Cyclic AMP-mediated Inhibition of Transcription of the Malic Enzyme Gene in Chick Embryo Hepatocytes in Culture

CHARACTERIZATION OF A CIS-ACTING ELEMENT FAR UPSTREAM OF THE PROMOTER*

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Glucagon, acting via cAMP, inhibits transcription of the malic enzyme gene in chick embryo hepatocytes. In transiently transfected hepatocytes, fragments from the 5′-flanking DNA of the malic enzyme gene confer cAMP responsiveness to linked reporter genes. The major inhibitory cAMP response element at −3180 to −3174 base pairs (bp) is similar to the consensus binding site for AP1. DNA fragments from −3134 to −3115, −1713 to −944, and −413 to −147 bp also contain inhibitory cAMP response elements. The negative action of cAMP is mimicked by overexpression of the catalytic subunit of protein kinase A, inhibited by overexpression of a specific inhibitor of protein kinase A, and inhibited by overexpression of the T3 receptor; these results indicate involvement of the classical eukaryotic pathway for cAMP action and suggest interaction between the T3 and cAMP pathways. Sequence-specific complexes form between nuclear proteins and a DNA fragment containing −3192 to −3158 bp of 5′-flanking DNA. In nuclear extracts prepared from cells treated with chlorophenylthiocyclic AMP and T3, the complexes have different masses than those formed with extracts from cells treated with T3 alone. Antibodies to c-Fos or ATF-2 inhibit formation of the complex formed by proteins from cells treated with chlorophenylthiocyclic AMP and T3 but not by those from cells treated with T3 alone. These results suggest an important role for c-Fos and ATF-2 in glucagon-mediated inhibition of transcription of the malic enzyme gene.

Malic enzyme (ME) (EC 1.1.1.40) catalyzes the oxidative decarboxylation of malate to pyruvate and CO2, simultaneously generating NADPH from NADP+. In avian liver, most of the NADPH used in the de novo synthesis of long chain fatty acids is generated by malic enzyme (1). Malic enzyme is a typical lipogenic enzyme; its activity in avian liver increases about 70-fold when newly hatched chicks are fed a diet high in carbohydrate (1) and decreases dramatically when animals are starved (2). In chicken embryo hepatocytes in culture, insulin plus T3 causes a 50-fold increase in malic enzyme activity and abundance of its mRNA; glucagon or cAMP blocks these effects (3, 4). Within 1 h after adding T3 to chick embryo hepatocytes, transcription of the malic enzyme gene increases by 30 to 40-fold; cAMP completely inhibits this increase (4). The T3-dependent increase in transcription of the malic enzyme gene is mediated by several T3 response elements (T3REs), with the major one between −3883 and −3858 bp upstream of the start site for transcription (5).

Several positive acting cAMP response elements have been described (6). Cyclic AMP stimulates gene expression by activating protein kinase A (PKA), which, in turn, phosphorylates members of the CREB/ATF family of transcription factors, thereby increasing their transactivation potential (7). The CREB/ATF family includes polypeptides encoded by at least seven distinct genes (6) that share the ability to bind, with different affinities, to CREs in the 5′-flanking DNA of genes activated by cAMP. The CREB/ATF proteins also dimerize with AP1 proteins, members of another family of leucine zipper proteins (8) for which the binding site differs from a consensus CRE by only one bp (9).

Much less is known about negative-acting cAMP-response elements. In the gene for L-type pyruvate kinase, the L4 element, −168 to −144 bp, binds major late transcription factor; it is the glucose/insulin response element and is required for inhibition by cAMP. Inhibition by cAMP also requires the contiguous L3 element, an element that binds hepatic nuclear factor 4 (10). Cyclic AMP also inhibits transcription of the genes for IL-2 and IL-2R in EL4 cells; the inhibition requires an AP1 site. In this case, cAMP increases the binding of Jun/Fos heterodimers to the AP1 site and alters the composition of Jun proteins that participate in the AP1 complex (11). A third example of inhibition of transcription by cAMP involves the hepatic gene for fatty acid synthase; insulin-induced transcription of this gene is inhibited by cAMP (12). The cis-acting element required for the inhibitory effect is an inverted CAAT box (13). The proteins that bind to this element have not been identified.

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C. M. contributed results for Figs. 4 and 6–10, statistically analyzed the transfection results, and wrote the manuscript. W. C. contributed the results for Figs. 1–3 and 5 and several experiments whose results are not shown. S. A. K. constructed the vectors in Fig. 1, set up the transfection system in our laboratory, and performed preliminary experiments for Figs. 1 and 3. A. G. G. supervised the planning, execution, and interpretation of the experiments and the writing of the paper.

