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Thyroid hormone regulates expression of a transfected α-myosin heavy-chain fusion gene in fetal heart cells

(thyroid hormone/chloramphenicol acetyltransferase assay/DNA transfection)

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ABSTRACT **In ventricular muscle, 3,5,3′-triiodo-L-thyronine (T_3) stimulates the expression of the α-myosin heavy-chain (α-MHC) gene. To test for gene elements required for induction, a fragment of the α-MHC gene containing 2.9 kilobases of 5′ flanking sequences and 420 base pairs of DNA 3′ to the transcription initiation site was linked to the coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene. The α-MHC fusion gene was introduced into primary cultures of fetal rat heart myocytes. Induction of the transfected gene was monitored by assaying CAT activity while endogenous α-MHC mRNA expression was measured by using a synthetic oligonucleotide probe complementary to sequences in the 3′ untranslated region of the mRNA. Without T_3, CAT activity was only slightly greater than background. When T_3 at a final concentration of 10 nM was added to the cultures, CAT activity was increased 8-fold by 48 hr. The response time and doses of T_3 required for induction of CAT activity and α-MHC mRNA in transfected cells were similar, suggesting that the synthetic and endogenous genes may have a common mechanism of control. When simian virus 40 enhancer and early promoter sequences were included in the construct, CAT activity was constitutively expressed, but it could be increased 7-fold by the addition of T_3. Several deletions were introduced into the 5′ flanking sequences of the α-MHC fragment and the effects on induction of CAT activity were examined. Progressive deletions of 5′ sequences from positions −947 to −374 reduced but did not eliminate induction of CAT activity, suggesting that more than one region may be required for optimal induction by thyroid hormone. The results indicate that DNA sequences required for efficient induction by T_3 are present in the 5′ flanking sequences of the α-MHC gene.**

Myosin, a major component of the contractile apparatus in vertebrate striated muscles, is comprised of two heavy chains each of M_r ≈200,000, and two pairs of light chains with M_r 16,000–22,000 (see refs. cited in refs. 1 and 2). Myosin catalyzes the hydrolysis of ATP, which serves as a source of energy for muscle contraction. Distinct myosin heavy chain (MHC) isoforms with different ATPase activities are found in cardiac muscle and skeletal muscles. The relative proportion of the isoforms present in each muscle type may be physiologically significant since the speed of muscle contraction has been shown to be related to myosin ATPase activity (3, 4).

The MHC genes are encoded by a highly conserved multigene family (1, 2). In the rat, at least seven MHCs are known to be expressed in striated muscles, including embryonic and neonatal skeletal muscle forms, two mature fast skeletal muscle forms, one expressed only in extraocular muscles, and two cardiac types, α and β (5). Expression of the various isoforms has been shown to be subject to developmental and innervational controls (5, 6). In addition, all of the MHC genes expressed in rat striated muscles are regulated by thyroid hormone in a highly complex manner (7–9).

In ventricular muscle, thyroid hormone causes a rapid accumulation of α-MHC mRNA while inhibiting the expression of β-MHC (10, 11). The hormone has been found to cause similar alterations in the expression of these genes in cultured rat heart cells (12, 13). The α- and β-MHC genes are closely linked within the genome ≈4 kilobases (kb) apart and are arranged in a 5′ to 3′ orientation that corresponds to the order of their developmental expression in ventricular myocardium (14). The inverse manner in which these genetically linked genes are regulated by thyroid hormone provides a particularly interesting system for study of hormonal regulation.

The actions of thyroid hormone in heart and other target tissues are thought to be mediated by binding of the hormone to DNA-associated receptors, which is associated with alterations in the expression of a number of genes (15). The molecular mechanisms that are responsible for control of these genes are poorly understood. However, studies of the expression of a number of genes responsive to steroid hormones, polypeptide hormones, and thyroid hormone have suggested that sequences in the 5′ flanking regions are important for the regulation of transcription (16–20). In the present study, we have investigated the DNA sequence requirements for induction by thyroid hormone of a chimeric gene containing the 5′ flanking sequences of the α-MHC gene. When the synthetic gene is introduced into primary fetal heart cell cultures, these sequences are sufficient to mediate hormone induction.

