Transient transfection of chick-embryo hepatocytes

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Nutritional state regulates the expression of many genes via alterations in the plasma levels of hormones or metabolic fuels. In many cases, transcription has been identified as the regulated step. The next objective in the experimental analysis of these transcriptionally regulated genes is to identify the cis-*acting sequence elements that confer regulation of a specific gene by a particular hormone or agent. One method for identifying* cis-*acting sequence elements involves transient expression of transgenes introduced into responsive cell types by a process called transfection. Promoter/regulatory sequences from the gene of interest are ligated to a reporter gene, and the chimeric DNA is added to cells in culture. Under appropriate conditions the DNA enters the cell, migrates to the nucleus, and is transcribed, but is not integrated into chromosomal DNA. Expression of the reporter gene is monitored to assess function of the putative promoter/regulatory DNA. The reporter gene codes for a protein that is not normally expressed in the responsive cell type. If this protein is an enzyme, then the amount of its activity is a measure of the ability of* cis-*acting elements in the promoter/regulatory DNA to regulate transcription. If a specific fragment of DNA can confer hormone responsiveness on the expression of the reporter gene, then sequences containing 5′ or 3′ deletions or mutations in suspected regulatory elements are used to identify the sequence elements more specifically. These DNA sequences are binding sites for regulatory proteins. The sequences identified in this "functional assay" can then be used in DNase I footprinting and gel mobility-shift assays to identify the proteins that bind to those elements.*

Keywords: hepatocyte; chicken; transfect; malic enzyme; fatty acid; transcription

Introduction

In this paper we provide a detailed description of the transient transfection assay that we used to identify *cis*-acting regulatory elements in the 5′-flanking DNA of the malic enzyme gene. We used chick-embryo hepatocytes in maintenance culture for these experiments because nutritional regulation of hepatic lipogenic enzymes such as malic enzyme is mimicked quantitatively by manipulating the hormonal and fatty acid milieu of cells incubated in a chemically defined, serum-free medium. In the absence of hormones or with only insulin in the medium, malic enzyme activity is low—about the same

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as that in livers from 19-day embryos. Addition of triiodothyronine alone causes a 30- to 50-fold increase in malic enzyme activity; addition of triiodothyronine plus insulin causes a greater than 100-fold increase. Glucagon, acting via cyclic AMP, and medium-chain fatty acids inhibit the increase in malic enzyme activity caused by insulin plus triiodothyronine.[1,2] These changes in enzyme activity are due primarily to regulation of transcription of the malic enzyme gene.[2,3]

Detectable levels of expression of several reporter genes can be achieved from transfected plasmid DNAs using liposome-mediated transfection of chick-embryo hepatocytes. In this transfection method, DNA binds to the liposome via ionic interactions and is taken up by the cells when the liposome fuses with the cell membrane.[4,5] The DNA is transported to the nucleus via an unknown pathway and is transcribed. We used a series of plasmid DNAs containing fragments of the 5′ flanking DNA of the malic enzyme gene linked to the chloramphenicol acetyltransferase gene to define specific hormone- and fatty acid-response elements that regulate transcription. Other plasmid DNAs are co-transfected with the test plasmid to control for transfection efficiency and to keep the total mass of transfected DNA constant. In addition to detailed descriptions of the procedures, we have discussed possible problems and provided examples of the results that can be obtained using this methodology.

Methods and materials

Reagents

Unless otherwise indicated all reagents were obtained from Sigma Chemical Co., St. Louis, MO USA.

Acetyl-CoA. Acetyl-coenzyme A was obtained from Promega (Madison, WI, USA) and diluted to 35 mg/mL in H_2O. Dispense portions into Eppendorf tubes and store at −20° C.

Chloramphenicol acetyltransferase (CAT) assay buffer. Tris-HCl (10 mmol/L, pH 7.8, EDTA (1 mmol/L).

^{14}C-Chloramphenicol. D-threo-[dichloracetyl-1,2-^{14}C]-chloramphenicol (40–60 mCi/mmol) in ethanol (New England Nuclear Corp., Boston, MA USA).