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The abbreviations used are: ME, malic enzyme; ATF, activating transcription factor; CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; CREB, cAMP response element binding protein; CBF, CRE-binding protein; CREM, cAMP response element modulator; CPT-cAMP, chlorophenylthiocyclic AMP; ICRE, inhibitory cAMP response element; PKA, protein kinase A; PKI, inhibitor of protein kinase A; RSV, Rous sarcoma virus; TR, thyroid hormone receptor; T3, 3,5,3′-triiodothyronine; TRβ, triiodothyronine response element; TK, thymidine kinase; bp, base pair(s); kb, kilobase pair(s); IL-2, interleukin-2; IL-2R, IL-2 receptor.
In this study, we have found that the 5’-flanking DNA of the gene for malic enzyme contains at least four cis-acting DNA sequences that are involved in responsiveness to the inhibitory action of cAMP. We have examined the function of the probable major inhibitory element and identified some of the nuclear proteins that bind to it.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim. Other enzymes were obtained from the indicated sources: TqQ DNA polymerase (Perkin-Elmer), T4 DNA ligase (Pharmacia LKB Biotechnology Inc.), Escherichia coli DNA polymerase I and calf intestinal phosphatase (Boehringer Mannheim). CPT-cAMP, 3,5'-3'-triiodothyronine, and cortisol were purchased from Sigma. Crystalline bovine insulin was a gift from Lilly. LipofectAce™ and Waymouth medium MD 705/1 were obtained from Life Technologies, Inc. [α-32P]dCTP (800 Ci/mmol) was purchased from Amersham Corp., and α-three-[3H]chloroacetate 1–2-14C]chloramphenicol was from NEN Life Science Products. β-Luciferin, potassium salt, was obtained from Analytical Luminescence Laboratory (San Diego, CA). Antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); all were raised against human protein for which the antigen was chicken in origin. All other chemicals were of reagent grade or of the highest purity commercially available.

Plasmid Constructions—Plasmid RSV-PKAc and pRSV-PKI were from R. A. Maurer (Oregon Health Sciences University) (14). Plasmid RSV-TRα was provided by H. H. Samuels (New York University) (15). The luciferase reporter plasmid, pXP1, was from S. K. Nordeen (University of Colorado Health Sciences Center). Plasmid RSV-LUC was constructed as described previously (5). B. Luckow and G. Schutz (Heidelberg, Germany) provided pBlCAT2 (Ref. 17; pTKCAT). Plasmid KSCAT (promoterless plasmid), p[ME−5800]/+31CAT, and the 5’-deletions thereof were constructed as described previously (5). For the TK constructs, we inserted various fragments of ME 5’-flanking DNA into the multiple cloning site 5’ of the HSV TK promoter in pBlCAT2 (Ref. 17; pTKCAT).

Plasmid [ME-T3RE2]TKCAT was constructed by inserting a 36-bp oligonucleotide containing T3RE2 (major T3RE) of the malic enzyme gene (5) into the NdeI and HindIII sites of pTKCAT. To make p[ME-T3RE2]−1713/−944TKCAT and p[ME-T3RE2]−413/−147TKCAT, we first amplified the −1713 to −944 and −413 to −147 fragments by polymerase chain reaction using oligonucleotides containing BamHI sites and then subcloned them into pTKCAT and BamHI site of p[ME-T3RE2]TKCAT. For p[ME−3474/−2715]TKCAT, we isolated the −3474 to −2715 fragment from p[ME−5800]/+31CAT by digesting with BglII. After blunt ending with T4 DNA polymerase, the resulting mixture was digested with HindIII. The resulting 758-bp fragment was subcloned into pTKCAT that had been digested by BamHI, blunt-ended with T4 DNA polymerase, and digested by HindIII.

In all cases, the inserts were confirmed by nucleotide sequence analysis using the Sequenase DNA sequencing kit (version 2.0, U.S. Biochemical Corp.). The magnitude of the cAMP effect is similar to that for the 6 μg of protein was mixed with 12 μl of binding buffer containing 20,000 cpm of 32P-labeled probe, 2 μg of poly(dI-dC), 0.01% Nonidet P-40, 0.8 μg of bovine serum albumin, 5% (v/v) glycerol, and 5 μg of salmon sperm DNA with or without a 100-fold molar excess of competitor oligonucleotide. The reaction was incubated for 15 min at room temperature. Antibody experiments used the same incubation conditions except that 1 μl of IgG (1 μg) was incubated with the reaction mixture for an additional 15 min at room temperature. The reaction mixture was then subjected to electrophoresis on 5% polyacrylamide gels at 150 V in 25 mM Tris-HCl, 0.19 mM glycine, 1 mM EDTA at 4 °C (24). Gels were dried and subjected to autoradiography.

RESULTS

Cis-acting Elements that Confer Responsiveness to cAMP Are Located within the 5.8 kb of DNA Upstream of the Start Site of the Malic Enzyme Gene—When chick embryo hepatocytes were transfected with 5.8 kb of 5’-flanking DNA of the malic enzyme gene linked to the CAT gene (p[ME−5800]/+31CAT), CAT activity was increased more than 40-fold by T3 and inhibited by 96% by CPT-cAMP (Fig. 1). CPT-cAMP did not have a statistically significant effect on luciferase activity in cells transfected with RSV-LUC. Thus, 5.8 kb of 5’-flanking DNA contains both positive acting T3REs, as previously reported (5), and one or more inhibitory cAMP response elements (ICREs). The magnitude of the cAMP effect is similar to that for the luciferase reporter construct. cAMP may be located in this fragment of DNA.

