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Genetics

Hormonal modulation of a gene injected into rat heart *in vivo*

(cardiac gene transfer/transfection/gene regulation/thyroid hormone/myosin heavy chain)

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ABSTRACT **We demonstrate gene transfer into rat heart *in vivo* by the direct injection of plasmid DNA. Injection of gene constructs driven by retroviral and cellular promoters resulted in detectable levels of reporter gene activities. The cellular promoter and 5′ flanking sequence (positions −613 to +32) were derived from the rat α-myosin heavy chain gene whose expression *in vivo* is restricted to cardiac muscle and is positively regulated by thyroid hormone. After DNA injection, activity of the firefly luciferase gene coupled to the myosin heavy chain promoter and regulatory sequence was detected in heart but not in skeletal muscle and was significantly increased in response to thyroid hormone treatment. Consequently, expression of injected genes can be targeted to specific cell types *in vivo* and can be modulated by the hormonal status of the animal. This approach provides a means of mapping the elements of genes that regulate their responses to complex stimuli that cannot be modeled *in vitro*.**

Gene transfer into somatic cells of adult animals has been achieved by several means including the introduction of DNA complexed with carriers and infection with viral vectors (1–5). Reporter genes driven by a retroviral promoter have been expressed after direct injection of plasmid DNA into mouse skeletal muscle (6). This straightforward means of gene transfer suggests an approach to somatic cell gene therapy and offers a way of studying gene regulation *in vivo* that is simpler and less costly than the generation of transgenic animals. For example, the mammalian heart has been shown to respond to various hormonal and hemodynamic stimuli with marked alterations in the expression of specific genes (for a review, see ref. 7). Consequently, the direct injection of DNA into the heart of an intact animal might provide a system with which to study the regulation of cardiac genes in various physiologic and pathologic states that cannot be modeled in tissue culture. Further, some cis-acting regulatory elements have been observed to influence gene expression differently *in vitro* than *in vivo* (8).

To address these issues, we asked whether an injected gene could be expressed in cardiac tissue and, if so, whether a gene coupled to a cellular promoter could be expressed at detectable levels and regulated appropriately. Such a finding would suggest an approach for targeting the expression of injected genes to specific cell types and for modulating their expression. The cellular promoter chosen for this study was derived from the rat α-cardiac myosin heavy chain (α-MHC) gene. *In vivo*, the expression of this gene is restricted to the heart (9) and its activity has been shown both *in vivo* (10) and *in vitro* (11, 12) to be positively regulated by thyroid hormone. By co-injecting rat cardiac and skeletal muscles with reporter genes linked to the α-MHC promoter and to a second viral promoter as an internal control, we demonstrated that the heart can be transfected *in vivo* with greater efficiency than skeletal muscle. Furthermore, we showed that the directly injected α-MHC promoter is active in heart, but not in skeletal muscle, and that its activity is regulated by the thyroid hormone status of the animal.

MATERIALS AND METHODS

Plasmids. pα-MHCluc contains the firefly luciferase coding region coupled to the rat α-MHC 5′ flanking sequence, base pairs −613 to +32 (13). The latter, which includes a putative thyroid hormone response element (12), had been subcloned into ptz19R (United States Biochemical) as an *Xba* I–blunted *Eco*RI fragment. A *Hin*dIII–*Kpn* I fragment of this plasmid was subcloned into pXP2 (14), a promoterless luciferase vector, to construct pα-MHCluc. pRSVCAT, in which the coding sequence of the chloramphenicol acetyltransferase (CAT) gene is spliced to the long terminal repeat of the Rous sarcoma virus (RSV), has been described (15). p0-CAT (pCAT-Basic Vector; Promega) is a promoterless CAT construct.

Animal Models. Adult female Wistar rats were anesthetized with inhaled methoxyfluorane and 0.1 ml/100 g (body weight) of ketamine (50 mg/ml) and zylazine (10 mg/ml) intramuscularly. Animals receiving cardiac injections underwent exteriorization of the heart through a left thoracotomy (16) followed by injection of 50 μl of a solution containing each plasmid at 2 μg/μl as indicated in 20% (wt/vol) sucrose and 2% (vol/vol) Evans blue into the apex of the left ventricle through a 27-gauge needle. After expression of air from the chest, animals were ventilated briefly on a small-animal respirator and the incision was closed. In other rats, the belly of the adductor magnus muscle was injected under direct visualization.

