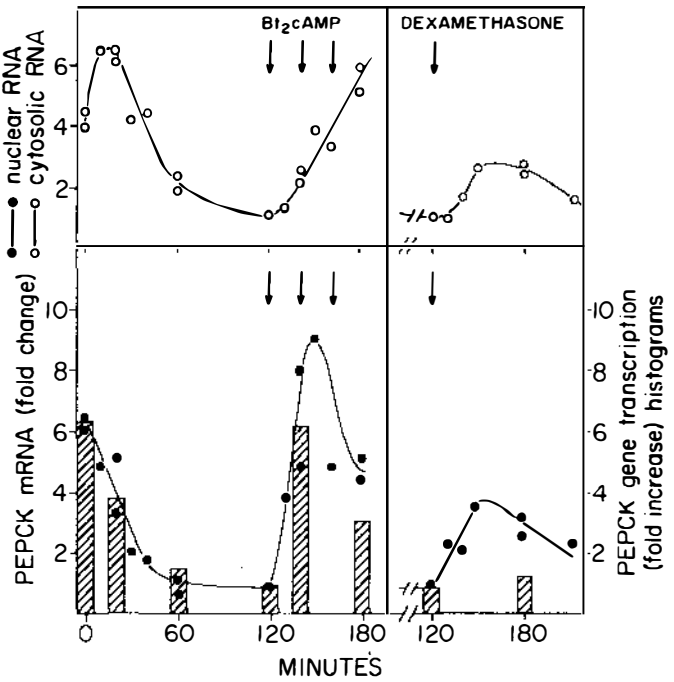

Figure 2 Time course of change in the rates of PEPCK gene transcription and in the sequence abundance of enzyme RNA in isolated rat liver nuclei and cytosol following glucose feeding or after treatment with dibutyryl cyclic AMP. *Closed circles,* nuclear RNA; *open circles,* cytosolic RNA. The procedures used to make these measurements are given in detail in (63). Taken from (63), with permission of the *Proc. Natl. Acad. Sci. USA.*

transcription of the PEPCK gene have been identified in a series of elegant genetic engineering experiments (12, 100, 126, 128). In the first set of experiments, 620 bp of DNA from the 5'-end of the PEPCK gene (including 547 bp of 5'-flanking sequence) were attached to the structural gene for herpes simplex thymidine kinase. This chimeric gene was transfected into FAO-2B cells that had been made thymidine kinase-deficient. FAO-2B cells are derived from a minimum deviation hepatoma and express many of the characteristics of normal differentiated hepatocytes, including a PEPCK gene that is stimulated by cyclic AMP and glucocorticoids and inhibited by insulin. Cells that contained the chimeric gene integrated into their genomic DNA were selected in "HAT" medium, a condition in which survival of cells requires expression of thymidine kinase enzyme activity. If the chimeric gene contained the 620 bp of 5'-DNA in a reverse orientation, it was not possible to select cells in HAT medium, presumably because they could not express the herpes simplex thymidine kinase. In cells containing the chimeric gene with the 5'-flanking sequence in the correct orientation, thymidine kinase activity and concentrations of both the endogenous PEPCK mRNA and herpes simplex thymidine kinase mRNA were increased by cyclic AMP (126). These experiments indicate that the 620 bp of 5'-sequence used in these experiments contains both a promoter element and a cyclic AMP regulatory element.

In the experiments described above, the chimeric gene contained both promoter and regulatory elements from the PEPCK gene. To identify more precisely the sequences responsible for cyclic AMP and glucocorticoid responsiveness, independent of the PEPCK promoter element, a new set of chimeric genes was constructed in which the structural part of the gene for herpes simplex thymidine kinase was attached to its own promoter element. This new series of chimeric genes contains different amounts of 5'-flanking sequence of the PEPCK gene ligated either 5' or 3' of the thymidine kinase gene. The sequence required for cyclic AMP responsiveness is found within a 47-bp region of 5'-flanking DNA. A 12-bp core sequence within this region shows significant similarity with sequences in four other cyclic AMP-regulated genes. Like many sequence elements that regulate the rate of transcription, the 47-bp cyclic AMP regulatory element is effective in either orientation and at a significant distance from the transcription start-site, even as far as the 3'-end of the reporter gene. Two glucocorticoid regulatory elements also were identified, but the sequences that specify the inhibitory response to insulin were not found (Figure 3) (12, 100, 128).

Malic Enzyme

Malic enzyme (EC 1.1.1.40) (ME) catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2, simultaneously generating NADPH from NADP. One of the principal uses of cytosolic NADPH generated by this