METHODS

Construction of Chimeric Plasmids. A recombinant phage (λMHC18) containing 10 kb of the 5′ flanking region of the rat α-MHC gene as well as 2.3 kb of sequences 3′ to the transcription initiation site was isolated from a rat genomic library (21). Screening was carried out with a ^{32}P-labeled synthetic oligonucleotide probe (5′ GGGCTGGAGCGCTGAGAGC 3′) made to be complementary to a 19-base-pair (bp) region immediately downstream from the TATA consensus sequence of the α-MHC gene (14). A 3.3-kb *Hin*dIII fragment from λMHC18 containing 2.9 kb of 5′ flanking DNA as well as 420 bp of DNA 3′ to the transcription initiation site was isolated by agarose gel electrophoresis and subcloned into the unique *Hin*dIII site at the 5′ end of the chloramphenicol acetyltransferase (CAT) coding sequences in the plasmids pSV0CAT and pSV2CAT (Fig. 1). Both of these plasmids contain the entire coding sequence of the prokaryotic CAT gene minus its promoter, followed by two fragments from simian virus 40 (SV40), which contain splice sites and a polyadenylylation signal (22). The pSV0CAT construct

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Abbreviations: MHC, myosin heavy chain; CAT, chloramphenicol acetyltransferase; T_3, triiodothyronine; SV40, simian virus 40.

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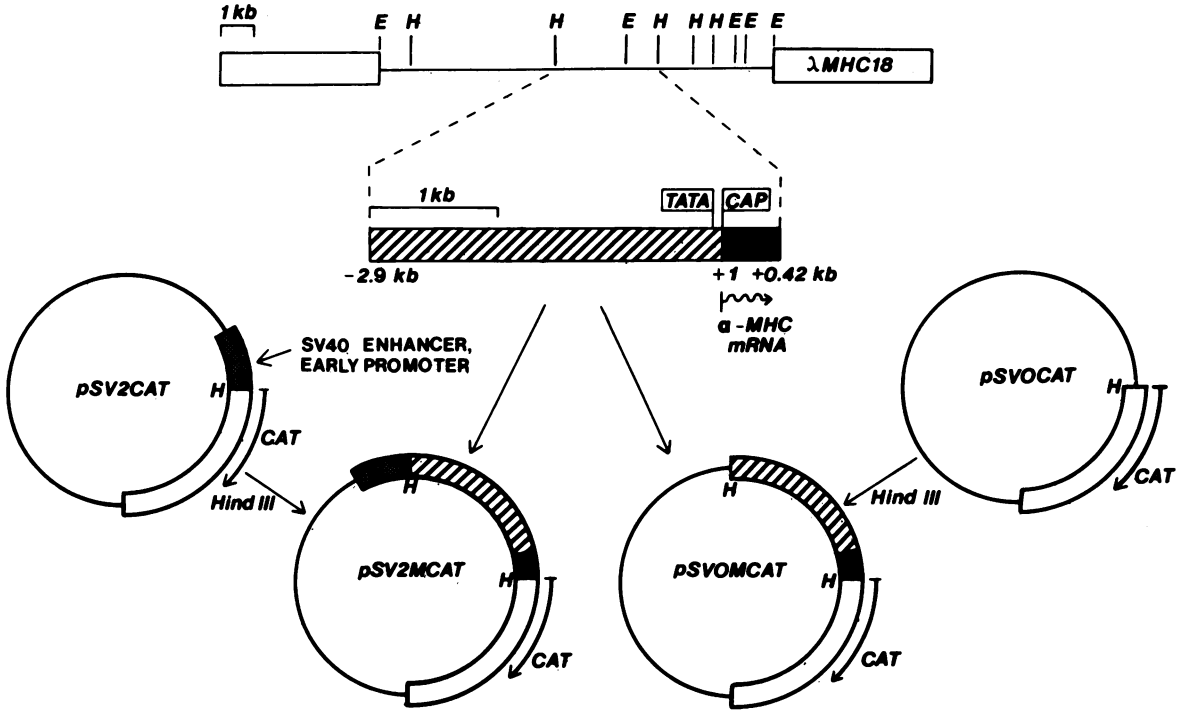