Homogenization buffer. Tris-HCl (10 mmol/L, pH 7.8, EDTA (1 mmol/L), dithiothreitol (1 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), trypsin inhibitor (10 μg/mL). PMSF is unstable in aqueous solutions; it should be dissolved in isopropanol and added to the lysis buffer immediately before use.

Luria-Bertani medium.[6] Add bacto-tryptone (10 g), bacto-yeast extract (5 g) and NaCl (10 g) to 950 mL of deionized H_2O and mix until the solutes have dissolved. Adjust the pH to 7.0, bring it to a final volume of 1 liter and sterilize in an autoclave.

Liposome reagent. TransfectACE was obtained from GIBCO/BRL (Grand Island, NY, USA).

100X Mg solution. $MgCl_2$ (0.1 M), β-mercaptoethanol (4.5 M), store at room temperature in a tightly sealed container.

$NaPO_4$ buffer, pH 7.5. Sodium phosphate (0.1 mmol/L), pH 7.5, sterilize in an autoclave and store at room temperature. This buffer can be made and stored as a 10× solution and diluted immediately before use.

ONPG. Dissolve 4 mg/mL of *O*-nitrophenyl-β-D-galactopyranoside in $NaPO_4$ buffer. This can be frozen at −20° C.

Phosphate-buffered saline (PBS), pH 7.4[7] Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of H_2O. Adjust the pH to 7.4, add

H_2O to 1 liter and sterilize it in an autoclave. PBS can be made and stored as a 10× solution at room temperature.

Plasmid DNAs Plasmid RSV-CAT contains the CAT gene, the SV40 small *t* intron, and polyadenylation signals. It is linked to the 3′ end of the Long Terminal Repeat of the Rous Sarcoma Virus.[8] Plasmid CMV-βGAL contains the β-galactosidase (βGAL) gene linked to the 3′ end of the CMV promoter in pUC19.[9] Bluescript KS(+) was purchased from Stratagene (La Jolla, CA, USA).

The CAT gene, small *t* intron, and polyadenylation signals were excised from pRSV-CAT and subcloned into Bluescript KS(+). The resulting plasmid was named pKSCAT. To construct pME5.8CAT, DNA from the malic enzyme gene was inserted between the *Eco*RI and *Hind*III endonuclease sites of pKSCAT. Plasmid ME5.8CAT DNA contained 5.8 kb of 5′ flanking DNA plus 31 bp of DNA corresponding to the 5′ untranslated part of malic enzyme mRNA linked to the CAT gene.

Plasmid DNAs containing 5′ deletions were prepared by deleting DNA between convenient restriction sites. The 5′ end of pME4.1CAT DNA corresponds to a Sph I site about 4.1 kb 5′ of the start site for transcription. This construct is the same as pME5.8CAT, except that DNA between −5.8 kb and −4.1 kb has been deleted. The 5′ end of pME0.41CAT DNA corresponds to a PstI site 412 bp upstream of the start site for transcription. This construct is the same as pME5.8CAT, except that DNA between −5.8 kb and −0.41 kb has been deleted.

Use of convenient restriction sites is only one of several methods for constructing 5′, 3′, and internal deletions in the target DNA. A second method uses the polymerase chain reaction and generates a target DNA fragment with unique restriction endonuclease sites at each end. This target fragment and the cloning plasmid are digested with the appropriate enzymes. The fragments are then purified and ligated to one another. A third method is to ligate double-stranded synthetic oligonucleotides containing the sequence of interest into the cloning plasmid. For additional methods and further information on the cloning of plasmids, see Sambrook et al.[10]

In addition to the plasmid DNAs to be tested for regulatory elements of interest, one needs control DNAs that will be co-transfected with the test plasmid. First, plasmid DNAs containing unregulated promoters are used to control for variations in transfection efficiency. Both the test plasmid and the control plasmid are expected to enter the same cells. The ratio of cellular uptake of test DNA to that of the control DNA should be the same in co-transfections of different batches of cells. Differences in the amount of expression from the gene with the unregulated promoter are used to correct for differences in the amount of test DNA that enters the cell and is expressed. Second, reporter genes without promoters are used as controls for background in the assays of reporter gene products. Finally, one needs DNA that contains neither reporter nor promoter to equalize the total mass of DNA added to each plate of cells. If the background is low enough, a promoterless reporter gene will serve this purpose.