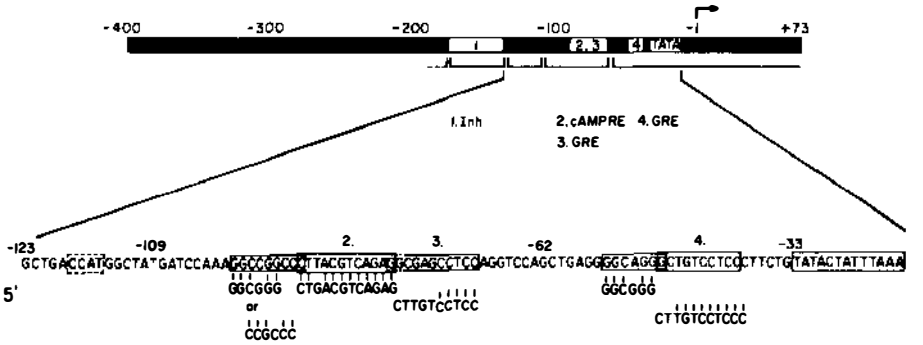

Figure 3 Regulatory elements in the PEPCK promoter. The positioning of the regulatory elements in the PEPCK promoter *(black bar)* are shown by brackets. The arrow at −1 identifies the start-site of transcription of the PEPCK gene. The numbers in the black bar indicate the approximate positioning of the regulatory elements. Each domain is identified by the following abbreviations: (*1*) Inh, inhibitory element; (*2*) cAMP-RE, cyclic AMP regulatory element; (*3* and *4*) GRE, glucocorticoid regulatory element. Sequence homologies for several regulatory domains are shown in the lower half of the figure. GGCGGG is the putative binding site for the transcription factory SP1. Sequences from the rat presomatostatin gene are aligned below the cyclic AMP regulatory element on the PEPCK gene. Finally, the glucocorticoid regulatory elements in the PEPCK genes are aligned relative to the glucocorticoid regulatory element from the human metallothionein II_A gene. Additional details can be obtained from (100). Taken from (100), with permission of the *J. Biol. Chem.*

enzyme is the de novo synthesis of long-chain fatty acids. ME responds to dietary and hormonal manipulations in much the same way as L-PK. Enzyme content of liver is low in starved animals and high in fed animals, especially if the diet is high in carbohydrate (30). The concentration of ME is regulated by controlling its rate of synthesis (65, 103, 113). The latter correlates well with abundance of ME mRNA, as determined by assay of functional mRNA (39, 101, 113) or hybridizable mRNA (41, 108, 124). This correlation indicates regulation at pretranslational steps.

Steps involved in the dietary regulation of the accumulation of ME mRNA have been identified in neonatal ducklings and adult rats. A 20-fold increase in mRNA level caused by feeding a carbohydrate-rich diet is accompanied by a 2- to 3-fold increase in transcription of the ME gene and a 65–80% decrease in the rate constant for degradation of mature ME mRNA (Figures 4 and 5) (34). The net effect of the two changes may be sufficient to account for the observed 20-fold increase in mRNA concentration. However, changes in the rates of other processes cannot be ruled out. In rat liver, the increased ME mRNA level caused by feeding is accompanied by decreased degradation of the mature mRNA but no change in the transcription rate of the gene for this enzyme (22). It is unclear whether or not the change in degradation rate constant is sufficient to account quantitatively for the change in abundance of mRNA.

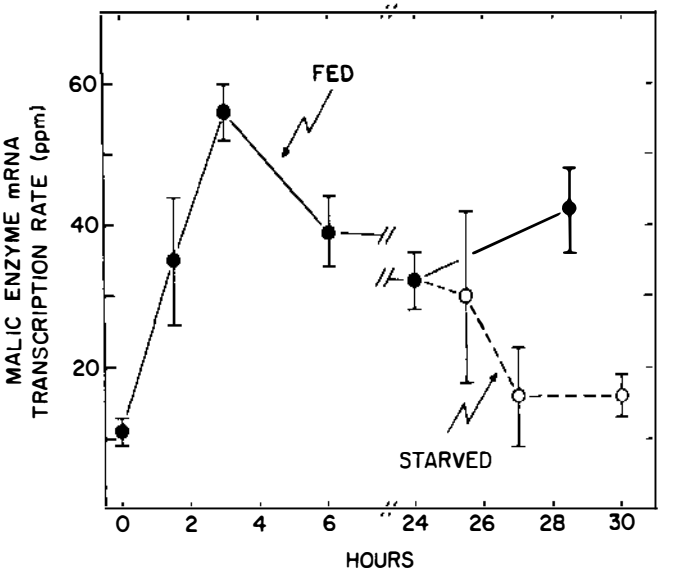

Figure 4 The effects of feeding and starvation on transcription of the ME gene. Ducklings were fed ad libitum for 10–14 days and starved for 48 hr before beginning these experiments. The ducklings were then fed *(closed circles)* or starved *(open circles)* as indicated. Nuclei isolated from the treated ducklings were used for the measurements of the relative rates of transcription of the ME gene (34). The results are expressed as means ± S.E. of 4 to 8 experiments (one duckling per experiment). Replotted from data presented in (34), to which the reader is referred for more detail. Reproduced with the permission of *Ann. NY Acad. Sci.*

ME concentration is low in diabetic and thyroidectomized animals, and is restored to normal or supranormal levels by administration of insulin and T3, respectively (30). In avian hepatocytes in culture, T3 stimulates a greater than 50-fold increase in the amount of ME (36, 38, 40). In serum-free media, insulin has little effect by itself but amplifies the effect of T3, such that the total increase is more than 100-fold (38). Insulin and T3 also stimulate accumulation of ME in rat hepatocytes in culture, but to a smaller extent than observed in vivo (75, 107, 122) or in chick-embryo hepatocytes.

The small effect of insulin in hepatocytes from chick embryos or rats contrasts with the profound decrease in the level of ME induced by diabetes and the importance of insulin in the accumulation of ME caused by refeeding starved animals carbohydrate-rich diets. Thus, factors other than insulin may be involved in the decreased ME concentration in diabetic animals. Glucagon is one possibility because its circulating level is high in diabetic rats and lowered on treatment with insulin (20). In addition, glucagon, acting via cyclic AMP, blocks the induction of ME and its mRNA caused by insulin plus T3 in hepatocytes in culture (38, 76, 124, 132).