We next tested a series of 5’-deletions of p[ME−5800]/+31CAT to localize the ICRE(s) (Fig. 1). Deletion of the DNA from −5800 to −3845 bp caused an increase in responsiveness to cAMP. This deletion removes part of the T3 response region (5) and may contain sequences that dampen the ability of cAMP to inhibit transcription. When the DNA from −3845 to
### Effects of T3 and cAMP on cells transfected with constructs containing different 5′-deletions of the 5.8 kb of 5′-flanking DNA of the malic enzyme gene linked to CAT.

| Deletion | Relative CAT activity | Fold change | Percentage of ICT3 |
|----------|-----------------------|-------------|--------------------|
|           | IC | ICT3 | ICT3A | ICT3/IC | ICT3A/ICT3 X 100 | n |
| -5800    | 3.9 ± 1.1 | 110 ± 10 | 4.0 ± 0.7 | 40 ± 8 | 40.2 ± 1.1 | 6 |
| -4135    | 4.2 ± 1.5 | 100 | 4.2 ± 0.8 | 47 ± 10 | 4.2 ± 0.8 | 9 |
| -3845    | 16 ± 3 | 87 ± 4 | 1.3 ± 0.2 | 6.5 ± 1.1 | 1.5 ± 0.3 | 6 |
| -3474    | 30 ± 8 | 58 ± 10 | 0.6 ± 0.2 | 2.2 ± 0.6 | 0.9 ± 0.2 | 6 |
| -2715    | 9 ± 3 | 3.1 ± 0.4 | 0.2 ± 0.02 | 0.6 ± 0.3 | 7.8 ± 1.6 | 6 |
| -944     | 2.0 ± 0.4 | 1.5 ± 0.1 | 0.5 ± 0.04 | 0.8 ± 0.1 | 36 ± 0.6 | 6 |
| -413     | 1.2 ± 0.3 | 0.9 ± 0.1 | 0.5 ± 0.07 | 0.9 ± 0.2 | 55 ± 13 | 6 |

**Note:** The degree of inhibition by cAMP was much greater when pME−3474/+31CAT were transfected with construct that expresses a specific inhibitor of the catalytic subunit (PKi or Walsh inhibitor) (25). When 1 or 2 μg of pRSV-PKi was cotransfected with pME−5800/+31CAT, inhibition by exogenously added cAMP was completely blocked; in fact, activity was higher than that in the absence of cAMP. In addition, overexpression of pRSV-PKi in the absence of cAMP, with or without T3, stimulated CAT activity. This suggests that these cells contain a significant level of free catalytic subunit of PKA in the absence of added cAMP. We conclude that the negative effect of cyclic AMP on transcription of the malic enzyme gene is mediated by the classical eukaryotic signaling pathway that involves PKA-mediated phosphorylation of target proteins. T3 and cAMP Can Function Independently but Have Interacting Effects on Promoter Activity—In the absence of T3, cyclic AMP caused 53% and 75% decreases in CAT activity in cells transfected by p[ME−5800/+31CAT]. This result is consistent with the inhibition of promoter activity caused by overexpression of the free catalytic subunit of PKA and suggests that cAMP-mediated inhibition of transcription of the malic enzyme gene does not inhibit TR function per se.

The degree of inhibition by cAMP was much greater when promoter activity of the malic enzyme gene was induced by T3 than in the absence of T3 (Fig. 3); this may be due to greater sensitivity to cAMP in T3-treated cells. Alternatively, the lower cAMP responsiveness of the malic enzyme gene in cells that were not treated with T3 may be due to a combination of the lower level of promoter activity in the absence of T3 and a
The constitutive basal level of activity that is independent of T3 or cAMP.

The conclusion that cAMP functions independently of T3 is supported by the results of a series of experiments in which different artificial and natural T3REs were linked to TKCAT and tested for responsiveness to T3 and cAMP. One construct contained five copies of a consensus T3RE (5'-AGGT-CANNNAGGTCA-3') linked to TKCAT (26); a second construct contained a palindromic T3RE linked to TKCAT (27). Hepatocytes transfected with each of these constructs gave robust responses to T3 but did not respond to cAMP (results not shown). Similarly, cells transfected with T3RE2 of the malic enzyme promoter were not responsive to cAMP. However, when the T3RE was replaced with a GAGA box, cAMP-responsive cells were obtained.

FIG. 2. The effect of overexpression of the catalytic subunit of PKA or the peptide inhibitor of PKA on T3 responsiveness. Chick embryo hepatocytes were isolated and incubated in culture as described in the legend to Fig. 1 and under "Experimental Procedures." Cells were transiently transfected using lipofectACE TM (40 μg/plate). The experimental groups were p[ME-5800/+31]CAT (5.8CAT) with no additional expression plasmid or p[ME-5800/+31]CAT with pRSV-PKAc (A) or pRSV-PKi (B). Corrected CAT activities were calculated as described in the legend to Fig. 1. Relative CAT activities were then calculated by setting the corrected CAT activity for T3-treated hepatocytes transfected with p[ME-5800/+31]CAT to 100 and adjusting all other activities proportionately. The results are the means ± S.E. of four experiments (n), each one using an independent batch of hepatocytes. Two independently prepared batches of each plasmid were used. A, CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME-5800/+31]CAT were 4.7 ± 1.3% (mean ± S.E., n = 4) conversion/15 h/mg of protein and 4.8 ± 1.6 × 10^6 (mean ± S.E., n = 4) light units/mg of protein, respectively. B, CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME-5800/+31]CAT were 3.6 ± 0.6% (mean ± S.E., n = 4) conversion/15 h/mg of protein and 0.5 ± 0.1 × 10^6 (mean ± S.E., n = 4) light units/mg of protein, respectively.