FIG. 1. Construction of recombinant plasmids, pSV0MCAT and pSV2MCAT, containing the α-MHC promoter region linked to the CAT coding sequence. A genomic α-MHC clone (λMHC18) was isolated and the 3.3-kbp *Hin*dIII fragment containing 2.9 kbp of 5′ flanking region (hatched segment) and 421 bp 3′ to the transcription initiation (cap) site (solid segment) was cloned into the unique *Hin*dIII site in the pSV0CAT and pSV2CAT vectors (22). The pSV2CAT vector contains the SV40 enhancer and early promoter, which are shown as a stippled segment. H, *Hin*dIII; E, *Eco*RI.

does not produce significant CAT activity when introduced into cell culture systems (22, 23). The pSV2CAT construct is identical, except for the addition of the SV40 enhancer and early promoter sequences, and has been shown to produce significant CAT activity in several eukaryotic cell systems (24, 25).

Deletion mutants of pSV0MCAT were constructed by using restriction sites within the 3.3-kb *Hin*dIII fragment. Deletions at −972 and −857 were made by digestion at *Spe* I and *Pvu* II sites, respectively. The deletion at −612 utilized an *Eco*RI site. The −1700/−374 deletion was made by digesting pSV0MCAT at the unique *Bgl* II and *Bst*EII sites at −1700 and −374, removing the internal fragment, and recircularizing the plasmid. The final deletion was made by digestion of the pSV0MCAT at the unique *Bst*EII site (−374), followed by a 5-min digestion with BAL-31 exonuclease and religation. The deletions were verified by restriction analysis and, in some cases, by DNA sequencing. All plasmids were analyzed periodically by agarose gel electrophoresis to ensure that >50% of the DNA was in the supercoiled form. Phage and plasmid DNA were isolated and subcloned by standard recombinant DNA procedures (26).

Cell Culture Experiments. Primary myocytes were cultured from 18-day gestational age fetal rat hearts using methods similar to those described earlier (12). Digested cells were differentially plated to remove nonmyocytes and the myocytes were plated at a density of 5×10^6 cells per 100-mm plate, which had been coated with collagen and fibronectin. The medium used was Ham's F-12K plus epithelial growth factor (20 ng/ml) and insulin/transferrin/selenium (Collaborative Research; Waltham, MA). Cultures consisted almost entirely (>95%) of myocytes that beat vigorously. Myocytes were transfected with 15 μg of DNA per plate using the $CaPO_4$ precipitation method (27). The medium was changed after 24 hr and triiodothyronine (T_3) or diluent was added to the cultures. After 48 hr, cell extracts were prepared and assayed for CAT activity as described by Gorman *et al.* (22). Acetylation of chloramphenicol was monitored by autoradiography of thin-layer chromatograms run on plastic sheets coated with silica gel. Spots containing acetylated chloramphenicol were cut out and quantitated by liquid scintillation counting. The data were expressed as nmol of chloramphenicol acetylated per 100 μg of protein after 2 hr of incubation, except as noted. Protein concentrations of the extracts were determined by the method of Bradford (28). The protein concentrations did not vary significantly in plates that had been treated with T_3 and in untreated control plates. Autoradiograms were exposed for 2 days.

The L_6E_9 cells were cultured by the procedures of Nadal-Ginard (29) to achieve relatively pure cultures of myotubes and myoblasts. In the case of the myotubes, >85% of the nuclei could be localized to fused cells. Due to the length of the transfection and hormone treatment protocols, some fusion did occur in the myoblast cultures, but in these experiments the percentage of nuclei in fused cells was <25%. The Rat 1 and 3T3 cell lines were cultured as recommended by the American Type Culture Collection. All transfections and CAT assays were performed as described for the primary myocytes.

Assay of Endogenous α-MHC Expression. Dot-blot assays for endogenous α-MHC mRNA were carried out according to Gustafson *et al.* (7, 9) using a synthetic oligonucleotide probe complementary to the 3′ untranslated region of the message. Total cellular RNA was prepared from each plate by the guanidinium isothiocyanate/hot phenol method (26). Samples containing 3 μg of total cellular RNA were spotted directly onto nitrocellulose filters in duplicate. Filters were hybridized using procedures for synthetic oligonucleotide probes as described earlier (7, 9). Spots were cut out of the filters and the radioactivity was quantitated by liquid scintillation counting. Data are expressed as percentage maximal response, which occurred 48 hr after T_3 addition.