In addition to insulin, T3, and glucagon, other potential regulators include

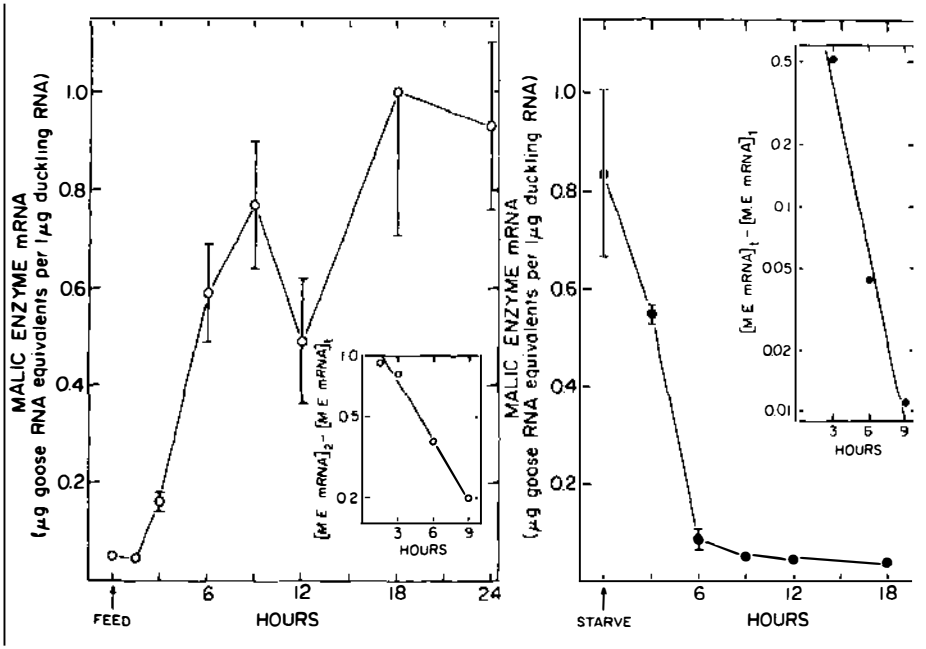

Figure 5 The effects of feeding *(left panel)* and starvation *(right panel)* on the abundance of ME mRNA in duckling liver. At the beginning of the experiment shown in the left panel, the birds were 2 days old and unfed. For the experiments shown in the right panel, the birds were 11 days old and were fed 8 days, starved 2 days, and refed 1 day prior to beginning the experiment. Both sets of experiments were started at 10 A.M. This dietary regimen ensured a high level of ME mRNA at the beginning of the starvation experiment. Total RNA was extracted, fixed to GeneScreen (New England Nuclear), and hybridized to a single-stranded, ^{32}P-labeled ME cDNA. In the main plots, each point is the mean±S.E. of three experiments (one duckling per experiment). The insets are semilog plots of the approaches of ME mRNA concentration to new steady states ($[M.E.\ mRNA]_1$, the steady-state concentration of ME mRNA in the starved state; $[M.E.\ mRNA]_t$, the concentration at any time, t, during the approach to steady state; $[M.E.\ mRNA]_2$, the steady-state concentration in the fed state). There were essentially no changes in ME mRNA concentrations when starved birds continued to starve or refed birds continued to feed. Taken from (34), with permission of the *J. Biol. Chem.*

growth hormone, epidermal growth factor, and simple sugars. Growth hormone and epidermal growth factor inhibit accumulation of ME in hepatocytes in culture (97, 133). The relevance of these effects to control of ME level by diet is unknown. In diabetic rats, a diet rich in fructose increases accumulation of ME and its mRNA (23, 32, 55). In this respect, ME behaves like other "lipogenic" enzymes, which are increased in amount when fructose is fed to diabetic rats. In rat hepatocytes in culture, glucose, fructose, and other simple carbohydrates stimulate increased accumulation of lipogenic enzymes (76, 107). Dichloroacetate, an activator of pyruvate dehydrogenase, also stimulates accumulation of ME in rat hepatocytes in culture, which suggests that

the active intermediate may be generated in mitochondria (76). In avian hepatocytes, neither glucose nor fructose increases ME level, with or without insulin in the medium. In the presence of T3, glucose augments induction by about 50% (Goodridge, unpublished results; 37). The mechanisms whereby glucose or fructose, or metabolic intermediates derived therefrom, control expression of malic enzyme remain obscure.

Hormonally induced changes in ME concentration are due to altered synthesis rates, which, in turn, correlate with altered levels of ME mRNA. This means that regulation is exerted primarily at pretranslational steps (24, 38, 55, 65, 71, 101, 113, 124). Mechanisms involved in the increase in abundance of ME mRNA caused by T3 and the decrease caused by glucagon (cyclic AMP) have been analyzed in avian hepatocytes in culture (3). T3 causes only a 2-fold increase in transcription of the ME gene, despite increasing the amount of ME mRNA by more than 14-fold. Thus, regulation is primarily post-transcriptional. Accumulation of ME mRNA after addition of T3 displays sigmoidal kinetics and takes 36–48 hr to come to a new steady-state. Furthermore, accumulation of ME mRNA is blocked when protein synthesis is inhibited with puromycin. The T3-induced increase in the level of ME mRNA (21) and another thyroid-sensitive mRNA, S11 (125), in thyroidectomized rats also is due only partly to increased transcription. The response of S11 mRNA is sigmoidal, takes about 36 hr to reach the new steady state, and is blocked by cycloheximide, an inhibitor of protein synthesis. Thus, T3 may stimulate the synthesis of an intermediate that regulates accumulation of ME and other mRNAs, primarily at post-transcriptional steps.