Preparation of supercoiled DNA. Transform *Escherichia coli* with plasmid DNA and grow in Luria-Bertani medium. Lyse the resulting bacteria with alkali. Purify supercoiled plasma DNA by two consecutive density-gradient centrifugations. Form the gradient by centrifuging DNA, TE, cesium chloride (1 g/mL), and ethidium bromide (0.8 mg/mL) in a vertical rotor at 175,000*g* for 20 hr. Supercoiled plasmid DNA forms a band in the gradient and can be removed from the gradient.

Extract ethidium bromide from the sample containing the DNA by four extractions with butanol. Remove the cesium chloride from the sample by spin dialysis through a microconcentrator (Amicon, Lexington, MA USA). Resuspend the DNA in sterile H_2O and stored at −20° C. Quantify the DNA concentration by measuring its absorbance at a wavelength of 260 nM.[11] For experiments using nicked plasmid DNA, extract the band corresponding to nicked DNA from the density gradient and prepare samples in the same manner used for the supercoiled DNA. For further information on transformation of bacteria, and isolation and purification of plasmid DNAs, see Sambrook et al.[10,12]

TE buffer, pH 8.0.[13] Tris-HCl (10 mmol/L), pH 8.0, ethylenediaminetetraacetate (1 mmol/L), pH 8.0 (EDTA), sterilized in an autoclave.

Supplies and equipment

Disposable plastic ware includes Eppendorf centrifuge tubes, sterile polystyrene tubes (15 and 50 mL), sterile serological polystyrene pipettes (10 and 25 mL), plastic cell scrapers, pipette tips (200 and 1000 μL), Centricon 30 (Amicon).

Equipment includes high-speed, refrigerated centrifuge with adapters for Eppendorf tubes, ultracentrifuge with a vertical rotor, spectrophotometer (UV and visible), automatic micropipettors, vortex mixer, water bath, scintillation spectrometer, spectrophotometer, incubator/shaker, luminometer, thin layer chromatography (TLC) developing chamber and silica gel TLC plates (250 μmol/L layer). The use of some pieces of this equipment may depend on the method of DNA preparation and specific assays for enzyme, protein and DNA.

Transfection procedure

Two 35 mm plates were used per point to reduce variation between experiments. DNA and TransfectACE concentrations and the DNA to TransfectACE ratio must be optimized for each cell type. An optimal amount of DNA will allow consistent and rapid assays. For chick hepatocytes, we used 30 μg of TransfectACE and 5 μg DNA per 35 mm plate. As little as 3.0 μg total DNA per 30 μg TransfectACE and as much as 40 μg of TransfectACE per 35 mm plate can be used on the chick hepatocytes; higher levels of TransfectACE will cause these cells to detach from the plate. When plasmid DNAs containing alternate promoter sequences are to be tested, all test plasmids are transfected at the same molar concentration. The total mass (μg) of transfected DNA per plate should be constant. Aseptic technique must be maintained throughout the transfection procedure.

The following is the protocol for a typical transfection experiment.

Day 0. Prepare isolated hepatocytes. Embryonated eggs from white Leghorn chickens are incubated in an electric forced-draft incubator. Hepatocytes are isolated from the livers of 19-day-old chick embryos.[14]

Distribute hepatocytes to tissue-culture plates. Isolated hepatocytes are incubated in Waymouth medium MD 705/1 (Gibco/BRL) containing penicillin (60 μg/mL), streptomycin (100 μg/mL), insulin (50 nmol/L) (Eli Lilly and Co., Indianapolis, IN USA) and corticosterone (1 μmol/L) on 35 mm plastic tissue-culture dishes at 40° C in an atmosphere of 5% CO_2 in air. Each plate contains 1.5–1.8 × 10^6 cells and 2 mL of medium. The hepatocytes are allowed to attach to the dishes for about 20 hr before any further manipulations.

