Overexpression of the α-Thyroid Hormone Receptor in Avian Cell Lines

EFFECTS ON EXPRESSION OF THE MALIC ENZYME GENE ARE SELECTIVE AND CELL-SPECIFIC*

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The role of the α-thyroid hormone receptor (TRα) in regulation of transcription of the gene for chicken malic enzyme was analyzed in fibroblast cell lines normally unresponsive to triiodothyronine (T₃). The gene for this transcription factor was introduced stably and overexpressed using a replication-competent retroviral vector. In chick embryo fibroblasts (CEF), overexpression of TRα decreased malic enzyme activity by 90% in the absence of T₃. Addition of T₃ almost completely restored malic enzyme activity to the level of similarly treated control CEF infected with virus lacking TRα. These TRα-induced changes in malic enzyme activity were mediated by alterations in transcription of the malic enzyme gene. Similar results were obtained when transcriptional activity of TRα was analyzed using a transient co-transfection system. Thus, the unliganded TRα is a transcriptional repressor of the malic enzyme gene; binding of T₃ to the receptor abolishes this repression.

In contrast, stable overexpression of TRα in QT6 cells had no effect on malic enzyme expression in the absence or presence of T₃. Nuclear T₃ binding was equally high in CEF and QT6 cells overexpressing TRα. These findings suggest that cell-specific factors control the ability of TRα to regulate the malic enzyme gene. Overexpression of TRα in CEF had no effect on the expression of fatty acid synthase and acetyl-CoA carboxylase, lipogenic enzymes that are stimulated by T₃ in hepatocytes in culture. Thus, gene-specific factors also may control the transcriptional activity of TRα.

Malic enzyme catalyzes the oxidative decarboxylation of malate to pyruvate and CO₂, simultaneously generating NADPH from NAD⁺. This reaction is the primary source of reducing equivalents for de novo fatty acid biosynthesis in chicken hepatocytes (1). Malic enzyme is subject to nutritional and hormonal regulation. In newly hatched unfed chicks, malic enzyme activity is low; feeding a high-carbohydrate mash diet stimulates enzyme activity about 70-fold (2). The diet-induced increase in malic enzyme activity can be mimicked in chick embryo hepatocytes in culture. Triiodothyronine (T₃) stimulates a 40-fold or greater increase in malic enzyme activity in this culture system (3). Insulin has no effect by itself, but amplifies the effect of T₃ by 3-fold. Glucagon, acting through cyclic AMP, almost completely blocks the effects of T₃ or insulin plus T₃. All of these diet and hormone-induced changes in enzyme activity are due to alterations in the rate of synthesis of the enzyme which, in turn, are correlated with changes in mRNA abundance for malic enzyme, indicating that regulation is pretranslational (4). Alterations in the rate of transcription initiation, as measured by nuclear run-on assay, account for most of the changes in abundance of malic enzyme mRNA (5, 6). Transcription of the chicken malic enzyme gene is not responsive to diet or hormones in non-hepatic tissues indicating that tissue-specific factors are involved in the regulation of this gene (5).

Determination of the mechanisms responsible for the nutritional, hormonal, and tissue-specific regulation of malic enzyme transcription initially requires the identification of the trans-acting factors that interact with cis-acting regulatory elements. High-affinity nuclear T₃ receptors synthesized in reticuloocyte lysates bind specifically to a thyroid hormone response element (T₃RE) in the 5′-flanking DNA of the rat malic enzyme gene in vitro (7). In addition, the T₃-induced stimulation of transcription of malic enzyme in chick embryo hepatocytes is both rapid (<1 h) and insensitive to inhibitors of protein synthesis (6). Together, these findings indicate that a nuclear T₃ receptor(s) directly interacts with the malic enzyme gene to modulate its transcription rate.

The function of individual factors involved in the hormonal regulation of transcription can be analyzed via transfection analyses. One approach is to introduce and overexpress genes for transcription factors in cells that are unresponsive to hormones. The activity of the factor is determined by monitoring the expression of an endogenous regulated gene if permanent transfection is employed or a "reporter" gene ligated to the promoter/regulatory region of a regulated gene if transient co-transfection is used. This strategy assumes that the diminished hormonal responsiveness of the cells employed in the assay is caused, in part, by a lack of activity of the transcription factor being tested. By employing multiple cell types, this approach can be used to assess the role of cell-specific factors in modulating the activity of a transcription factor.

In the present study, we have analyzed the regulation of the gene for malic enzyme by the α-subtype of the chicken nuclear T₃ receptor (TRα), the predominant form of the

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1 The abbreviations used are: T₃, 3,5,3′-triiodo-L-thyronine; TRα, nuclear T₃ receptor, α subtype; CEF, chick embryo fibroblasts; CAT, chloramphenicol acetyltransferase; T₃RE, thyroid hormone response element; kb, kilobase(s).
receptor expressed chick embryo hepatocytes and fibroblasts (8, 9). Both permanent transfection and transient co-transfection methods have been used. Retroviral vector-mediated gene transduction was employed to introduce stably and overexpress TRα in avian cell lines in which malic enzyme is normally unresponsive to hormones. Three cell types were employed to analyze the role of gene-specific factors in regulation of nuclear T3 receptor function. Finally, the gene regulatory activity of TRα was compared with that of v-erbA, a mutated version of the receptor which comprises one of the two oncogenic loci in avian erythroblastosis virus (10).