FIG. 3. Effect of cAMP on malic enzyme promoter activity in the absence of T3. Chick embryo hepatocytes were isolated, incubated in culture, and transfected as described in the legend to Fig. 1 and under "Experimental Procedures." Columns 1–4, CAT activity normalized by luciferase activity. The results were expressed as the percentage of [14C]chloramphenicol converted to acetylated chloramphenicol per mg of soluble protein and then corrected for differences in transfection efficiency by dividing by luciferase activity of the same extract. Cells transfected with p[ME-5800/+31]CAT and p[ME-3474/+31]CAT were treated with insulin and corticosterone alone or those hormones plus CPT-cAMP, T3, or CPT-cAMP plus T3. Column 5, normalized CAT activity in cells incubated with insulin, corticosterone, and T3 divided by that in control cells incubated with insulin and corticosterone. Column 6, normalized CAT activity in cells incubated with insulin, corticosterone, and CPT-cAMP divided by that in cells incubated with insulin and corticosterone, multiplied by 100. Column 7, normalized CAT activity in cells incubated with insulin, corticosterone, T3, and CPT-cAMP divided by that in cells incubated with insulin, corticosterone, and T3, multiplied by 100. Column 8, number of experiments. The results are the means ± S.E. of five or eight experiments. CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME-5800/+31]CAT were 4.2 ± 0.6% (mean ± S.E., n = 8) conversion/15 h/mg of protein and 9 ± 2.4 × 10^6 (mean ± S.E., n = 8) light units/mg of protein, respectively. Abbreviations are as defined in the legend to Fig. 1.
cAMP-mediated Inhibition of Transcription of the ME Gene

| Relative CAT activity | Fold change | Percentage of ICT3 |
|----------------------|-------------|-------------------|
| IC | ICT3 | ICT3A | ICT3/IC | ICT3A/ICT3 |
| 3474 | -2715 | TKCAT | 172.1 ± 6 | 100 | 2.9 ± 0.4 | 6.6 ± 0.5^a | 2.02 ± 0.4^a | 10 |
| -3260 | -2715 | TKCAT | 122.0 ± 2 | 130 ± 19 | 2.71 ± 0.9 | 12.2 ± 0.9 | 7.2 ± 0.8^b | 78.2 ± 0.8^c | 6 |
| -3474 | -2715 | TKCAT | 2.9 ± 0.6 | 10 ± 7 | 6.2 ± 1.4 | 7.2 ± 0.9^b | 7.2 ± 0.8^b | 78.2 ± 0.8^c | 6 |
| -3259 | -3115 | TKCAT | 1.1 ± 0.4 | 2.2 ± 1.3 | 1.0 ± 0.4 | 2.1 ± 0.8 | 7.2 ± 0.9^b | 78.2 ± 0.8^c | 6 |
| -3474 | -3114 | TKCAT | 66.6 ± 0.2 | 450 ± 70 | 6.1 ± 2.0 | 7.7 ± 0.9^b | 1.4 ± 0.0^c | 7 |
| -3474 | -2930 | TKCAT | 2.7 ± 0.6 | 28 ± 0.6 | 4.0 ± 0.0 | 1.3 ± 0.2 | 200 ± 0.2 | 10 |
| -2929 | TKCAT | 2.7 ± 0.6 | 28 ± 0.6 | 4.0 ± 0.0 | 1.3 ± 0.2 | 200 ± 0.2 | 10 |

**A**

**B**

**Fig. 4. Effects of T3 and cAMP on cells transfected with constructs containing fragments of three different regions of malic enzyme 5′-flanking DNA linked to TKCAT.** Chick embryo hepatocytes were isolated, incubated in culture, and transfected as described in the legend to Fig. 1 and under “Experimental Procedures.” Left, constructs used in this experiment. TK, thymidine kinase promoter; T3RE2, major T3 response element located between −3883 and −3858 bp in the 5′-flanking DNA of the malic enzyme gene. Numbers in large type represent the extremities of the tested fragments; numbers in small type represent the ends of internal deletions. Columns 1–3, CAT activity was normalized by luciferase activity. The results were calculated and are presented as described in the legend to Fig. 1. Column 4, relative CAT activity in cells incubated with insulin, corticosterone, and T3 divided by that in control cells incubated with insulin and corticosterone. Columns 5, relative CAT activity in cells incubated with insulin, corticosterone, T3, and CPT-cAMP divided by that in cells incubated with insulin, corticosterone, and T3, multiplied by 100. Column 6, number of experiments. A, the relative CAT activity for T3-treated hepatocytes transfected with p[ME−3474/−2715]TKCAT was fixed at 100, and the other values were adjusted proportionately. The results, calculated as described in the legend to Fig. 1, are the means ± S.E. of 6–10 experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME−3474/−2715]TKCAT were 8.7 ± 2.6% (mean ± S.E., n = 10) conversion/15 h/mg of protein and 7.6 ± 1.9 × 10^6 (mean ± S.E., n = 10) light units/mg of protein, respectively. Statistical significance of comparisons made within a column is as follows: a, p < 0.01 versus TKCAT; b, p < 0.05 versus TKCAT; c, p < 0.02 versus TKCAT. B, the relative CAT activity for T3-treated hepatocytes transfected with p[ME(T3RE2)−1713/−944]TKCAT was fixed to 100, and the other values were adjusted proportionately. The results, calculated as described above, are the means ± S.E. of five or six experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME(T3RE2)−1713/−944]TKCAT were 2.2 ± 0.7% (mean ± S.E., n = 6) conversion/15 h/mg of protein and 12.3 ± 2.5 × 10^6 (mean ± S.E., n = 6) light units/mg of protein, respectively. Statistical significance of comparisons made within a column is as follows: d, p < 0.05 versus p[ME(T3RE2)TKCAT]. Abbreviations are as defined in the legend to Fig. 1.