Day 1. Prepare transfection medium. For each 35 mm plate, we add 30 μg TransfectACE and a total of 5 μg plasmid DNA per 2 mL medium. The amount of each plasmid DNA in a typical experiment is 0.5 μg pCMV-βGAL, 2.5 μg pME5.8CAT, and 1.5 μg pBluescript KS(+). If an equimolar amount of another test DNA is more or less than 2.5 μg, we adjust the added amount of pBluescript KS(+) DNA. Amounts of DNA for both control and test plasmids should be kept as low as possible to minimize competition for rate-limiting transcription factors. Dilute the liposome reagent and DNA separately in 1 ml each of medium. Use polystyrene tubes (15 mL) because the liposome/DNA complexes may stick to polypropylene. Mix each tube gently by 10 inversions. Where possible, dilute the DNA and TransfectACE in batches. This reduces variability between plates, assuming accurate volumetric addition of reagents and media. Add the diluted TransfectACE mixture to the DNA mixture and mix by 10 inversions. Then, allow the DNA/liposome complexes to form for 10 min at room temperature. Aspirate the initial plating medium and then pipette 2 mL of medium containing the DNA/TransfectACE mixture onto the cells. Use polystyrene pipettes. Incubate the liposome-DNA complex with cells for 24 hr. (The incubation time for optimum transfection may vary with cell type.)

Day 2. Remove the medium containing the DNA/liposome mixture and replace it with medium containing appropriate hormones or agents.

Day 4. Harvest the cells 48 hr after addition of hormones or agents. Wash the cells twice with 1 mL PBS. Add 1 mL of PBS to the plate and scrape the cells into this solution with a plastic cell scraper. Transfer the PBS and cells from each plate into a 1.5 mL Eppendorf tube. If harvesting two plates per point, use 0.5 mL of PBS per plate and combine both plates into one Eppendorf tube. Keep the harvested cells and soluble protein on ice at all times. Centrifuge the cells for 5 min at 4° C at 135*g*. Carefully aspirate the PBS and replace it with homogenization buffer (200 μL per plate). At this point the samples can be frozen at −70° C and stored until analyzed.

Lyse cells with three cycles of freezing on dry ice and thawing at 37° C. Mix the thawed samples on a vortex mixer after each cycle. Minimize the time that samples

are at 37° C because the products of some reporter genes are not stable for extended periods at that temperature. All samples should be treated as consistently as possible.

Centrifuge the cell lysate at 27,000*g* for 10 min in a fixed angle rotor to sediment nuclei and membranes. Keep the cell pellet if cellular DNA will be measured.[15] The supernatant solution contains soluble protein and will be subjected to various assays.

Distribute samples of each extract into several tubes to be assayed at a later time. Typically, we apportion 5–10 μL for protein, 30–40 μL for βGAL, and 150 μL for CAT assays. Save the remaining extract for re-assaying as necessary. These amounts of extract can be adjusted to meet the requirements of the particular experiment.

Freeze samples at −70° C until assayed. Process samples as rapidly as possible before the −70° C step.

Analysis of samples

Protein is analyzed according to Bradford.[16]

β-galactosidase activity is measured according to Sambrook et al.[17] Mix 30 μL cell extract, 3 μL 100X Mg solution, 200 μL $NaPO_4$ buffer, and 66 μL ONPG. Incubate the reaction mixture at 37° C for 30 min or until a yellow color develops. Stop the reaction by adding 0.5 mL of Na_2CO_3 and place the samples on ice. Read the optical density of the samples at a wavelength of 420 nm. Include a sample without cell extract as a blank, and a sample with extract from untransfected cells as a negative control. βGAL activity is expressed as units of optical density or OD_{420} per μg protein.