Glucagon has no effect on transcription of the ME gene, despite inhibiting accumulation of ME mRNA by more than 95%. Glucagon also causes ME mRNA to decrease with a half-life of 1.5 hr or less, whereas inhibition of transcription by α-amanitin or actinomycin D causes it to decrease with a half-life of 8–11 hr. Thus, cyclic AMP, the intracellular mediator for glucagon, may act post-transcriptionally to regulate ME mRNA, in part at least, by increasing the degradation rate constant for ME mRNA. We have speculated that cyclic AMP inhibits activity of the T3-induced protein intermediate (3).

Fatty Acid Synthase

Fatty acid synthase (EC 2.3.1.85) (FAS) is a multifunctional polypeptide that, using acetyl-CoA as a primer, catalyzes the formation of long-chain saturated fatty acids from malonyl-CoA and NADPH. In vertebrates, the enzyme is a homodimer with subunits of M_r = 260,000 (119). The gene for goose FAS is unique, but transcribed into two mRNAs of about 11,800 and 10,400 nucleotides (2). The rat gene also appears to be a unique gene that is transcribed into two mRNAs, both of which are somewhat shorter in length than those of

the goose (129). Dietary and hormonal regulation of activity is primarily via changes in enzyme concentration (119).

The effects of dietary manipulation on the amount of FAS in liver are similar to those observed with other lipogenic enzymes. Enzyme content is high in animals fed a diet with a high carbohydrate content and is low in starved animals. In both mammals and birds, changes in enzyme concentration are attributable to altered rates of synthesis of FAS (18, 28, 33, 117, 134). Increased synthesis of FAS caused by refeeding starved animals is accompanied by comparable increases in the levels of functional (29) or hybridizable mRNA (40, 80, 81, 87). The rate of transcription of the FAS gene increases about 10-fold when starved ducklings are refed (2). A similar change in the level of FAS mRNA suggests that regulation is primarily transcriptional. In contrast, control of ME mRNA accumulation by feeding has a large post-transcriptional component (34).

The level of hepatic FAS is decreased profoundly by diabetes and restored by treatment of diabetic animals with insulin (10, 19, 62, 118). In starved diabetic animals, refeeding glucose does not increase FAS unless insulin is given during the refeeding period. If fructose is refed, the requirement for insulin is circumvented (60, 118). Refeeding fructose without administration of insulin is almost as effective as refeeding glucose with insulin. Treatment of rats with glucagon or cyclic AMP causes the level of FAS to decrease in normally fed rats (117) and blocks the increase caused by refeeding a high-carbohydrate diet (62). These actions of glucagon raise the possibility that the elevation of circulating glucagon in diabetic animals mediates the effect of insulin deprivation (20). Glucagon and/or insulin regulate the concentration of FAS in rats by controlling its rate of synthesis (19, 60, 62, 117).

Thyroid hormone and dietary carbohydrate are synergistic in their regulation of several lipogenic enzymes, including FAS (74). The steps at which thyroid hormone regulates FAS level in rats have not been evaluated. However, the similarity of the effects of T3 on expression of FAS, ME, and other lipogenic enzymes suggests regulation at similar steps, e.g. at a pre-translational step(s).

In chick-embryo hepatocytes in culture, the amount of FAS is increased about 2-fold by insulin alone, about 2-fold by T3 alone, and about 15-fold by the combination of insulin and T3 (28). The effects of insulin and T3, acting individually, on the level of FAS in rat hepatocytes are similiar to those observed in avian hepatocytes (107). The effects of insulin plus T3 were not reported. In avian hepatocytes, enzyme concentration is controlled by regulating the rate of enzyme synthesis (28). There is, however, a discrepancy between enzyme synthesis and amount of hybridizable mRNA. In the absence of T3, insulin has no effect on accumulation of FAS mRNA despite a 3-fold increase in enzyme synthesis. With T3 present, insulin increases mRNA level

by 2-fold and enzyme synthesis by 14-fold (123). Thus, T3 regulates enzyme synthesis at pretranslational steps, whereas insulin may regulate the efficiency of translation of FAS mRNA.

The mechanisms involved in the T3-induced increase in FAS mRNA are similar to those for ME mRNA. After adding T3, FAS mRNA accumulates with sigmoidal kinetics and takes 36–48 hr to achieve a new steady state (Figure 6). Puromycin blocks the increase (Figure 7). These data suggest a peptide intermediate in the action of T3 on accumulation of FAS mRNA, presumably the same intermediate that regulates the amount of ME mRNA. Because feeding controls expression of FAS at a transcriptional step, whereas regulation of ME has a large post-transcriptional component, it will be