Two protocols were used in experiments involving thyroid hormone manipulations. (*i*) Rats were rendered hypothyroid by the administration of propylthiouracil (500 mg/liter in drinking water) (17) for 3 weeks, at which point their hearts were co-injected with 100 μg of pRSVCAT and 100 μg of pα-MHCluc. The animals were then divided into two groups: one was continued on propylthiouracil and a second received 3,5,3′-triiodothyronine [T3; 200 μg/100 g (body weight), intraperitoneally] 2 hr after the cardiac injection and daily for 5 days. Five days after the injection, both groups were sacrificed. A third group consisted of age-matched euthyroid animals that were injected with DNA and sacrificed in parallel. (*ii*) All animals were euthyroid at the time of intracardiac DNA injection. After this injection, one group received T3 according to the above schedule but the remain-

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Abbreviations: CAT, chloramphenicol acetyltransferase; MHC, myosin heavy chain; T3, 3,5,3′-triiodothyronine.

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der did not. In this experiment, animals were sacrificed 4 days after the injection.

Tissue Homogenization. At the time of sacrifice, heart and skeletal muscles were resected and washed in ice-cold phosphate-buffered saline. The atria and great vessels were trimmed and the basal third of the ventricles were fast-frozen in liquid nitrogen for subsequent RNA analysis. The apical two-thirds of the ventricles and approximately a 3-cm length of skeletal muscle centered around a suture marking the injection site were weighed, minced into 0.5–1.0 ml of ice-cold homogenization buffer as described (18) except for the omission of Triton X-100, and homogenized with a Tekmar Tissumizer (Tekmar, Cincinnati) for 45 sec. After centrifugation at 6000 × *g* for 10 min at 4°C, supernatants were transferred to new tubes and their volumes were measured.

CAT Assays. Each homogenate (5–20% of the homogenate, constant within a given experiment) was assayed for CAT activity (19). In some experiments, as noted, specimens were first heated at 65°C for 10 min and then clarified by centrifugation at 10,000 × *g* for 5 min. Reaction mixtures were incubated for 2 hr at 37°C. Percent chloramphenicol conversion was determined by excising appropriate areas of the thin layer chromatography plate and measuring radioactivity in Econofluor (DuPont/NEN).

Luciferase Assays. Each homogenate (5–20% of the homogenate, constant within a given experiment) was assayed for luciferase activity (18) with the following modifications. Triton X-100 was omitted from the homogenization buffer but added prior to the luciferase reactions. The buffer (18) containing D-luciferin (Analytical Luminescence Laboratory, San Diego) was used at 100 μl per assay. Light production was measured for 20 sec and integrated over time by a Monolight model 2010 luminometer (Analytical Luminescence Laboratory).

Northern Blot Analysis. Total RNA (15 μg) (20) was size-fractionated on a 0.8% denaturing agarose gel (21) and blotted onto a nylon membrane (Biotrans; ICN). The blot was hybridized sequentially with isoform-specific oligonucleotides complementary to the 3′ untranslated regions of α-MHC [nucleotides 5860–5883 (22)] and β-MHC [nucleotides 5846–5869 (23)], according to standard methods (21) at 55°C overnight and then washed in 2× SSC (20× SSC = 3 M NaCl/0.3 M sodium citrate)/0.2% SDS for 5 min at 55°C. Autoradiography was performed with XAR film (Kodak) for the exposure times indicated.

Serum Thyroxine Determinations. Thyroid-hormone-manipulated animals and controls underwent determination of serum thyroxine levels several hours prior to intracardiac DNA injections and at the time of sacrifice by radioimmunoassay using a kit (Pantex, Santa Monica, CA) previously validated with rat plasma.