EXPERIMENTAL PROCEDURES

Cell Culture—Chick embryo fibroblasts (CEF) were obtained from SPAFAS, Inc. (Norwich, CT). Quail QT6 cells were kindly provided by C. Moscovic (Gainesville, FL) (11). Both cell lines were routinely cultured in DEMEM/M199 (Duobecco's modified Eagle's medium (25 mM glucose) and Medium 199 (GIBCO/Bethesda Research Laboratories (BRL)) in a 1:1 ratio (v/v), containing 10,000 units/liter penicillin G, 10 mg/liter streptomycin sulfate, and 25 μg/liter amphotericin B) supplemented with 5% fetal bovine serum. Primary cultures of chick embryo hepatocytes were prepared as described previously (12) and maintained in serum-free Waymouth medium MD705/1 containing 50 mM insulin (gift from Eli Lilly Corp.). Incubation of all cell types was at 49°C in a humidified atmosphere of 5% CO2 and 95% air. Where indicated, thyroid hormone was removed from fetal bovine serum by treatment with AG-1X-8 ion exchange resin (13). Construction of a Retroviral Vector Expressing the Gene for Chicken TRα—A HindIII restriction fragment (1250 base pairs) containing the coding sequence of the chicken TRα (c-erbAα) gene was excised from a plasmid, pFLA1 (kindly provided by B. Vennstrom) (14), and subcloned into the adaptor plasmid, CLA-12 (15), to form CLA-12-c-erbαα. c-erbαα and polylinker sequences in this plasmid are flanked by Clal sites. The Clal restriction fragment of CLA-12-c-erbαα was then subcloned into the replication-competent avian retroviral vector, RCAS (subgroup A)(15), at the Clal site to form RCAS-c-erbαα. Orientation of the insert was determined by digestion with restriction endonucleases. The supercoiled forms of the plasmids, RCAS and RCAS-c-erbαα were purified twice by CsCl gradient centrifugation (16) and used to transfect cells.

Stable Introduction of TRα and c-erbα into Avian Cell Lines—CEF and QT6 cells were cotransfected with RCAS-c-erbαα using lipofectin (22). Based on reverse transcriptase activity (18) in the medium, the extent of infection of cells by recombinant retrovirus was maximal after 8–12 days of culture. Mass cultures of infected CEF were employed in experiments. Individual clones of QT6 cells infected with RCAS-c-erbαα virus were isolated via dilution on 96-well plates. QT6 cell lines employed in this study were derived from two rounds of clonal isolation.

The replication-incompetent retroviral vector, XJ12 (kindly provided by J. Samaratia) (21), was used to introduce and express v-erbA in CEF. Mass cultures of XJ12-infected CEF were produced by cotransfection with pR-H 2 helper virus DNA. Control cells were infected with TXN3' which is similar to XJ12 except that it lacks v-erbA sequence. XJ12- and TXN3'-infected CEFs were selected in regular growth medium containing 200 μg/ml G418 (GIBCO/BRL). Malic enzyme activity (19) and protein (20) were measured by the indicated methods.

Isolation of RNA and Quantitation of mRNA Levels—RNA was extracted from cells by the guanidium thiocyanate/phenol/chloroform method (22). Total RNA was treated with formaldehyde and subjected to electrophoresis in 9% agarose gels. The separated RNAs were transferred to "GeneScreen" (Du Pont-New England Nuclear) and hybridized with 32P-labeled DNA probes labeled by "random priming" (Amersham Multiprime DNA Labeling Kit) according to the manufacturer's instructions. Membranes were hybridized and washed as described (23). Washed filters were subjected to autoradiography at −70°C with Kodak XAR-5 film and intensifying screens. Exposed films were scanned at 633 nm in an LKR Ultrason XL densitometer.

Quantitation of Nuclear T3 Receptor Concentration—Nuclear binding of 3H-T3 was measured in intact cells as described by Samuels et al. (24) with minor modifications. CEF and QT6 cells were incubated 16 h with DMEM/M199 supplemented with 0.5% fetal bovine serum depleted of thyroid hormone. The medium was replaced, and the monolayers were washed three times with serum-free DMEM/M199. The cells were incubated with 1.2 nm 3H-T3 (Du Pont-New England Nuclear) in serum-free medium for 4 h. The assay measures total T3 receptor levels because this concentration of T3 (1.2 nm) has been shown to inhibit 90% of the receptors. After the incubation nuclei were isolated as described (25). Nonspecific binding was determined by incubating cells with a 1000-fold excess of nonradioactive T3. Nonspecific binding was subtracted from total binding to obtain specific binding. Radioactivity was measured in a γ spectrometer. DNA was assayed by the method of Labara et al. (26).