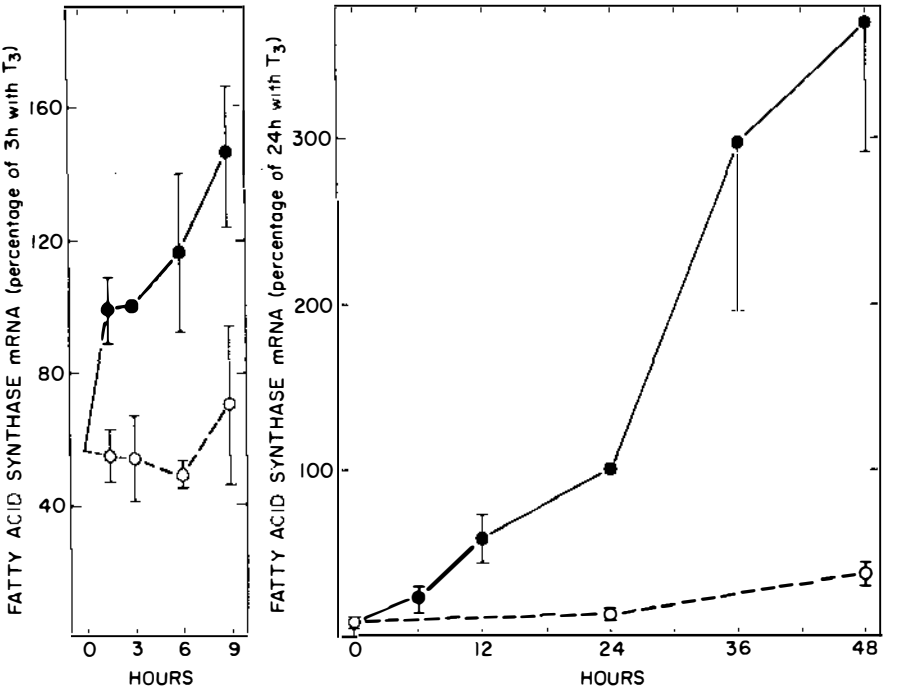

Figure 6 Concentration of FAS mRNA as a function of time after adding T3. Hepatocytes were isolated and incubated in chemically defined medium containing insulin (300 ng per ml). The medium was changed to one of the same composition at about 20 hr of incubation. At about 44 or 68 hr of incubation, T3 (1 μg/ml, *solid circles, solid lines*) or nothing *(open circles, dashed lines)* was added to the medium. Total RNA was isolated and assayed for FAS mRNA (123). In the left panel, the results are expressed as a percentage of the value for cells incubated for 3 hr with T3±S.E. of 2 to 5 experiments. An approximate value for time zero was determined by averaging the values for control samples at 1.5, 3, and 6 hr. In the right panel, the results are expressed as a percentage of the value for cells incubated for 24 hr with T3±S.E. of 4 or 5 experiments. Taken from (123), with permission of the *J. Biol. Chem.*

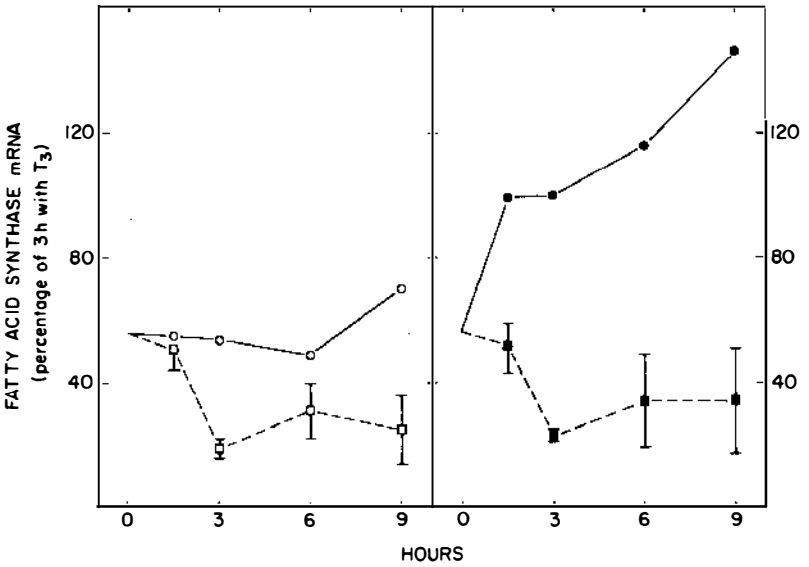

Figure 7 Puromycin blocks the accumulation of FAS mRNA stimulated by T3. Hepatocytes were isolated and incubated in chemically defined medium containing insulin (300 ng/ml). The medium was changed to one of the same composition at 20 hr. At about 44 hr (time zero), T3 (1 μg/ml, *solid symbols*) or nothing *(open symbols)* was added to the medium. Puromycin (1 mM, *squares, dashed line*) or nothing *(circles, solid line)* was added 30 min prior to adding the T3. Total RNA was isolated and assayed for FAS mRNA (123). The effect of puromycin is expressed as a percentage of the corresponding control ± S.E. (n = 2 to 5) with controls set at the values given in the left panel of Figure 6. Taken from (123), with permission of the *J. Biol. Chem.*

interesting to identify the step at which T3 controls the increase in FAS mRNA.

Glucagon decreases the amount of FAS in avian hepatocytes treated with insulin plus T3 (28). Comparable decreases in FAS synthesis and amount of FAS mRNA suggest regulation at pretranslational steps (123). The decreases in levels of FAS and FAS mRNA caused by glucagon are much smaller than the decreases in ME or ME mRNA. There is another difference in the responses of ME and FAS to glucagon. In the first day of culture, glucagon stimulates FAS activity (40), an effect not seen with ME. By three days, however, FAS activity in glucagon-treated hepatocytes is much lower than that in control cells (28, 40). This dual effect of glucagon—stimulation early in the incubation period followed by later inhibition—may explain the lack of effect of glucagon on FAS activity in explants of chick-embryo liver incubated for 48 hr (51).