RESULTS

Transfection Experiments. The role of the 5′ genomic sequences in the induction of α-MHC expression by thyroid hormone was evaluated by measuring CAT activity after transfection of plasmids containing chimeric myosin genes into primary cultures of fetal cardiac myocytes. A representative experiment is shown in Fig. 2. As controls, the pSV0CAT and pSV2CAT vectors, prior to the insertion of the α-MHC fragment, also were transfected. The pSV0CAT plasmid showed very little CAT activity in either the absence or presence of T_3. By contrast, when pSV0MCAT, which contained the 5′ α-MHC sequences, was introduced into the system, addition of T_3 to give a final concentration of 10 nM for 48 hr caused an 8-fold increase in CAT activity but showed little activity in the absence of T_3. When the pSV2CAT vector, which contained SV40 enhancer and early promoter sequences, was introduced into the cells, a large amount of CAT activity was observed. In this case, however, T_3 did not cause additional activity. When pSV2MCAT was introduced into the cells, there was a higher basal level of CAT activity than seen with pSV0MCAT. Nevertheless, when T_3 was added to the system an ≈7-fold induction of CAT activity was seen. These results indicate that the DNA sequences required for induction by T_3 are present in the 3.3-kb fragment of the α-MHC gene used in these experiments.

To determine whether other cell types contain the regulatory factors required for induction of the α-MHC gene, the plasmids pSV0CAT, pSV2CAT, pSV0MCAT, and pSV2-MCAT were transfected into L_6E_9 cells, a permanent line derived from rat skeletal muscle, and two nonmuscle cell lines, Rat 1 and 3T3 (Fig. 3). The pSV2CAT plasmid gave high levels of CAT activity in all cell lines examined except 3T3, which has been shown to express low levels of CAT (24). However, introduction of pSV0MCAT and pSV2MCAT showed no significant CAT activity in any cell line other than primary myocytes in either the presence or absence of thyroid hormone.

Time Course of Induction and Dose–Response Characteristics. Duplicate plates of primary myocytes were transfected with the pSV0MCAT plasmid and analyzed either for CAT activity or for expression of the endogenous α-MHC gene at selected time intervals after addition of T_3 (Fig. 4). Since the synthetic oligonucleotide probe used in the α-MHC mRNA assay was made to be complementary to the unique 3′ untranslated region of the α-MHC mRNA, hybridization signals from transfected cells represented expression of only the endogenous gene. The time required for thyroid hormone induction of CAT activity paralleled that required for stimulation of the endogenous α-MHC mRNA expression with only a slight lag in CAT activity, which would be expected for activity produced by the protein of an induced gene.

The regulation of the introduced and endogenous α-MHC genes was further examined by comparing the concentration of T_3 required for induction. As shown in Fig. 5, CAT activity and endogenous α-MHC mRNA increased in a highly dose-dependent manner. In both cases, the EC_{50} was ≈1 nM.

Deletion Mutations. The location of functionally significant thyroid hormone regulatory sequences was determined by construction of a number of deletions in the 5′ flanking sequences of pSV0MCAT. The resulting recombinants were analyzed for induction of CAT activity by thyroid hormone after transfection into myocytes. As shown in Fig. 6, deletions of the sequences upstream from positions −972, −857, or −612 resulted in the reduction of T_3-inducible CAT activity to 67%, 50%, and 57% respectively, of the activity obtained with the complete pSV0MCAT construct. When sequences between −1700 and −374 were deleted, thyroid hormone induction of the chimeric gene was reduced even further to ≈22% of control. A small deletion between −437 and −314 was found to have no significant effect on CAT activity. None of the recombinants showed measurable CAT activity in the absence of T_3. We interpret these data to suggest that there may be more than one hormone-sensitive element within the 5′ flanking region, broadly located between nucleotides −612 and −437 and between −1700 and −972.

DISCUSSION

Thyroid hormone has been shown to influence the expression of a number of genes (15), but the mechanism by which this occurs has remained elusive. In the present study, we have used gene transfer techniques to show that DNA sequences in the 5′ flanking region of the α-MHC gene are sufficient to mediate thyroid hormone induction. We also compared activation of the introduced synthetic genes with induction of their endogenous counterpart by monitoring CAT activity together with α-MHC mRNA levels. The similarities in the time course of induction and dose–response characteristics of the transfected and endogenous genes strongly suggest that they are regulated by similar factors.