The enzyme gene linked directly to TKCAT also failed to respond to cAMP (Fig. 4). Thus, a T3 response by itself is not sufficient to make transcription of the malic enzyme gene responsive to cAMP.

Plasmid [ME−5800+/+31]CAT and an expression vector for chicken TRa were cotransfected into hepatocytes. At many T3REs, TR is a repressor in the absence of T3 (15). Overexpression of TRa caused the expected decrease in basal activity (IC-treated cells) and an increase in T3-induced activity (Fig. 5); inhibition by cAMP decreased from ~95 to ~40%.

**Functional Inhibitory cAMP Response Elements: Localization of Several and Identification of One—**Three regions appear to contain ICREs (Fig. 1). DNA fragments containing the putative ICREs were subcloned upstream of the TK promoter in pTKCAT. A 35-bp oligonucleotide containing T3RE2 of the malic enzyme gene (5) was inserted upstream of each of the ICRE-containing fragments that lacked a T3RE. This ensured a high level of promoter activity in T3-treated cells (Fig. 4). When hepatocytes were transfected with p[ME−3474/−2715]TKCAT, CAT activity was normalized by luciferase activity. The results were calculated and are presented as described in the legend to Fig. 1 and under “Experimental Procedures.” TK, thymidine kinase promoter; T3RE2, major T3 response element located between −3883 and −3858 bp in the 5′-flanking DNA of the malic enzyme gene. Numbers in large type represent the extremities of the tested fragments; numbers in small type represent the ends of internal deletions. Columns 1–3, CAT activity was normalized by luciferase activity. The results were calculated and are presented as described in the legend to Fig. 1. Column 4, relative CAT activity in cells incubated with insulin, corticosterone, and T3 divided by that in control cells incubated with insulin and corticosterone. Columns 5, relative CAT activity in cells incubated with insulin, corticosterone, T3, and CPT-cAMP divided by that in cells incubated with insulin, corticosterone, and T3, multiplied by 100. Column 6, number of experiments. A, the relative CAT activity for T3-treated hepatocytes transfected with p[ME−3474/−2715]TKCAT was fixed at 100, and the other values were adjusted proportionately. The results, calculated as described in the legend to Fig. 1, are the means ± S.E. of 6–10 experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME−3474/−2715]TKCAT were 8.7 ± 2.6% (mean ± S.E., n = 10) conversion/15 h/mg of protein and 7.6 ± 1.9 × 10^6 (mean ± S.E., n = 10) light units/mg of protein, respectively. Statistical significance of comparisons made within a column is as follows: a, p < 0.01 versus TKCAT; b, p < 0.05 versus TKCAT; c, p < 0.02 versus TKCAT. B, the relative CAT activity for T3-treated hepatocytes transfected with p[ME(T3RE2)−1713/−944]TKCAT was fixed to 100, and the other values were adjusted proportionately. The results, calculated as described above, are the means ± S.E. of five or six experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME(T3RE2)−1713/−944]TKCAT were 2.2 ± 0.7% (mean ± S.E., n = 6) conversion/15 h/mg of protein and 12.3 ± 2.5 × 10^6 (mean ± S.E., n = 6) light units/mg of protein, respectively. Statistical significance of comparisons made within a column is as follows: d, p < 0.05 versus p[ME(T3RE2)TKCAT]. Abbreviations are as defined in the legend to Fig. 1.

For further localization of the 5′-most ICRE(s), we constructed and tested a series of deletions of p[ME−3474/−2715]TKCAT (Fig. 4A). Deletion of the 5′-end to −3260 bp or the 3′-end to −2929 bp did not affect either T3 or cAMP responsiveness of hepatocytes transfected with these constructs. When the DNA from −3259 to −3115 bp was deleted from the parent plasmid, T3 responsiveness was essentially unchanged, but inhibition by cAMP decreased from 98 to 22%, indicating that an ICRE was located in this DNA fragment. When the DNA from −3114 to −2930 bp was deleted, T3 responsiveness decreased to 2-fold, and inhibition by cAMP decreased to 23%. This result suggests that a T3RE is located in this region. The decrease in responsiveness to cAMP could have been due to 1) the presence of a second ICRE in this region, 2) an ICRE that was deleted by the 5′-most ICRE(s), we constructed and tested a series of deletions of the plasmid, and 3) a decrease in responsiveness to cAMP due to the deletion of other regulatory elements.
CAMP-mediated Inhibition of Transcription of the ME Gene

| Relative CAT activity | Fold change | Percentage of ICT3 |
|-----------------------|-------------|--------------------|
| IC        | ICT3   | ICT5A  | ICTMC  | ICT5AICT3 X 100 | n |
| p[ME-5800+31]CAT     | 2.65±0.06  | 100    | 5.5±0.9 | 43±7       | 5.5±2.09 | 4 |
| p[ME-5800+31]CAT + RSV-TRα | 0.94±0.3  | 180±7  | 110±12  | 350±160    | 59±5    | 4 |

FIG. 5. Effect of overexpression of TRα on CAMP inhibition of T3-induced promoter activity. Chicken embryo hepatocytes were transfected with p[ME-5800+31]CAT with or without pRSV-TRα (0.02 μg/plate) as described in the legends to Figs. 1 and 2. Left, constructs used in this experiment. Columns 1–3, relative CAT activity normalized by luciferase activity. The relative CAT activities for T3-treated hepatocytes transfected with p[ME-5800+31]CAT alone were normalized to 100, and the other values were adjusted proportionally. Columns 4–6, presentation of the results is similar to that described in the legend to Fig. 1. The results are the means ± S.E. of four experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME-5800+31]CAT were 3.5 ± 1.5% (mean ± S.E., n = 4) conversion/15 h/mg of protein and 1.2 ± 0.2 × 10⁶ (mean ± S.E., n = 4) light units/mg of protein, respectively. The abbreviations are as defined in the legend to Fig. 1.

overlaps both of these deletions, or 3) the deletion of the T3RE localized between −3114 and −2930 bp. T3-induced activity in the absence of a T3RE is little different from basal activity and may be too low to permit detection of a larger response to cAMP.