CAT activity is determined according to a modification of Gorman et al.[8] Heat a portion of the cell extract at 60° C for 30 min to inactivate inhibitory activities that interfere with the assay. This step should be optimized for each type of cell extract. Centrifuge at 12,000*g* for 10 min. Transfer the supernatant solution to an Eppendorf tube for storage. Discard the precipitate. Transfer a predetermined amount of the heat-treated extract to a clean tube for analysis. Add CAT homogenization buffer to the tube. The total volume should be 139 μL. Mix 10 μL acetyl-CoA solution with 1 μL ^{14}C-chloramphenicol (0.1 μCi) for each sample, and add to the cell extract. Incubate at 37° C for 15 hr. Shorter incubation times may be used if all samples have very high activities. Add 1 mL ethyl acetate to each tube and mix for 30 sec. Centrifuge the sample for 30 sec at 600*g* to separate the layers. Transfer the ethyl acetate phase to a clean tube. Discard the aqueous phase. Evaporate to dryness under vacuum. Redissolve the sample in 30 μL of ethyl acetate. Mix the tube for 60 sec and centrifuge for 30 sec at 600*g*. Buy or prepare thin layer chromatography (TLC) plates with 250 micron layers of silica gel. Apply the samples to the origin, and allow them to evaporate to dryness. Prepare a chamber for developing the TLC plates. It should contain 200 mL of chloroform:methanol (95:5). Place the TLC plate in the chamber, and allow the solvent front to move approximately 80% of the distance to the top of the plate. Remove the TLC plate from the chamber and allow to dry at room temperature (10 min). Expose the TLC plate to X-ray film (Kodak X-OMAT AR, Eastman Kodak Co., Rochester, NY, USA). Develop the film, and align it with the TLC plate. Three spots usually appear in each lane. The spot nearest the origin is the substrate, ^{14}C-chloramphenicol. The more rapidly migrating spots are 3- and 1-acetyl chloramphenicol. Occasionally a fourth spot corresponding to diacetyl chloramphenicol is observed close to the solvent front. Presence of diacetyl chloramphenicol indicates the reaction is beyond the linear range of the assay. To quantify the CAT activity, cut or scrape the radioactive spots from the TLC plates and measure the amount of radioactivity in a liquid scintillation spectrometer. CAT activity is calculated as a ratio of radioactivity in acetylated chloramphenicol to total radioactivity in all forms of chloramphenicol per μg of nonheat-treated protein. CAT activity is corrected for transfection efficiency by normalization to βGAL activity. The final value is expressed as (CAT activity)/(βGAL activity).

Chick-embryo hepatocytes contain low levels of activities that acetylate chloramphenicol or hydrolyse βGAL, so every transfection experiment includes a set of untransfected plates or plates transfected with the promoterless pKSCAT DNA that are analyzed with the experimental samples. These background CAT or βGAL activities are subtracted from the respective enzyme activities of each of the experimental samples.

We used longer assay times and higher concentrations of acetyl-CoA than those described by Gorman et al.[8] The malic enzyme promoter does not generate high levels of CAT activity, especially in cells incubated without triiodothyronine or when using test DNAs that do not contain a triiodothyronine response element. Increasing the length of the incubation time increases sensitivity of the assay. Acetyl-CoA levels are increased to assure adequate substrate during the entire assay.

Table 1 Effect of nicked DNA on gene expression

DNA	βGAL activity (OD420/μg protein)	CAT activity (% conversion/μg protein)	Normalized CAT activity
pRSVCAT supercoiled pCMVβGAL supercoiled	0.180	12.7	70.5
pRSVCAT nicked pCMVβGAL supercoiled	0.020	1.4	70.0

Plasmid RSVCAT (0.5 μg), pCMVβGAL (0.5 μg), and pBluescript KS(+) (4.0 μg) were added to each plate. In two plates, the pRSVCAT DNA was supercoiled, while in the other two plates the pRSVCAT DNA was nicked. βGAL DNA was supercoiled in all plates. The cells were transfected and harvested in the normal manner except that each plate was analyzed separately. Protein, βGAL and CAT activities were determined as described. Data presented is the average of two plates.

Several alternative methods for CAT assays have been described.[18-20] There are also alternative reporter genes. They include human growth hormone and luciferase, both of which are available commercially. Growth hormone is secreted into the medium, and its production can be monitored by radioimmunoassay without killing the cells.[21] Luciferase is a rapid, simple, and sensitive enzymatic assay. Vertebrate cells lack endogenous luciferase activity.[22]

Sources of variation

A major source of variation in uncorrected CAT activity is differences in transfection efficiency between experiments.[23] This may be due to variation in the quality or purity of different DNA preparations, in handling of the DNA/liposome mixture during transfection, in different batches of liposome reagent, or in different preparations of cells. The use of a second plasmid DNA that contains an unregulated promoter and a different reporter gene allows us to correct CAT activity for differences in transfection efficiency. In our experiments we expressed CAT activity relative to βGAL activity.