Nuclear T3 binding was measured in chick embryo hepatocytes 24 h after the cells were placed into culture. The same procedure described for CEF and QT6 cells was used except that incubation prior to the binding assay was in serum-free medium. The medium was changed prior to addition of 3H-T3.

Run-on Assays of Transcription Rates—Nuclei were isolated from CEF by the method of Milstead et al. (27) with the modification that the nuclear storage buffer was 50% glycerol, 50 mM HEPES, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 5 mM diithiothreitol, and 0.125 mM phenylmethylsulfonyl fluoride. The nuclear run-on assay has been described previously (28).

DNA Probes—A near full-length cDNA for duck malic enzyme (pDME1) has been described (29) and chicken CDNAs for fatty acid synthase (30), glyceroldehyde-3-phosphate dehydrogenase (31), β-actin (32), and TRα (14) were generously provided by Drs. Gordon G. Hammes (Duke University), Robert Schwartz (Baylor College of Medicine), Don W. Cleary (Johns Hopkins University), and Birgitta Vennstrom (Karolinska Institute), respectively. The probe for Rous sarcoma virus gag was kindly provided by Dr. H.-J. Kung (Case Western Reserve University). The cDNA probe for chicken acetyl-CoA carboxylase was generated by amplification of sequences 6012–6964 (33) by the polymerase chain reaction using a cDNA copy from total chicken liver RNA with avian reverse transcriptase. The genomic DNA probes for chicken malic enzyme used in nuclear run-on assays were subclones of genomic DNA that had been isolated from bacteriophage λ libraries (5, 6). M.E.-4.8-5' (4.8 kb in pUC 19) is an EcoRI fragment from λ clone 20B and is derived from the 5' end intron of the malic enzyme gene. M.E.-4.8-3' (4.8 kb in pUC 19) is an EcoRI fragment of λ clone 2B and contains exon 8 from the middle of the mRNA as well as surrounding intronic DNA. M.E.-2.3 2.3 kb in M13 mp18) is a HindIII-EcoRI fragment of λ clone 1 and contains intron DNA and exon 12 from the 3' third of the mRNA.

Transient Transfection Experiments—CEF and QT6 cells were seeded at 1.5 × 104 cells per 6-well plate in DMEM with 20% fetal bovine serum until 70% confluent. Twenty-four hours before transfection, medium was changed to DMEM/M199 containing 5% fetal bovine serum depleted of thyroid hormone. The cells were incubated in this medium throughout the experiment. Cells were transfected with plasmid DNA using the calcium phosphate method (34). Transfected DNAs were pMBS3-CAT (test/reporter plasmid), pCMV-β-galactosidase (internal transfection standard) (35), pRSV-chicken-erbAα (thyroid hormone receptor expression vector provided by H. Samuels) (38) and pRS-v-erbAα (v-erbA expression vector provided by K. Damm and R. Evans) (37). pUC-19 DNA was added as an internal control to maintain a constant amount of DNA in each transfection. Exposure to the calcium phosphate/DNA precipitate was for 5 h followed by a 30 s glycerol shock. Twelve hours later, the transfected cells were trypsinized and distributed to 100 × 20-mm tissue culture plates. Hormones were added to the medium at this time. After 48 h of incubation, cells were harvested and extracts were prepared for CAT (38, 39) and β-galactosidase (16) assays. CAT activity was expressed relative to β-galactosidase activity to correct for differences in transfection efficiency between samples.

RESULTS

Development of Avian Cell Lines That Stably Overexpress Chicken TRα—The replication-competent retroviral vector, RCAS-c-erbAα, was used to introduce stably and overexpress the chicken TRα gene in CEF and QT6 cells. To determine
whether CEF and QT6 cells integrated the intact provirus, total RNA from cells was isolated and subjected to Northern analysis. Transcription of the integrated recombinant provirus should produce three RNA species; all originate in the 5'-long terminal repeat, which contains a promoter/enhancer, and terminate in the 3'-long terminal repeat, which provides a polyadenylation signal. The full-length genomic RNA transcript is co-linear with the integrated provirus and serves as the mRNA for the gag and pol genes. Alternative processing results in separate subgenomic transcripts which serve as the mRNAs for the env and c-erbBα genes. Hybridization of RNA to a c-erbBα cDNA probe detected three transcripts of sizes similar to those expected for the genomic RNA (8.5 kb), env mRNA (4.3 kb), and c-erbBα mRNA (2.0 kb) expressed from the intact provirus (data not shown).