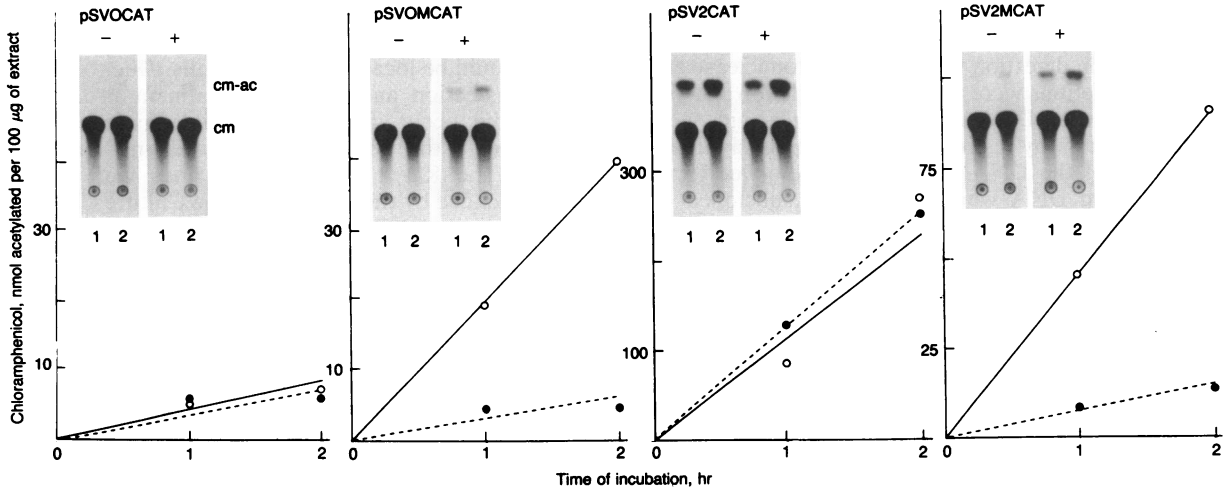

FIG. 2. Hormone responsiveness of CAT activity in transfected cells. CAT activity was assayed after transfection with the four plasmids described in Fig. 1 into primary 18-day fetal rat heart myocytes in the presence (○) or absence (●) of 10 nM T_3 for 48 hr as outlined in *Methods*. (*Insets*) Autoradiograms of thin-layer separations of [^{14}C]chloramphenicol (cm) and its acetylated derivatives (cm-ac) from the assay mixture after 1 and 2 hr of incubation.

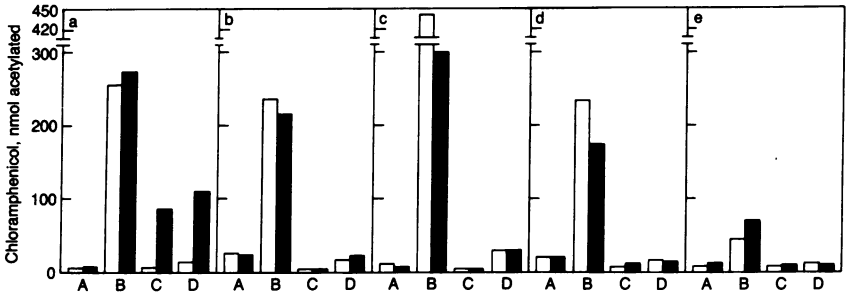

FIG. 3. Analysis of CAT activity after transfection of MHC/CAT recombinant plasmids into primary myocyte cultures and cultures of permanent cell lines derived from skeletal muscle (L_6E_9) and nonmuscle (Rat 1 and 3T3) cells. Transfections were performed with pSV0CAT (A), pSV2CAT (B), pSV0MCAT (C), and pSV2MCAT (D). After 24 hr, duplicate cultures were treated for 48 hr with 10 nM T_3 (solid bars) or diluent (open bars). (*a*) Primary cardiomyocytes; (*b*) L_6E_9 myoblasts; (*c*) L_6E_9 myotubes; (*d*) Rat 1 fibroblasts; (*e*) 3T3 fibroblasts.