Nicking of the DNA or contamination of DNA by ethidium bromide, RNA, or protein affects the quality or purity of the DNA and may interfere with transfection efficiency. For example, the use of nicked plasmid DNA decreased both βGAL and CAT activities (*Table 1*). The activities of both reporter genes were decreased when either of the plasmid DNAs was nicked (data not shown for nicked pCMV-βGAL DNA). When CAT activity was corrected for transfection efficiency, however, CAT activity per unit of βGAL activity was the same in the two sets of transfections. These and other experiments demonstrate the importance of correcting CAT activity from a test promoter for differences in transfection efficiency by co-transfection with a reporter gene linked to an unregulated promoter.

Even though correcting for differences in transfection efficiency decreases the variability within an experiment, there may still be differences in the amount of expression of the reporter genes between experiments. Differences between experiments are eliminated by normalizing the corrected CAT activities. In our experiments we set the activity for one plasmid DNA (the control) under one hormonal condition at 100 (pME5.8CAT in the presence of insulin and triiodothyronine). For each experiment, activities of the mutant DNAs with and without test agents are expressed relative to the control DNA.

In the experiments shown in *Table 1*, the use of nicked DNA substantially lowered both enzyme activities. Despite the lowered activity, it was possible to obtain valid values for corrected CAT activity because both genes have strong promoters. If weaker promoters were used, the presence of nicked DNA would have eliminated detectable promoter activity. Thus, use of nicked DNA may not be especially important if the promoter/regulatory DNAs drive high levels of expression of the reporter genes. If the transgenes have low promoter activities, however, achieving maximal levels of expression by using supercoiled DNA may be very important. In general, the use of nicked DNA should be avoided because there is evidence that covalently closed circular DNA may be required for hormone responsiveness.[24]

Despite corrections for transfection efficiency and normalization within individual experiments, differences in different preparations of plasmid DNA may lead to altered total activities or altered responses to a hormone or agent. In addition to using highly purified supercoiled DNA, this source of uncertainty can be reduced by assaying the function of several different preparations of each test DNA. Finally, variability between

different investigators in the same laboratory should be minimized by having everyone adopt a set of standard procedures for both transfection and enzyme assays.

Choice of transfecting agent and application to other systems

Lipofection was chosen for our transfection experiments because it is easy, relatively non-toxic to hepatocytes, and does not interfere with regulation of expression of endogenous malic enzyme. DEAE-dextran-mediated transfection[25,26] also is feasible, but it is more cumbersome. Calcium phosphate precipitation is an inexpensive and widely used procedure for introducing DNA into cells[27,28] that we use routinely and effectively for chick-embryo fibroblasts.[29] In our laboratory, however, calcium phosphate has not been effective in promoting the efficient uptake and expression of plasmid DNA into chick-embryo hepatocytes (Wilson, S. B. and Goodridge, A.G., unpublished results). These experiments were done using an alternative hepatocyte isolation procedure.[3] We have not attempted to use calcium phosphate-mediated transfection with the current isolation procedure. Other laboratories used calcium phosphate as a transfection method with rat hepatocytes[30] and chick-embryo hepatocytes.[31] Electroporation is another commonly used method for introducing DNA into cells in culture.[32] We have used this method to introduce DNA into the rat liver cell-line, ARL1 (Potts, B., Savaiano, D., and Goodridge, A. G., unpublished results). For this method, cells must be removed from the tissue-culture plates and suspended in medium during electroporation. The monolayer formed by primary hepatocytes in serum-free medium is more easily disturbed and more difficult to reestablish than that of cells adapted to permanent culture conditions. As a consequence, we have not tried to use electroporation to introduce DNA into chick-embryo hepatocytes in culture.

Lipofection can be used with a range of different cell-types in culture.[4,5] Our laboratory has used it successfully with chick-embryo fibroblasts, for example (Hillgartner, F. B. and Goodridge, A.G., unpublished results). In cell-types other than hepatocytes, serum may be required for growth and/or viability; it should be omitted while the liposome reagent is present because serum inhibits both formation of the DNA/liposome complex and the transfection process.[33] This was not a problem for our experiments because we used serum-free, chemically defined medium. Despite the fact that lipofection can be used for many different types of cells in culture, a less expensive method such as calcium phosphate precipitation or electroporation may be a better choice if preliminary investigation indicates that such a method stimulates uptake of DNA into the cell type of interest.