RNA was also hybridized to a gag cDNA probe that should detect only genomic RNA transcripts derived from the provirus. In CEF infected with RCAS-c-erbBα, gag cDNA hybridized to a single transcript (8.5 kb) identical in size to the genomic RNA transcript detected by the c-erbBα probe, suggesting that most of the proviruses in these cells did not contain major insertions or deletions (data not shown). In RNA from QT6 cells infected with RCAS-c-erbBα, however, gag hybridized to two transcripts (8.5 and 7.2 kb). The larger transcript was identical in size to the genomic RNA transcript that hybridized to c-erbBα and probably was transcribed from the intact provirus. The smaller transcript did not hybridize to c-erbBα and probably originated from a provirus in which the entire c-erbBα insert had been deleted.

To obtain separate sets of QT6 cells that integrated only intact provirus, or provirus lacking the c-erbBα insert, individual cells from the mixed population of RCAS-c-erbBα-infected QT6 cells were isolated and cloned. Two QT6 cell lines (clones 3 and 4) expressed a predominant viral genomic RNA transcript that hybridized to both gag and c-erbBα. The sizes of the genomic transcript and the two subgenomic mRNAs that hybridized to c-erbBα were similar to those expected from expression of the intact retroviral transcription unit (data not shown). QT6 cell clones 3 and 4, therefore, contained intact undeleted proviruses and were named RCAS-c-erbBα-QT6-3 and RCAS-c-erbBα-QT6-4, respectively. Two other QT6 cell lines (clones 1 and 2) did not express exogenous c-erbBα sequences. In these cells, gag hybridized to a single transcript (7.2 kb) that was similar in size to the genomic RNA transcript observed in CEF infected with the parent vector RCAS (data not shown). Thus, QT6 cell clones 1 and 2 contained proviruses from which the c-erbBα insert had been deleted and were named RCAS-QT6-1 and RCAS-QT6-2, respectively.

Nuclear T3 binding was measured in intact cells to determine whether CEF and QT6 cells infected with RCAS-c-erbBα overexpressed TRα (Table I). Specific nuclear T3 binding in a mixed population of CEF infected with RCAS-c-erbBα was increased 8- and 14-fold relative to RCAS-infected CEF and uninfected CEF, respectively. Nuclear T3 receptor concentrations in RCAS-c-erbBα-QT6-3 and RCAS-c-erbBα-QT6-4 were increased approximately 25-30-fold compared with RCAS-QT6-1, RCAS-QT6-2, or a mixed population of uninfected QT6 cells. Nuclear T3 receptor levels were similar in CEF and QT6 cells expressing RCAS-c-erbBα and approximately 7-fold higher than those in chick embryo hepatocytes. Conversely, nuclear T3 binding was reduced in uninfected CEF (−48%), uninfected QT6 cells (−76%), RCAS-QT6-1 (−71%) and RCAS-QT6-2 (−71%) compared with chick embryo hepatocytes. Nuclear T3 receptor concentrations in RCAS-infected CEF and chick embryo hepatocytes were similar.

Effects of Overexpression of TRα on Malic Enzyme Activity—CEF infected with RCAS or RCAS-c-erbBα were incubated in serum-free medium for 2 days to deplete endogenous thyroid hormone. This was followed by a 4-day incubation with or without added hormones in medium supplemented with thyroid hormone-depleted fetal bovine serum (5%). In the absence of hormones, malic enzyme activity in RCAS-c-erbBα-CEF was only 9% of that in similarly treated RCAS-CEF (Table II). Incubation of either cell type with insulin (50 nM) had no effect on malic enzyme activity. Addition of T3 (1.5 μM) caused an 11-fold increase in enzyme activity in RCAS-c-erbBα-CEF. This contrasts with a 1.4-fold stimulation of enzyme activity by T3 in RCAS-CEF. In both cell types, addition of insulin plus T3 had the same effect on enzyme activity as treatment with T3 alone. Malic enzyme activity in uninfected CEF subjected to the above hormonal manipulations was similar to that in RCAS-infected CEF (data not shown).

In a separate experiment, we determined the kinetics of the increase in malic enzyme activity caused by T3. In both cell types, activity appeared to approach a new steady state about 4 days after T3 was added (data not shown). After 6 days with T3, malic enzyme activity in RCAS-c-erbBα-CEF was 78% of that in similarly treated RCAS-CEF. The concentration of T3 that resulted in maximum malic enzyme activity (5 nM) was the same in both RCAS- and RCAS-c-erbBα-CEF (data not shown).

In the preceding experiment, an effect of insulin on malic enzyme activity, either by itself or in combination with T3, may have been masked by the presence of insulin or a factor(s) with insulin-like activity in fetal bovine serum depleted of thyroid hormone. The effects of insulin on malic enzyme activity in RCAS- or RCAS-c-erbBα-CEF was also explored in cells incubated with or without added hormones in serum-free medium. Incubation of RCAS-c-erbBα-CEF with insulin plus T3 caused a significantly greater increase (11-fold) in malic enzyme activity than treatment with T3 alone (7-fold) (Table II). Thus, under serum-free conditions, insulin amplified the effect of T3 on malic enzyme activity in RCAS-c-erbBα-CEF but had little effect on malic enzyme activity by itself. Serum depleted of thyroid hormones must contain a factor(s)
Effects of overexpression of TRα on malic enzyme activity in chick embryo fibroblasts incubated in the absence or presence of hormones