Although hormonal control of several eukaryotic genes has been demonstrated after introduction into different cell types (16–20), the present results demonstrate hormonal control of a transfected muscle-specific gene. Since primary heart cell cultures were used in this study, it is unlikely that the transfected genes are incorporated into the genomic DNA. It appears, therefore, that neither the native chromosomal environment of the endogenous gene nor chromosomal integration are absolute requirements for thyroid hormone regulation of gene expression. Possibly, there may be regulatory factors that recognize specific DNA sequences within the 5′ flanking region of the gene and confer thyroid hormone sensitivity. An obvious candidate for such a role would be the nuclear thyroid hormone receptor described by Oppenheimer and Samuels (for review, see ref. 15).

Analysis of the effects of thyroid hormone on a number of deletions in the chimeric α-MHC gene has allowed us to begin to define DNA sequences in the 5′ flanking region that are required for hormonal induction. Deletion analysis suggests that multiple elements may be required for efficient induction of the α-MHC gene by thyroid hormone, probably located in the region upstream of the cap site between positions −1700 and −972, and another located downstream from −612. In this regard, it should be pointed out that deletion analysis would be unable to distinguish between loss of activity resulting from excision of thyroid hormone-sensitive elements and that caused by the removal of elements involved in tissue-specific or basal expression of the α-MHC gene, particularly if these elements are interspersed (or are the same). The presence of multiple regulatory elements would not be unique to thyroid hormone-regulated genes, having been demonstrated in regulation of mouse mammary tumor virus by glucocorticoids (16). Multiple regulatory elements also are thought to be involved in the regulation of the human cardiac α-actin gene (30) and perhaps are a common feature in the control of many eukaryotic genes.

Expression of the α-MHC gene has been shown to be highly tissue specific in the rat, occurring only in ventricular and atrial muscles (8, 9). The inability to detect CAT activity after introduction of plasmids containing α-MHC promoter sequences into skeletal muscle and nonmuscle cells is in keeping with these earlier observations. It appears that the tissue specificity of expression of this gene is retained *in vitro* and suggests that the required regulatory factors may not be expressed in other cell types. These results also suggest that negative regulatory processes such as DNA methylation probably are insufficient to explain the lack of expression of the α-MHC gene in these cells, since the transfected DNA would not be subject to these controls.

The loss of thyroid hormone inducibility with mutants containing deletions in the 5′ flanking region of the fusion gene

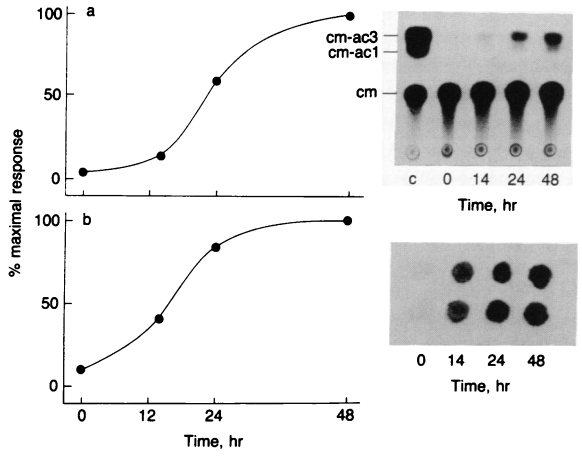

FIG. 4. Time course of changes in CAT activity (*a*) and endogenous α-MHC mRNA levels (*b*) after addition of T_3 at a final concentration of 10 nM to primary cultures of cardiac myocytes that had been transfected with the pSV0MCAT plasmid. At the times indicated after addition of the hormone, cells from duplicate plates were analyzed either for CAT activity or for α-MHC mRNA by dot-blot assay. Zero time point refers to values obtained from untreated plates. A control reaction containing purified chloramphenicol acetyltransferase (Pharmacia) in place of cell extract is shown in lane C. Spots were excised either from the dot-blot filters or the thin-layer chromatography plates and quantitated by liquid scintillation counting. Data are expressed as percentage maximal induced activity.