Each of the fragments containing a potential ICRE was subcloned upstream of the TK promoter in pTKCAT and transfected into hepatocytes (Fig. 6). The major T3RE of the malic enzyme gene was inserted upstream of the −3259/−3115-bp fragment. This was not necessary for the −3114/−2930-bp fragment, because it contains a T3RE. In cells transfected with p[ME(T3RE2)]−3259/−3115|TKCAT, CAT activity was strongly suppressed by cAMP (98%). In contrast, when the cells were transfected with p[ME−3114/−2930]|TKCAT, there was no change in CAT activity in response to cAMP despite the fact that CAT activity was stimulated 12-fold by T3. These results indicate that at least one ICRE is localized between −3259 and −3115 bp in the 5′-flanking DNA of the gene for malic enzyme. They also confirm the presence of a T3RE between −3115 bp in the 5′-flanking DNA of the gene for malic enzyme.

To identify proteins that bind to this DNA fragment, we used antibodies (IgG) raised against mammalian forms of several candidate proteins (Fig. 10). AP1 is a Fos/Jun heterodimer. The Fos antibody used in this experiment (k-25) reacts with all members of the Fos gene family: c-Fos, FosB, Fra-1, and Fra-2. The antibody used for Jun is specific for the c-Jun isoform and does not cross-react with JunB or JunD. AP1 also can contain specific complex of lower molecular weight may represent degradations products or complexes missing a component of complexes a or b.

To identify proteins that bind to this DNA fragment, we used antibodies (IgG) raised against mammalian forms of several candidate proteins (Fig. 10). AP1 is a Fos/Jun heterodimer. The Fos antibody used in this experiment (k-25) reacts with all members of the Fos gene family: c-Fos, FosB, Fra-1, and Fra-2. The antibody used for Jun is specific for the c-Jun isoform and does not cross-react with JunB or JunD. AP1 also can contain heterodimers that include nuclear factors of the CREB/ATF family (28, 29). The ATF-1 antibody (25c10g) cross-reacts with ATF-1, CREB, and CREM. The antibody for ATF-2 is specific for ATF-2. We also tested an antibody raised against CREB-binding protein (CBP), a potential CREB co-activator (30). TRα binds to the major T3RE of the malic enzyme gene (5). We tested for TR binding to this element because the T3 and cAMP signaling systems appear to interact (Fig. 4). Formation of complex a with nuclear proteins extracted from T3-treated cells was not inhibited by any of the antibodies (Fig. 10). When the analysis was performed with nuclear proteins from cells treated with T3 plus cAMP, formation of complex b was inhibited completely by IgG raised against c-Fos and ATF-2 but not by any of the other IgGs (Fig. 10). These results suggest that...
**Fig. 6. Effects of T3 and cAMP on cells transfected with constructs containing the −3259- to −3115-bp and −3114- to −2930-bp regions of 5′-flanking DNA of the malic enzyme gene linked to TKCAT.** Chick embryo hepatocytes were isolated, incubated in culture, and transfected as described in the legend to Fig. 1 and under "Experimental Procedures." Left, constructs used in this experiment. A, columns 1–3, the relative CAT activity for T3-treated hepatocytes transfected with p[ME(T3RE2)−3259/−3115]TKCAT was fixed to 100, and the other values were adjusted proportionately. The results, calculated as described in the legend to Fig. 1, are the means ± S.E. of three or six experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME(T3RE2)−3259/−3115]TKCAT were 17.9 ± 2.3% (mean ± S.E., n = 6) conversion/15 h/mg of protein and 16.2 ± 1.5 × 106 (mean ± S.E., n = 6) light units/mg of protein, respectively. Columns 4–6, presentation of the results is similar to that described in the legend to Fig. 1. Statistical significance of comparison made within a column is as follows: a, p < 0.05 versus p[ME(T3RE2)TKCAT. B, columns 1–3, the relative CAT activity for T3-treated hepatocytes transfected with p[ME−3114/−2930]TKCAT was fixed to 100, and the other values were adjusted proportionately. The results, calculated as described in the legend to Fig. 1, are the means ± S.E. of four or six experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[ME−3114/−2930]TKCAT were 6.4 ± 1.6% (mean ± S.E., n = 6) conversion/15 h/mg of protein and 17.6 ± 5.1 × 106 (mean ± S.E., n = 6) light units/mg of protein, respectively. Columns 4–6, presentation of the results is similar to that described in the legend to Fig. 1. Statistical significance of comparison made within a column is as follows: b, p < 0.05 versus p[ME(T3RE2)TKCAT. Abbreviations are as defined in the legend to Fig. 1.