RESULTS

Expression of Genes Directly Injected into Rat Heart. The hearts and adductor magnus muscles of adult female rats were co-injected with two plasmid DNAs, 100 μg of pRSVCAT and 100 μg of pα-MHCluc. pRSVCAT directs the synthesis of CAT under the control of the RSV long terminal repeat, which is a constitutively active promoter in many mammalian cell types. pα-MHCluc directs the synthesis of firefly luciferase under the control of the rat α-MHC promoter and 5′ flanking sequence (positions −613 to +32). The endogenous α-MHC gene is expressed only in cardiac muscle *in vivo* (9). pRSVCAT was co-injected with pα-MHCluc to control for differences in DNA uptake and/or expression both between the two tissues and among individual animals. As negative controls, both heart and skeletal muscles were also co-injected with 100 μg of pRSVCAT and 100 μg of pXP2 (14), the latter being a construct containing the luciferase gene with no promoter sequence. Five days after injection, muscle homogenates were assayed for CAT and luciferase activities.

CAT activities greater than 0.25% were detectable in 100% of hearts (n = 37) and 73% of skeletal muscles (n = 30) (Fig. 1). In contrast, the mean CAT activity resulting from injection of p0-CAT, a promoterless CAT vector, was 0.14%. This result confirms that the constitutive RSV promoter was active in both tissues. However, there was a striking difference in CAT activities between the two tissues. The heart expressed this gene at levels 10–100 times higher per mg of total protein than did skeletal muscle. Although the explanation for this differential expression has not yet been established, it was not caused by a tissue-specific inhibitor as reconstitution experiments using noninjected heart and skeletal muscle lysates and known amounts of exogenous CAT demonstrated similar dose–response curves (data not shown). These results demonstrate that the expression of an injected gene can be easily detected in heart muscle.

Tissue-Restricted Expression of an Injected Gene Coupled to a Cellular Promoter. Since α-MHC gene expression *in vivo* is limited to the heart (9), we hypothesized that a gene linked to the α-MHC promoter (pα-MHCluc) would be expressed in heart but not in skeletal muscle after direct DNA injection. No α-MHC-promoter-driven luciferase activity was seen in any of 19 injected skeletal muscles. However, the relatively less-efficient expression of pRSVCAT injected into skeletal muscle as compared to heart would make it very difficult to detect low levels of α-MHC-promoter-driven luciferase activity in skeletal muscles. To control for this variable, luciferase activities were normalized to CAT activities in each heart and skeletal muscle (Fig. 2). We included in our analysis only those skeletal muscles that had been successfully transfected; i.e., those with CAT activities greater than 0.25% conversion or approximately twice background, resulting in the inclusion of 100% of hearts and 78% of skeletal muscles. The luciferase and CAT activities measured in one experiment are shown in Table 1. The luciferase/CAT ratio, therefore, provides a relative measure of the levels of α-MHC promoter driven luciferase expression in the two tissues if it is assumed that reporter gene mRNA and protein turnover do not differ between the tissues. The activity of the α-MHC promoter was significantly ($P < 0.001$) higher in heart than in

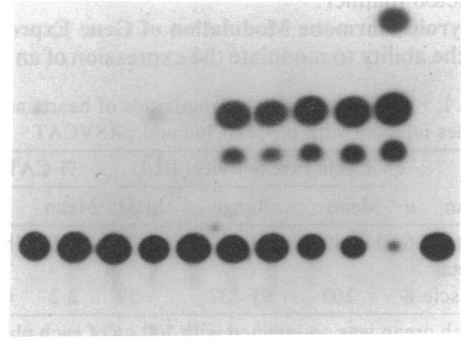

FIG. 1. Heart and skeletal muscles express injected genes. CAT activities in rat skeletal muscles (lanes S) and hearts (lanes H) co-injected with 100 μg of pRSVCAT and 100 μg of pα-MHCluc as shown. Twenty percent of each organ homogenate was assayed 5 days after DNA injection. The above autoradiogram resulted from a 12-hr exposure. Percent chloramphenicol conversion is indicated below each lane. Lanes + and − correspond to positive and negative controls consisting of homogenization buffer with or without partially purified *Escherichia coli* CAT.