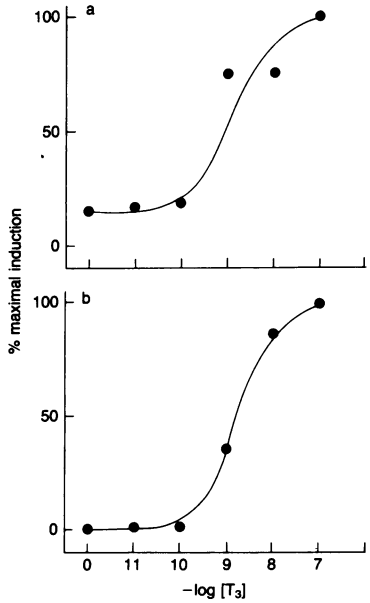

FIG. 5. Dose–response curves for induction of CAT activity (*a*) and endogenous α-MHC mRNA levels (*b*) by T_3 in transfected heart cells. Myocyte cultures were transfected with the pSV0MCAT plasmid. Duplicate cultures were treated for 48 hr with the concentrations of T_3 indicated and assayed either for CAT activity or endogenous α-MHC mRNA. Data are expressed as percentage maximal induction, which occurred at 0.1 μM. Zero dose refers to the values obtained from untreated plates.

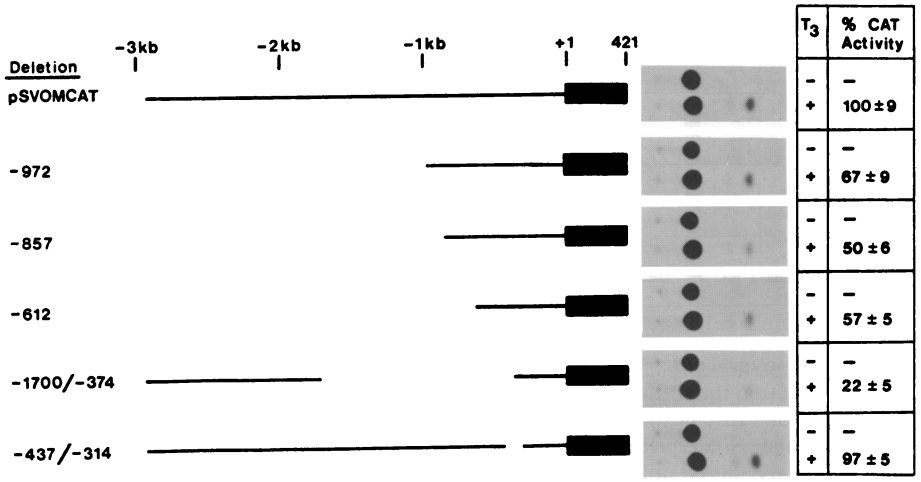

FIG. 6. Relative activity of α-MHC recombinants containing deletions in the 5′ flanking region. Deletions were constructed from the parent pSV0MCAT plasmid as described. The end points of all deletions are referred to in base pairs relative to the transcription start site (+1). Relative CAT activities were determined after 48 hr of treatment with 10 nM T_3. Sequences downstream from the transcription initiation site are indicated by solid bars. Relative CAT activities are expressed as a percentage of the activity of the parent pSV0MCAT plasmid in the presence of 10 nM T_3. Data represent mean activities (±SEM) of three to six plates from three separate experiments. None of the constructs showed measurable CAT activities in the absence of T_3.

suggests that the DNA elements are part of a positive regulatory system. Removal of a negative regulatory element would be expected to result in increased expression of the gene in the absence of thyroid hormone, which was not seen here. We propose, therefore, as the simplest interpretation of the results, that positive regulatory factor(s) under the control of thyroid hormone, possibly including the T_3 nuclear receptor, interact with upstream DNA control elements to activate transcription of the α-MHC gene. Since L_6 cells have been shown to contain functional high-affinity T_3 receptors (31) but are unable to regulate transfected MHC/CAT fusion genes in a hormone-dependent manner (Fig. 3), additional DNA-binding factors may be required to regulate the α-MHC gene.

Although it is difficult to exclude differential mRNA stability as a contributing factor to the results, the finding that expression of both the endogenous and introduced genes is essentially absent without addition of T_3 suggests that control of these genes resides, at least in part, at the level of transcription. This mechanism would be similar to that proposed for the actions of glucocorticoid hormones (16). Additional studies will be required to more clearly define the molecular basis for regulation of the α-MHC gene by thyroid hormone, but the present results indicate that the sequences located in the 5′ region of the gene are important in the regulatory process.

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