Liver Protein S14

When total polyadenylated RNA from the livers of normally fed, euthyroid rats is translated in a cell-free system in the presence of labeled methionine,

products of about 230 mRNAs are detected by two-dimensional gel electrophoresis (99). About 20 mRNAs are regulated by thyroid status (99). Changing rats from standard laboratory chow to a high-carbohydrate diet alters the levels of 10 of the 230 mRNAs; 9 of the 10 also are regulated by thyroid status (66). Two of the 9 mRNAs affected by both diet and thyroid status, S11 and S14, have been subjected to further analysis. After treatment of rats with T3, S11 and ME mRNAs accumulate with the same kinetics (125). Other similarities in the regulation of ME and S11 mRNAs were discussed earlier in the section on malic enzyme.

S14 mRNA responds rapidly to thyroid hormone. Maximum translational activity of S14 mRNA is achieved within 4 hr after injecting T3 into thyroidectomized rats, and a significant increase occurs at 20 min (66, 98). These changes in translational activity are due to comparable changes in the mass of S14 mRNA (53). Increased levels of nuclear precursors to S14 mRNA are detected as soon as 10 min after injecting hypothyroid rats with T3 (85).

Protein S14 also has a tissue distribution similar to that of the lipogenic enzymes. The concentrations of S14 mRNA in adipose tissue and lactating mammary gland are 10- and 4-fold higher, respectively, than that in the liver of euthyroid rats. Brain, heart, kidney, lung, spleen, testes, and pituitary have low rates of lipogenesis and a level of S14 mRNA less than 7% of that in liver (52). Furthermore, diet and thyroid hormone regulated S14 mRNA in adipose tissue in the same way that they controlled lipogenic enzymes in this tissue. In rat hepatocytes in culture, accumulation of S14 mRNA is stimulated by T3 and glucose and inhibited by glucagon, consistent with a function of S14 protein in lipogenesis (73, 112).

Liver protein S14 is a relatively abundant cytosolic protein (53). Its sequence was deduced from that of the cloned cDNA. The mRNA codes for a protein of 150 amino acids, M_r = 17,010. The gene, which also has been cloned, is present in a single copy per haploid genome, but encodes two mRNAs differing by a 170-nucleotide extension at the 3' end (67). The protein does not share significant sequence similarity with any protein in the data bank of the National Biomedical Foundation.

Levels of S14 mRNA and its nuclear precursor RNA are elevated 5- to 6-fold in euthyroid rats and 9- to 12-fold in hyperthyroid rats relative to those in hypothyroid controls. However, the transcription rate of the S14 gene is increased only 50% in euthyroid and hyperthyroid as opposed to hypothyroid rats (86). A single injection of T3 into hypothyroid rats causes a small, transient increase in transcription of the S14 gene at 30 min, but the levels of S14 mRNA and nuclear precursor RNA increase to a much greater extent throughout the first 4 hr of treatment. These results indicate a post-transcriptional site for the regulation of S14 mRNA accumulation caused by thyroid hormone. The increase in abundance of nuclear RNA precedes the increase in mature cytoplasmic mRNA (86). In addition, in hepatocytes in

culture, T3 stimulates accumulation of S14 mRNA but does not affect its half-life (73). Together, these results suggest that T3 regulates the production of the mature mRNA by controlling the stability of nuclear precursors. Because the "run-on" assay measures initiation of transcription, the rate of transcription elongation also could be regulated.

Inhibition of protein synthesis with cycloheximide blocks the response of S14 mRNA to T3 (98). This is similar to the finding that inductions of ME, FAS, and S11 required on-going protein synthesis. In the case of S14 mRNA, however, response to T3 is very rapid and accumulation of mRNA is linear with little or no lag period (53, 86). This argues against the synthesis of an intermediate protein that, in turn stimulates accumulation of S14 mRNA. In this case, a rapidly turning-over protein may be required for the induction by T3.

Other Enzymes Involved in Fatty Acid Synthesis

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), ATP-citrate lyase (EC 4.1.3.8), and acetyl-CoA carboxylase (EC 6.4.1.2) are four additional enzyme proteins involved in the de novo synthesis of long-chain fatty acids. For the most part, levels of these enzymes are increased by carbohydrate-rich diets, insulin, and T3, and are decreased by starvation and glucagon in the same manner as those for L-PK, ME, and FAS. Enzyme concentrations are controlled by regulating rates of enzyme synthesis. Cloned cDNAs have been isolated for each of these enzymes and used to show that rates of enzyme synthesis are, in turn, correlated with abundances of the enzyme mRNAs (4, 58, 79, 109). Regulation is thus primarily at pretranslational steps. Further analysis of these interesting genes has not yet been reported.

3-Hydroxy-3-methylglutaryl Coenzyme A Reductase

Animal cells require cholesterol for growth. This cholesterol is obtained from the receptor-mediated uptake of plasma lipoproteins or from the de novo synthesis of cholesterol from acetyl-CoA (35). The pace-setting step in the multienzyme pathway by which cholesterol is synthesized is 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase), an integral membrane glycoprotein of the endoplasmic reticulum that catalyzes the conversion of HMG CoA to mevelonate (9a). In rat liver, the concentration of HMG-CoA reductase varies over a range of more than two orders of magnitude (15). The highest levels of enzyme are observed after feeding a diet supplemented with cholestyramine and mevinolin, the lowest levels after administration of mevalonolactone, cholesterol, or hydroxysterols. Cholesterol or metabolites related to cholesterol decrease the level of HMG-CoA reductase in hepatocytes in culture (27, 54) and in normal Chinese hamster ovary (CHO) cells (105).