**DISCUSSION**

The chicken malic enzyme gene is expressed primarily in hepatocytes and is subject to regulation by various hormones and nutritional states (2). Agents that increase intracellular levels of cAMP markedly decrease transcription of the gene for malic enzyme in chicken embryo hepatocytes in culture (4); four different regions in the 5′-flanking DNA of the malic enzyme gene appear to be required for the full cAMP inhibition endowed by 5.8 kb of 5′-flanking DNA. In this report, we have focused on a specific, AP1-like site at −3180 to −3174 bp of the malic enzyme gene. What do we know about the other ICREs? The region from −3134 to −3115 bp (63% inhibition by cAMP) contains a CRE-like element (2 mismatches with respect to the consensus element, TGACGTCA) (6) between −3127 and −3120 bp. Both the −1713- to −944-bp and −413- to −147-bp fragments endowed about 75% inhibition by cAMP. Each of these fragments contains one or more AP1 consensus sites (TGAG/G/TCA) (9). The functional roles of these downstream AP1-like sites remains to be determined.

The AP1-like site (TAATCTA) centered at −3177 bp contains one mismatch with respect to a site (TAAGTCA) that is involved in cAMP-mediated inhibition of transcription of the IL-2 and IL-2R genes in EL4 cells (11). The AP1 sites of the IL-2 and IL-2R genes bind Fos/Jun heterodimers; cAMP increases their binding activity and alters the type of Jun protein in the AP1 complex. AP1 consists of a collection of structurally related transcription factors that belong to the Fos and Jun families. These proteins form a variety of homo- and heterodimers and constitute an important group of signal-regulated transcription factors, BZip proteins. The consensus sequence for an AP1 binding site (TGA/G/C/TCA) is similar to that for a CRE consensus sequence (TGA/GTCA). CREs bind other members of the BZip protein family: CREB and ATF (31). Factors that bind to the AP1 site also form heterodimers with the members of the CREB/ATF family (28, 29). Of the various members of these two gene families for which we assayed, only c-Fos and ATF2 were found in the complexes that bound to the AP1-like site implicated in responsiveness to cAMP; they were present in the complex only when nuclear extracts were from cAMP-treated hepatocytes. It has been reported that c-Fos cannot heterodimerize with ATF-2 (31). However, others have reported the formation of a c-Fos/ATF-2 complex in *vitro* but did not characterize its DNA binding site (32).

To our knowledge, we are the first to report the formation of a complex containing c-Fos and ATF-2 in intact cells. This unusual complex may explain why the AP1-like sequence char-
Characterized in this report does not match perfectly with the consensus site for the binding of Jun/Fos heterodimers. The two thymidines and the cytidine in an AP1 site (TGAGTCA) are important for the binding of the Jun/Fos heterodimer (33). In our sequence, these three bases are conserved. However, replacement of the first guanosine with an adenosine (as in our sequence) abolishes the binding of the Jun/Fos complex.

Our results confirm that cAMP inhibits transcription of the malic enzyme gene via the classical PKA signaling pathway. In response to the binding of cAMP to regulatory subunits of PKA, the catalytic subunits are released and catalyze phosphorylation of target proteins (34, 35). In the nucleus, the phosphorylation states of transcription factors and related proteins appear to modulate function and expression of cAMP-responsive genes directly (6). How does the activation of PKA lead to the formation of a c-Fos/ATF-2 complex on the AP1-like site of the malic enzyme gene?

**Fig. 7.** Effects of T3 and cAMP on cells transfected with constructs containing the −3259 to −3115 bp region and deletions thereof of malic enzyme 5′-flanking DNA linked to TKCAT. Chick embryo hepatocytes were isolated, incubated in culture, and transfected as described in the legend to Fig. 1 and under “Experimental Procedures.” Left, constructs used in this experiment. Columns 1–3, The relative CAT activity for T3-treated hepatocytes transfected with p[MET3RE2TK]CAT was fixed to 100, and the other values were adjusted proportionately. The results are the means ± S.E. of six or eight experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with p[MET3RE2TK]CAT were 3.5 ± 1% (mean ± S.E., n = 8) conversion/15 h/mg of protein and 6.9 ± 1.7 × 10^6 (mean ± S.E., n = 8) light units/mg protein, respectively. Columns 4–6, presentation of the results is similar to that described in the legend to Fig. 1. Statistical significance of comparisons made within a column is as follows: a, p < 0.05 versus p[MET3RE2]TKCAT. Abbreviations are as defined in the legend to Fig. 1.

| Relative CAT activity | Fold change | Percentage of ICT3 |
|----------------------|-------------|-------------------|
| IC | ICT3 | ICT3/C | ICT3/C |
| -3259 | -3115 | 14 ± 4 | 680 ± 110 | 14 ± 5 | 55 ± 7 | 1.9 ± 0.6^a | 6 |
| -3224 | -3115 | 7.2 ± 7.7 | 1300 ± 180 | 22 ± 9 | 290 ± 110^a | 1.8 ± 0.4^a | 6 |
| -3192 | -3115 | 2.5 ± 0.4 | 360 ± 50 | 4.8 ± 1.3 | 160 ± 30 | 1.6 ± 0.4^a | 6 |
| -3161 | -3115 | 1.7 ± 0.4 | 44 ± 6 | 9.5 ± 2.9 | 32 ± 6^a | 24 ± 5^a | 6 |
| -3134 | -3115 | 4.0 ± 1.2 | 56 ± 8 | 24 ± 10 | 31 ± 14^a | 37 ± 11^a | 6 |
| -3180 | -3174 | 2.8 ± 0.6 | 100 | 100±10 | 59 ± 7 | 100±10 | 8 |