Chick embryo fibroblasts infected with RCAS or RCAS-c-erbAα were incubated in serum-free medium for 2 days to deplete endogenous thyroid hormone. This was followed by a 4-day incubation with or without hormones in serum-free medium or medium supplemented with thyroid hormone-depleted fetal bovine serum (5%). The media were changed to one of the same composition 2 days after the start of hormone treatment. Cells were harvested and malic enzyme activity (19) and protein (20) assayed by the indicated methods. The concentrations of the hormones were: T3, 1.5 μM; insulin, 50 nM. The experiment presented in this table is representative of three that were performed. Values for chick embryo fibroblasts are means ± S.E. (n = 3).

| Chick embryo fibroblasts | T3-depleted serum | Serum-free |
|--------------------------|-------------------|------------|
| RCAS                    | RCAS-c-erbAα      | RCAS       | RCAS-c-erbAα |
| None                    | 43 ± 2            | 4 ± 0.1    | 44 ± 2      | 5 ± 0.5 |
| Insulin                 | 43 ± 3            | 5 ± 0.1    | 56 ± 2      | 6 ± 0.1 |
| T3                      | 61 ± 0.3          | 45 ± 1     | 55 ± 4      | 37 ± 1  |
| Insulin + T3            | 67 ± 3            | 42 ± 1     | 62 ± 2      | 54 ± 1  |

Published data for malic enzyme activity in chick embryo hepatocytes were taken from Fischer and Goodridge (52) and have been confirmed in numerous experiments subsequent to that time. Values are means ± S.E. of eight experiments.

Effects of over-expression of the c-erbAα/nuclear T3 receptor on malic enzyme activity in QT6 cells incubated in the absence or presence of hormones

Individual clones of retrovirus-infected QT6 cells and a mixed population of uninfected QT6 cells were incubated in serum-free medium for 2 days. The medium was changed to one of the same composition supplemented with thyroid hormone-depleted fetal bovine serum (5%) and hormones were added. After 2 days of incubation, cells were harvested and malic enzyme activity was measured. The experiment presented in this table is representative of three that were performed. Values are means ± S.E. (n = 3). The concentrations of the hormones are the same as in Table II.

Table III

| Chick embryo hepatocytes* |
|--------------------------|
| RCAS                    | RCAS-c-erbAα      |
| None                    | 52 ± 0.4          |
| Insulin + T3            | 53 ± 1            |

* Published data for malic enzyme activity in chick embryo hepatocytes were taken from Fischer and Goodridge (52) and have been confirmed in numerous experiments subsequent to that time. Values are means ± S.E. of eight experiments.

Fig. 1. Effects of overexpression of the T3Rα on the abundance of the mRNAs for malic enzyme, fatty acid synthase, acetyl-CoA carboxylase, glyceraldehyde-3-phosphate dehydrogenase, and β-actin in chick embryo fibroblasts incubated in the absence or presence of T3. Chick embryo fibroblasts infected with RCAS or RCAS-c-erbAα were incubated for 2 days in serum-free medium. This was followed by incubation with insulin (50 nM) or insulin plus T3 (1.5 μM) in medium supplemented with thyroid hormone-depleted fetal bovine serum (5%). After 24 and 48 h of incubation, total RNA was isolated and subjected to Northern analysis as described under “Experimental Procedures.” RNA (20 μg/lane) was hybridized to 32P-labeled cDNAs for malic enzyme, fatty acid synthase, acetyl-CoA carboxylase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and c-erbAα. This experiment was conducted a total of three times with similar results. The sizes (in kilobases) of the mRNAs were: malic enzyme, 2.1; fatty acid synthase, 1.3; and c-erbAα, 2.0.

mRNA Levels—RCAS- and RCAS-c-erbAα-CEF were preincubated for 2 days in serum-free media followed by incubation with insulin or insulin plus T3 in medium containing thyroid hormone-depleted serum (5%). The abundance of malic enzyme mRNA in RCAS-c-erbAα-CEF, incubated with insulin for 24 h, was decreased by 90% relative to similarly treated RCAS-infected CEF (Fig. 1). Addition of T3 for 24 h stimulated 1.3- and 5-fold increases in malic enzyme mRNA in RCAS- and RCAS-c-erbAα-CEF, respectively, compared with corresponding cells treated with insulin alone. Similar results were obtained after 48 and 96 h of hormone treatment (data not shown for 96 h). Levels of malic enzyme mRNA in RCAS-c-erbAα-CEF after 24 h with insulin plus T3 were about 50% of those in similarly treated RCAS-CEF. Differences in malic enzyme mRNA levels between different CEF cell lines (Fig. 1) were correlated with similar differences in malic enzyme activity (Table II), indicating that the effects of T3Rα...
overexpression on malic enzyme activity in CEF were pre-
translational.