Cholestyramine and mevinolin probably work by lowering plasma and tissue concentrations of cholesterol. Cholestyramine complexes with bile acids in the intestinal tract, and mevinolin is a potent competitive inhibitor of HMG-CoA reductase. Mevalonic acid or its lactone (mevalonolactone) is the product of HMG-CoA reductase, which is then converted via specific phosphorylations to isopentenyl pyrophosphate, an intermediate in sterol biosynthesis. Thyroid hormone and insulin also increase the activity of HMG-CoA reductase, but these effects may be secondary to changes in blood or tissue cholesterol concentration brought about by these hormones (61).

Cholestyramine and mevinolin increase the rate of synthesis of HMG-CoA reductase in rat liver (26). Similarly, cholesterol or related compounds exert at least part of their inhibitory effects on the amount of reductase in CHO cells by controlling synthesis of this protein (105). In both rat liver and CHO cells, the rate of synthesis of HMG-CoA reductase is correlated with abundance of HMG-CoA reductase mRNA (14, 15, 68, 70). Cholestyramine plus mevinolin increases and mevalonolactone decreases transcription of the HMG-CoA reductase mRNA in rat liver (16). Taken together, these results suggest that cholesterol or a related metabolite regulates the transcription of the reductase gene.

Cloned cDNAs for HMG-CoA reductase were isolated from a library prepared from the mRNA of UT-1 cells, a cell line derived from CHO cells that has a 15-fold amplification of the gene for the reductase and produces more than 100 times the normal amount of reductase mRNA (70). These cloned cDNAs were used in many of the studies of mRNA level described above. They also were used to isolate two genomic clones containing the entire HMG-CoA reductase gene plus considerable 5'- and 3'-flanking DNA (93). The reductase gene is 25 kb in length and contains 20 exons. The promoter region of this gene lacks characteristic TATA and CCAAT boxes, features that probably account for the multiple transcription initiation sites (94). The promoter region is rich in G+C residues and contains five copies of the hexanucleotide sequence CCGCCC or its inverse complement GGGCGG. This hexanucleotide sequence is important for transcription of the herpes simplex thymidine kinase gene (78) and the early region genes of the SV40 virus (31). A *trans*-acting cellular factor, SP1, interacts with this region of the SV40 DNA (25) and is required for initiation of SV40 RNA synthesis in vitro.

Sequences responsible for promoter activity, and for mediating the inhibition of the transcription of the reductase gene by cholesterol, have been identified in a series of gene-transfer experiments using hybrid genes containing all or part of the 5'-flanking region of the reductase gene ligated to a reporter gene, chloramphenicol acetyltransferase (CAT) (91). Recombinant plasmid DNA was introduced into mouse L cells by calcium phosphate–mediated transfection. Hybrid genes containing reductase and CAT DNA

were cotransfected with the hybrid gene pSV3-Neo, which contains the regulatory region of SV40 DNA and the structural gene for resistance to the antibiotic, G418 (106). When two DNAs are transfected simultaneously, most cells that take up one DNA will take up both. Cells with the antibiotic resistance gene integrated into their DNA were selected by growth in G418. Sequences responsible for both promoter activity and cholesterol-mediated inhibition of transcription were distributed over 500 bp, extending 300 bp upstream of the transcription initiation sites. Any sizable deletion within this region decreased CAT expression (Figure 8). This region contains the five hexanucleotide repeats that occur in SV 40 and herpes simplex virus promoters. This hexanucleotide motif has been found in several genes that are expressed in relatively tissue-independent fashion (see 91). Every reductase plasmid that showed transcriptional activity also showed inhibition by sterols, which indicates that the sites responsible for promoter activity and inhibition of transcription are closely associated.

Summary

The mechanisms of the responses of an enzyme to different hormones and metabolites or several enzymes to a single hormone are surprisingly varied. There is neither an operon for lipogenic enzymes nor a common step at which hormones and metabolites coordinately regulate the expression of lipogenic genes. In bacteria, coordinated expression of several enzymes in a single metabolic pathway often is achieved by organizing the genes into operons. An operon is a group of genes linked together in a linear fashion and producing a polycistronic mRNA. *Trans*-acting factors regulate the transcription of these genes by interacting with promoter/regulatory sequences in the 5'-flanking region of the most 5'-ward of the genes. In vertebrate animals, however, coordinated control of gene transcription is not achieved by linking the individual genes, but by putting in the 5'-flanking regions of these genes a regulatory sequence that interacts with common *trans*-acting factors. Genes controlled by different hormones are expected to have regulatory elements for each hormone. The presence of glucocorticoid and cyclic AMP regulatory elements at the 5'-end of the PEPCK gene is consistent with this notion.

Transcription is not the only step at which hormones and metabolites control the pathways for gene expression. The levels of the mRNAs for L-PK, ME, S11, and S14 are increased by T3 at post-transcriptional steps. Glucagon also regulates the accumulation of ME mRNA post-transcriptionally. Neither the mechanism nor the sequence organization of regulatory elements is known for post-transcriptional control of gene expression.