**Fig. 8.** The effect of a block mutation in the −3192 to −3158 bp fragment. Chick embryo hepatocytes were isolated, incubated in culture, and transfected as described in the legend to Fig. 1 and under “Experimental Procedures.” Left, constructs that were used in this experiment. The wild-type (WT) plasmid contains the natural sequence of this putative ICRE, a consensus AP1 binding site. The mutant (MUT) plasmid contains a block mutation in the AP1 site (complementary sequence, as shown in the figure). Columns 1–3, the relative CAT activity for T3-treated hepatocytes transfected with the wild-type, p[ME(T3RE2)−3192/−3158]TKCAT, was fixed to 100, and the other values were adjusted proportionately. The results are means ± S.E. of 3–6 experiments (n). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with the wild-type, p[ME(T3RE2)−3192/−3158]TKCAT, were 8.6 ± 2.8% (mean ± S.E., n = 6) conversion/15 h/mg of protein and 9.2 ± 2.5 × 10^6 (mean ± S.E., n = 6) light units/mg protein, respectively. Columns 4–6, presentation of the results is similar to that described in the legend to Fig. 1. Statistical significance of comparisons made within a column is as follows: a, p < 0.05 versus p[MET3RE2]TKCAT. Abbreviations are as defined in the legend to Fig. 1.

| Relative CAT activity | Fold change | Percentage of ICT3 |
|----------------------|-------------|-------------------|
| IC | ICT3 | ICT3/C | ICT3/C |
| -3180 | -3174 | 2.8 ± 1.5 | 100 | 16 ± 7 | 71 ± 17 | 16 ± 7^a | 6 |
| -3192 | -3158 | 1.3 ± 0.5 | 19 ± 4 | 22 ± 4 | 19 ± 5^a | 130 ± 12 | 6 |
| -3180 | -3174 | 3.7 ± 1.0 | 107 ± 49 | 90 ± 8 | 32 ± 8 | 88 ± 12 | 3 |

In our sequence, these three bases are conserved. However, replacement of the first guanosine with an adenosine (as in our sequence) abolishes the binding of the Jun/Fos complex.

Our results confirm that cAMP inhibits transcription of the malic enzyme gene via the classical PKA signaling pathway. In response to the binding of cAMP to regulatory subunits of PKA, the catalytic subunits are released and catalyze phosphorylation of target proteins (34, 35). In the nucleus, the phosphorylation states of transcription factors and related proteins appear to modulate function and expression of cAMP-responsive genes directly (6). How does the activation of PKA lead to the formation of a c-Fos/ATF-2 complex on the AP1-like site of the malic enzyme gene via the classical PKA signaling pathway? In response to the binding of cAMP to regulatory subunits of PKA, the catalytic subunits are released and catalyze phosphorylation of target proteins (34, 35). In the nucleus, the phosphorylation states of transcription factors and related proteins appear to modulate function and expression of cAMP-responsive genes directly (6). How does the activation of PKA lead to the formation of a c-Fos/ATF-2 complex on the AP1-like site of the malic...
enzyme gene? c-Fos is phosphorylated after activation of protein kinase C, but phosphorylation by PKA has not been reported (36). ATF-2 also is phosphorylated in intact cells (37), phosphorylation increases its DNA binding activity and appears to be a primary determinant for formation of c-Jun/ATF-2 heterodimers. A stress-activated protein kinase appears to activate ATF-2 heterodimers. A stress-activated protein kinase appears to be involved. To our knowledge, phosphorylation of ATF-2 by PKA has not been reported. We speculate that the rapid cAMP-mediated inhibition of transcription of the malic enzyme gene (37RE, lanes 5 and 9). The major complexes are designated a and b. Apparent complexes of lower molecular weight may represent degradation products or complexes missing a component of complexes a or b. Lane 1, no nuclear extract. Abbreviations are as defined in the legend to Fig. 1.

Overexpression of TRs in chicken embryo hepatocytes decreased cAMP responsiveness, suggesting an interaction between the T3 and cAMP signaling pathways. TRs and AP1 can antagonize one another in vivo and in vitro by a mechanism that does not involve competition for the same binding site or interference from adjacent binding sites (40, 41). The ability of these factors to interfere with another’s regulation of gene expression appears to involve a TR-AP1 interaction rather than a TR-DNA interaction. The mechanism by which cAMP inhibits the T3-induced transcription of the malic enzyme gene by glucagon and cAMP persists for days in hepatocytes in culture (4). A cAMP-mediated inhibition of transcription of the malic enzyme gene by glucagon and cAMP is involved. To our knowledge, phosphorylation of ATF-2 by PKA has not been reported. We speculate that the rapid cAMP-mediated inhibition of transcription of the malic enzyme gene may involve a cascade of kinases and/or phosphatases. Inhibition of transcription of the malic enzyme gene by glucagon and cAMP persists for days in hepatocytes in culture (4). A cAMP-induced increase in transcription of the c-Fos gene (38, 39) may account for the long-term effect.

In summary, the 5′-flanking DNA of the chicken malic enzyme gene contains at least four sequences involved in cAMP responsiveness of this gene. An ICRE centered at −3177 contains an AP1-like sequence that appears to bind a c-Fos/ATF-2 dimer in a cAMP-dependent manner. This mechanism may explain the inhibition of transcription of the malic enzyme gene caused by starvation because starved animals have elevated concentrations of glucagon in the blood.

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