The abundance of c-erbAα mRNA transcribed from RCAS-
c-erbAα was unaffected by T3 treatment for 24, 48, and 96 h
(Fig. 1, data not shown for 96 h). Thus, the T3-induced
increase in malic enzyme expression in RCAS-c-erbAα-CEF
was not due to alterations in TRα expression.

Malic enzyme mRNA levels in RCAS-c-erbAα-QT6 cell
lines incubated in the absence of hormones were about 1.5-
fold higher than those of similarly-treated RCAS cell
lines (data not shown). Concentrations of malic enzyme mRNA in
cells not treated with hormones were similar among RCAS-
c-erbAα-QT6 cell lines and a mixed population of uninfected
QT6 cells. Treatment with insulin plus T3 had no effect on
the abundance of malic enzyme mRNA in any of the QT6 cell
lines. These results are consistent with malic enzyme activity
measurements (Table III).

**Specificity of the Effects of Overexpression of TRα in CEF—**
In chick embryo hepatocytes, T3 stimulates the accumulation of
mRNAs for acetyl-CoA carboxylase and fatty acid synthase
without affecting the abundance of mRNAs for glyceralde-
hyde-3-phosphate dehydrogenase and β-actin (40, 41, 42).
Incubation of RCAS- or RCAS-c-erbAα-CEF with insulin plus
T3 for 24 or 48 h had no effect on the mRNA levels of acetyl-
CoA carboxylase, fatty acid synthase, glyceraldehyde-3-phos-
phate dehydrogenase, and β-actin relative to corresponding
cells treated with insulin alone (Fig. 1). The concentrations of
these mRNAs also were similar between CEF cell types.
Thus, effects of overexpression of TRα are selective for malic
enzyme.

In RCAS-QT6 cell lines, T3 treatment had no effect on
fatty acid synthase, acetyl-CoA carboxylase, glyceraldehyde-
3-phosphate dehydrogenase, and β-actin mRNA levels (data
not shown). Similar results were observed for RCAS-c-erbAα-
QT6 cell lines with the exception that T3 treatment caused a
2-fold stimulation in acetyl-CoA carboxylase mRNA abun-
dance (data not shown).

**Transcription—** To determine the mechanism by which
overexpression of TRα controlled malic enzyme mRNA levels,
transcriptional activity was measured using the nuclear run-
on assay and a DNA probe from 3′ region on the malic enzyme
gene (M.E.-2.3). Transcription of the malic enzyme gene in
RCAS-c-erbAα-CEF incubated with insulin 1 or 24 h was
20 or 15%, respectively, of that of similarly-treated RCAS-
CEF (Fig. 2). Treatment of RCAS-c-erbAα-CEF with insulin
plus T3 for 1 or 24 h stimulated malic enzyme transcription by
3- or 4-fold, respectively, relative to cells of the same
genotype treated with insulin. Transcription of the malic enzyme
gene in RCAS-CEF was not affected by T3. In RCAS-
c-erbAα-CEF incubated with insulin plus T3 for 1 or 24 h,
rates of transcription of the malic enzyme gene were about 80
or 60%, respectively, of those in similarly-treated RCAS-
CEF. Results obtained from run-on assays using genomic DNA
probes spanning the 5′ (M.E.-4.8-5′) and middle (M.E.-4.8-
3′) regions of the malic enzyme gene were similar to those
described above using M.E.-2.3 (data not shown). Rates of
transcription of the fatty acid synthase, β-actin, and glycerc-
aldehyde-3-phosphate dehydrogenase genes were similar in
the different cell types and were not altered by T3.

To confirm that the effects of exogenous TRα on malic
enzyme expression were mediated by changes in transcription and
to examine further the mechanisms of cell type-specific expression of the malic enzyme gene, we measured the pro-
mitter/regulatory activity of 5′-flanking DNA from the malic
enzyme gene in a transient transfection system. pME5.8-CAT
is a chimeric DNA comprised of a continuous piece of DNA

![Fig. 2. Effects of overexpression of TRα on transcription of genes for malic enzyme, fatty acid synthase, β-actin, and glyceroldehyde-3-phosphate dehydrogenase in chick embryo fibroblasts incubated in the absence or presence of T3. Chick embryo fibroblasts infected with RCAS or RCAS-c-erbAα were incubated for 2 days in serum-free medium. This was followed by incubation with insulin (50 nM) or insulin plus T3 (1.5 μM) in medium supplemented with thyroid hormone-depleted fetal bovine serum (5%). After 1 and 24 h of incubation, cells were harvested, nuclei isolated, and transcription run-on assays performed as described under "Experimental Procedures." Independent experiments were performed at each time of hormone treatment. Identical strips were hybridized with equal amounts of 32P-labeled nascent RNA (35 × 106 and 25 × 106 cpm for 1 and 24 h, respectively) from nuclei of fibroblasts treated with or without T3. The probe for malic enzyme (ME) was a genomic DNA. The probes for fatty acid synthase (FAS), β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were cDNAs. A DNA (A) was used as a negative control. Autoradiography for malic acid and A was for 21 days at 1 h of hormone treatment, 14 days at 24 h of hormone treatment. Autoradiography for FAS, β-actin, and GAPDH was for 3 days at 1 h of hormone treatment, 4 days at 24 h of hormone treatment. Experiments were carried out three times with similar results.