In the case of PEPCK and HMG-CoA reductase, the next steps will be to determine more precisely the sequences in the 5'-region that mediate hormone

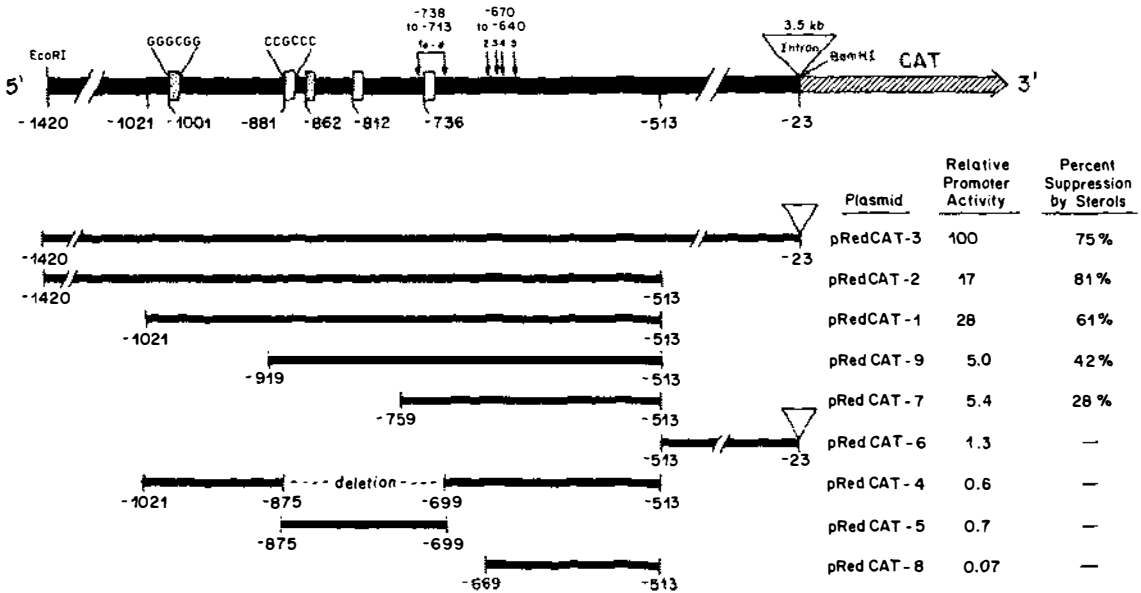

Figure 8 Structure and activity of reductase promoter-CAT chimeric genes. The DNA fragment from the 5′-end of the hamster HMG-CoA reductase gene used to construct pRedCAT-1 to −9 is represented at the top by the solid bar and bounded by the indicated restriction endonuclease sites. Nucleotide positions 11420 to −23 of the reductase promoter region are numbered in relation to the A of the methionine initiation codon (93). The exact endpoint of the EcoRI site at −1420 is estimated from partial sequencing and detailed restriction mapping. The ~3.5-kb intron is omitted from the numbering scheme. The hexanucleotide repeat sequences homologous to the SV40 promoter are represented by open (CCGCCC) or dotted (GGGCGG) boxes. Arrows denote the positions of the transcription initiation sites for HMG CoA reductase (93, 94). The CAT coding sequence is denoted by the cross-hatched bar, and the direction of CAT transcription is indicated by the arrow. The structure of each pRedCAT construct is shown. The columns on the right give average CAT enzymatic activity obtained in several experiments with each plasmid. The data are expressed as a percentage of the mean activity obtained with pRedCAT-3, the plasmid with the largest amount of reductase sequence. The mean suppression by 25-hydroxycholesterol plus cholesterol for each plasmid also is shown. Taken from (91), with permission of *Cell*.

sensitivity and feedback inhibition, respectively, and whether *trans*-acting factors are involved. For the other genes discussed, identification of the regulated step must precede identification of sequences that confer hormone or metabolite-sensitive regulation on a specific gene. In general, it is probable that the hybrid gene approach, so successful for PEPCK and HMG-CoA reductase, also will be effective in defining *cis*-acting hormone- or metabolite-regulatory elements in other genes. These techniques should be applicable to both transcriptional and post-transcriptional mechanisms.

Our long-term objective is to understand the molecular basis of each event that intervenes between the binding of hormone or metabolite to its appropriate receptor and altered enzyme level. The results of studies reviewed above represent great advances toward that objective. Nevertheless, a challenging part of the analysis still lies ahead. The identification in eukaryotic DNA of *cis*-acting hormone- or metabolite-regulatory sequences confirms a mechanism known to exist in prokaryotes. What is the nature of the *trans*-acting factors that interact with these sequences to regulate gene expression? How do *trans*-acting factors interact with different *cis*-acting regulatory sequences and with one another to achieve the multihormonal regulation so commonly observed in genes controlled by diet? DNA-protein binding assays (17, 83), DNA footprinting (92), and DNA band-shift assays (114) are procedures that may be useful to detect the binding of *trans*-acting factors to specific DNA sequences. In the band-shift assay, proteins that bind to specific DNA fragments are detected by their ability to retard the electrophoretic mobility of DNA fragments in nondenaturing agarose gels. Under appropriate conditions, this method detects proteins in crude extracts, which indicates that the band-shift assay will be useful in the purification of factors from crude extracts.

Definitive analysis of the interactions of factors with DNA or RNA sequences will be facilitated by the development of cell-free systems, and ultimately by the ability to reconstitute complete regulatory systems from purified components. At present, we lack cell-free assay systems that retain the regulatory properties of intact cells; this is a serious impediment to progress. An alternative approach may involve the reconstruction of regulatory systems in normally unresponsive intact cells using the genetic engineering and gene transfer techniques that have proved so valuable for the identification of hormone regulatory elements.

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