Representative results

Triiodothyronine stimulates and medium-chain fatty acids, such as hexanoate, inhibit expression of malic enzyme in chick-embryo hepatocytes in culture. Triiodothyronine stimulates and hexanoate inhibits both accumulation of malic enzyme mRNA and transcription of the malic enzyme gene, indicating that regulation is predominantly transcriptional.[2,3] We are using the methodology described in this paper to identify *cis*-acting sequences in the malic enzyme gene that confer responsiveness to triiodothyronine and hexanoate.

Table 2 contains examples of results obtained from this kind of analysis. In these experiments, we tested the effects of triiodothyronine and hexanoate on CAT activity in transfections with pME5.8CAT and two 5′ deletions. For each test DNA, βGAL activities varied from experiment to experiment, suggesting differences in transfection efficiency. Nevertheless, when CAT activity was corrected for transfection efficiency by expressing it per unit of βGAL activity, triiodothyronine and hexanoate regulated expression of CAT activity in each experiment with pME5.8CAT and pME4.1CAT. Triiodothyronine stimulated and hexanoate inhibited uncorrected CAT activity, but neither agent affected βGAL activity. When CAT activity in the presence of insulin plus triiodothryonine was normalized to 100, the degree of stimulation of promoter activity by triiodothyronine or inhibition by hexanoate was similar in each experiment.

When hepatocytes were transfected with pME0.41CAT, triiodothyronine did not stimulate and hexanoate did not inhibit normalized CAT activity. We conclude that the 5′ flanking DNA contains sequence elements that confer responsiveness to triiodothyronine and hexanoate on the malic enzyme gene. These elements are located between −4.1 kb and −0.41 kb

Table 2 Effect of T3 and hexanoate on the malic enzyme promoter

DNA	Additions	CAT Activity (% Conversion/ μg Protein)	βGAL Activity (OD/μg Protein)	CAT Activity / βGAL Activity	Normalized Activity
pME5.8CAT	None	0.0427	0.0452	0.945	6.76
		0.1407	0.0183	7.68	8.78
	T3	0.2841	0.0203	14.0	100
		1.417	0.0162	83.7	100
	T3, C6	0.0573	0.0190	2.58	18.5
		1.045	0.0316	33.0	37.8
pME4.1CAT	None	0.0027	0.0026	1.02	7.28
		0.0447	0.0100	4.47	5.12
	T3	0.0326	0.0021	15.7	112
		1.651	0.0089	186	213
	T3, C6	0.0067	0.0041	1.66	11.9
		0.6863	0.0092	75.0	85.9
pME0.41CAT	None	0.0186	0.0055	2.38	24.1
		0.1272	0.0071	18.0	20.6
	T3	0.0044	0.0029	1.51	10.8
		0.4440	0.0094	47.5	54.4
	T3, C6	0.0213	0.0074	2.86	20.4
		0.3824	0.0123	31.1	35.6

Chick-embryo hepatocytes were transfected with pME5.8CAT, pME4.1CAT, or pME0.41CAT DNA as described in the text. The cells were treated with insulin (50 nmol/L) and corticosterone (1 μmol/L) for the entire incubation period. Triiodothyronine (1.6 μmol/L) was added for the final 48 hr in culture. The cells were harvested, and two plates combined for each data point and assayed for protein, βGAL, and CAT activities. For the normalized activities, corrected CAT activities for pME5.8CAT in the presence of triiodothyronine were set at 100. Two experiments are presented. T3, triiodothyronine; C6, hexanoate.

upstream from the start site for transcription. We are completing more extensive analyses of the *cis*-acting elements that confer responsiveness of the malic enzyme gene to triiodothyronine and hexanoate. Our laboratory also is characterizing inhibitory cyclic AMP- and tissue-specific response elements in the 5′-flanking DNA of the malic enzyme gene. Results of these analyses will be reported in future papers.

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