from the malic enzyme promoter/regulatory region (~5800 to
+32) coupled to the gene for chloramphenicol acetyltransfer-
ase (CAT). T3 stimulates a 30-fold increase in expression of
transiently transfected pME5.8-CAT in chick embryo hepato-
cytes.3 The extent of this regulation is similar to T3-induced
changes in the transcription of the endogenous malic enzyme
gene in these cells (6). In RCAS-CEF transfected with
pME5.8-CAT, incubation with insulin plus T3 for 48 h had
no effect on CAT activity relative to cells of the same genotype
expressed TRα on pME5.8-CAT activity in CEF were not as
great as the changes detected by the nuclear run-on transcrip-
tion assay. This may have been caused by a depletion of
transcription factors due to the uptake of multiple copies of
pME5.8-CAT per cell. To test this hypothesis, pME5.8-CAT
was co-transfected with pRSV-chicken-c-erbAα, an expres-
sion plasmid for TRα (37). When pME5.8-CAT was co-
transfected with 10 μg of pRSV-chicken-c-erbAα, the T3-
induced stimulation in CAT activity in RCAS-CEF increased
to 50-fold (Fig. 3A). Similar results were observed for RCAS-
c-erbAα-CEF. In a dose-response experiment, transcription of
≥10 μg of pRSV-chicken-c-erbAα produced maximal T3-in-
duced changes in CAT activity in both cell lines (data not
shown). Increases in the T3-induced stimulation of CAT ac-
tivity caused by co-transfection of pRSV-chicken-c-erbAα
were due to a reduction in basal CAT activity in cells incu-
bated in the absence of T3. The results from these transient
expression experiments are consistent with those of the nu-
clear run-on transcription assay and confirm that TRα-in-
duced changes in malic enzyme mRNA levels in CEF primar-
ily result from alterations in transcription of the malic enzyme
gene.

In RCAS-QT6-1 and RCAS-c-erbAα-QT6-4 transfected

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3 S. A. Klautky and A. G. Goodridge, unpublished results.
activity in RCAS-QT6-1 and RCAS-c-erbAa-QT6-4, respectively, relative to corresponding cells treated with insulin alone. Transfection of ≥10 μg pRSV-chicken-c-erbAa produced the maximal T3-induced increase in CAT activity in both cell lines (data not shown). Thus, transient expression of TRα is able to confer T3 responsiveness on a co-transfected malic enzyme promoter/reporter gene in QT6 cells. The magnitude of this effect, however, was only 5–10% of that observed in similar co-transfection experiments using CEF (Fig. 3A).

Effects of v-erbA on Malic Enzyme Expression—The v-erbA gene is a mutated version of the chicken TRα gene that participates in the neoplastic transformation of cells by the avian erythroblastosis virus (10). The v-erbA protein lacks the ability to bind T3 but retains the wild-type receptor’s ability to bind to specific DNA sequences (43, 44). Unlike the wild-type receptor, the v-erbA protein is reported to be a constitutive repressor of genes induced by thyroid hormones (37, 45–47). We asked whether expression of v-erbA constitutively inhibited malic enzyme expression in CEF. The gene for v-erbA was stably introduced and expressed in CEF using the retroviral vector XJ12. Northern analysis confirmed the expression of v-erbA mRNA in XJ12-CEF, the abundance of which was similar to that of the provirally derived c-erbA transcript in RCAS-c-erbAa-CEF (data not shown). When XJ12-CEF were incubated in serum-free medium for 2 days followed by 4 days of incubation with insulin (50 nM) in medium supplemented with thyroid hormone-depleted fetal bovine serum, malic enzyme activity was unchanged relative to that of similarly treated control cells infected with virus lacking v-erbA sequence (42 ± 2 versus 38 ± 2 milliunits/mg protein, mean ± S.E., n = 3, respectively). Treatment with T3 caused similar increases in enzyme activity in XJ12-CEF (29%) and control CEF (33%). Thus, stable expression of v-erbA in XJ12-CEF had no effect on malic enzyme expression.

We next determined whether transient expression of v-erbA was able to modulate the activity of the malic enzyme promoter, since transient expression of TRα was more effective in regulating malic enzyme promoter activity than stable expression of this transcription factor. The v-erbA expression plasmid, pRS-v-erbA, was transiently transfected into RCAS-CEF and effects on pME5.8-CAT activity were determined. In cells transfected with 10 μg of pRS-v-erbA and incubated with or without T3 for 48 h, pME5.8-CAT activity was reduced 50% relative to similarly treated cells not transfected with v-erbA expression plasmid (Fig. 3C). Transfection with 20 μg of pRS-v-erbA caused a 75% decrease in pME5.8-CAT activity in the absence or presence of T3 (n = 1, data not shown). Thus, transient expression of v-erbA repressed malic enzyme promoter activity in a hormone-independent manner. This contrasts with the transcriptional repressor activity of the wild-type receptor which is dependent on the absence of hormone.

**DISCUSSION**

We have developed a retroviral vector-based stable expression system to analyze the mechanism by which the TRα regulates malic enzyme expression. This system has several advantages over a transient transfection system in the functional analysis of trans-acting factors. First, due to the high efficiency of infection, the activity of the stably overexpressed factor can be monitored by simply measuring the activity of a responsive endogenous gene. In contrast, due to the low efficiency of transfection, the activity of a transiently expressed factor must be assessed by measuring the activity of a co-transfected reporter gene coupled to a responsive promoter. Expression of an endogenous gene is a more physiological indicator of factor activity than expression of a pro-
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Dramatic effects of enhanced expression of \(T\alpha\) on these variables in CEF. Because CEF and QT6 cells infected with RCAS-\(c-erb\alpha\) expressed \(T\alpha\) at similar levels, the specific transcriptional activity of \(T\alpha\) must be decreased in QT6 cells relative to CEF. Results from transient transfection analyses support this conclusion. The responsiveness of pME5.8-CAT to \(T_3\) was increased 2-fold in RCAS-\(c-erb\alpha\)-CEF relative to CEF, whereas \(T_3\)-mediated regulation of this chimeric gene was not altered in RCAS-\(c-erb\alpha\)-QT6-4 relative to RCAS-QT6-1 (Figs. 3, A and B). Transient expression of pRSV-chicken-\(c-erb\alpha\) increased the \(T_3\)-induced stimulation of pME5.8-CAT expression to a maximum of about 50-fold in CEF. In contrast, the maximum \(T_3\)-induced stimulation of pME5.8-CAT activity in QT6 cells transfected with pRSV-chicken-\(c-erb\alpha\) was 5-fold. The increased ability of transiently \(T_3\)-responsive exogenous transgenic expression of \(T\alpha\) to modulate malic enzyme promoter activity in CEF and QT6 cells is probably due to a higher level of expression of the transcription factor per cell in the former system. In summary, the ability of the nuclear \(T_3\) receptor to regulate the malic enzyme gene is highest in chick embryo hepatocytes, followed by CEF and QT6 cells in the order of decreasing activity. Experiments are in progress aimed at determining the mechanism(s) responsible for cell-specific differences in \(T\alpha\) activity.

Comparison of basal and \(T_3\)-stimulated malic enzyme activities in CEF with those in chick embryo hepatocytes indicates that impaired \(T_3\)-responsiveness in the former cell type is caused by a decrease in both the transcriptional repressor and \(T_3\)-dependent de-repressor/activation activities of the endogenous nuclear \(T_3\) receptor (Table II). Malic enzyme activity in the absence of hormones is 6-fold higher in RCAS-CEF than in similarly treated chick embryo hepatocytes. \(T_3\)-induced stimulation of malic enzyme activity in RCAS-CEF is about 3% of that in chick embryo hepatocytes. Overexpression of \(T\alpha\) in CEF restores basal malic enzyme activity to the same low level observed in chick embryo hepatocytes and partially unmasks transcriptional activation by \(T_3\). Diminished activity of the endogenous nuclear \(T_3\) receptor in CEF is not due to decreased nuclear \(T_3\) receptor concentration, because RCAS-CEF exhibit similar levels of nuclear \(T_3\) binding as chick embryo hepatocytes (Table I). Thus, it is the specific transcriptional activity per unit \(T_3\) binding activity of the endogenous nuclear \(T_3\) receptor that is decreased in CEF.

The above observations suggest that cell-specific factors are involved in regulating the transcriptional activity of the nuclear \(T_3\) receptor. This hypothesis is supported by the finding that stable overexpression of \(T\alpha\) had different effects on malic enzyme expression in CEF and QT6 cells. Stable overexpression of \(T\alpha\) in QT6 cells did not confer \(T_3\) regulation on malic enzyme activity and had little or no effect on the basal expression of this enzyme. This contrasts with the dramatic effects of enhanced expression of \(T\alpha\) on these
protein can constitutively repress activity of the malic enzyme promoter in CEF. Interestingly, stable expression of v-erbA in XJ12-CEF had no effect on malic enzyme activity. Differences in stable and transient expression systems may due to

In summary, TRα possesses multiple activities with respect to the malic enzyme gene. Cell-specific as well as gene-specific factors are involved in controlling the activities of this transcription factor. The differential responsiveness of chick embryo hepatocytes, CEF and QT6 cells to T3 should facilitate identification and characterization of these factors. The retroviral vector-based stable expression system developed in the present study will be useful in future work aimed at analyzing the function of factors that regulate nuclear T3 receptor activity in avian cells.

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