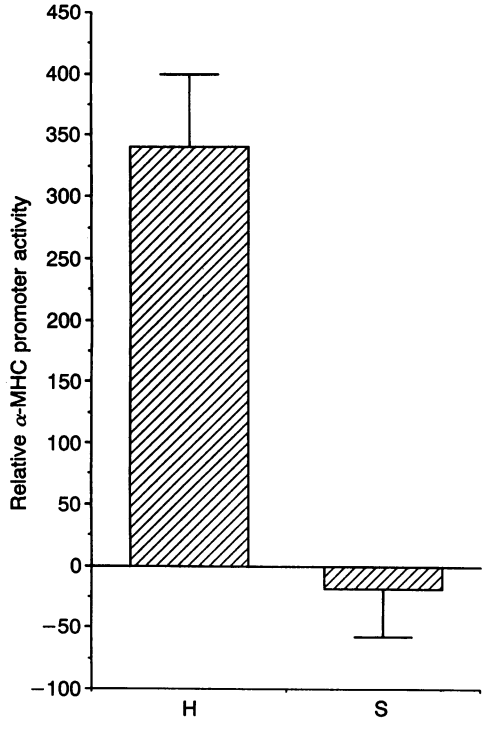

FIG. 2. Tissue-restricted expression of a gene injected into heart and skeletal muscles. Luciferase activities were normalized to CAT activities in DNA-injected hearts (bar H) and skeletal muscles (bar S) and expressed as relative α-MHC promoter activity [raw luminometer units (RLU)/% conversion]. Rat hearts and skeletal muscles were co-injected with each member of the following pairs of plasmids at 100 μg: pα-MHCluc/pRSVCAT (n = 6 hearts and 12 skeletal muscles) and pXP2/pRSVCAT (n = 3 hearts and 11 skeletal muscles). Five days later, organ homogenates were assayed for activities of CAT (15% of each homogenate preincubated at 65°C for 10 min) and luciferase (5% of each homogenate). Only those muscles with CAT conversions >0.25% or approximately twice background (n = 6 hearts and 8 skeletal muscles injected with pα-MHCluc/pRSVCAT and 3 hearts and 10 skeletal muscles injected with pXP2/pRSVCAT) were chosen for analysis. Data are expressed as mean ± SEM (P < 0.001).

skeletal muscle. These results demonstrate that α-MHC promoter directs detectable expression of a gene in a tissue-restricted manner.

Thyroid Hormone Modulation of Gene Expression. To assess the ability to modulate the expression of an injected gene as well as to determine the feasibility of mapping the regulatory elements of a gene, we studied the influence of thyroid hormone on the expression of injected pα-MHCluc DNA. α-MHC mRNA (10) and protein (24) are positively regulated by thyroid hormone *in vivo*. A construct bearing the same 5′ flanking region of the rat α-MHC gene used in the current study has been shown to confer thyroid hormone responsiveness to a reporter gene transfected into fetal cardiac myocytes when normalized to the expression of a cotransfected RSV-driven β-galactosidase construct (12). However, the regulatory elements of this gene have not been mapped *in vivo* nor has it been possible to assess the thyroid responsiveness of DNA constructs in the adult heart. To assess the ability of pα-MHCluc to respond to thyroid hormone, animals were made hyperthyroid by two models and their hearts were injected with reporter genes.

Table 1. Luciferase and CAT activities of hearts and skeletal muscles injected with pα-MHCluc and pRSVCAT

		Luciferase activity, RLU			% CAT conversion		
Organ	n	Mean	Range	SEM	Mean	Range	SEM
Heart	6	11,580	6,509–18,667	1975	38.6	18.8–88.7	10.7
Skeletal muscle	8	160	91–237	19	2.2	0.4–7.2	0.8

Each organ was co-injected with 100 μg of each plasmid. Animals were sacrificed 5 days after the injection. Luciferase activity was measured in raw luminometer units (RLU) in 5% of the homogenate above the machine background which was measured in noninjected organs. Background resulting from the promoterless luciferase construct was determined by injecting 100 μg of pXP2 and 100 μg of pRSVCAT resulting in 139 RLU (range, 63–265 RLU; SEM, 64 RLU; n = 3) and 133 RLU (range, 177–236 RLU; SEM, 19 RLU; n = 10) in 5% of the homogenates from hearts and skeletal muscles, respectively. % CAT conversion in 15% of the homogenate above background was measured. CAT background was determined in 10 skeletal muscles injected with 100 μg of p0-CAT (promoterless CAT construct) and 100 μg of pα-MHCluc (mean, 0.14%; range, 0.07–0.51%; SEM, 0.04%).

In one model, three groups of adult female Wistar rats were co-injected with 100 μg of pα-MHCluc and 100 μg of pRSV-CAT: hypothyroid animals, hypothyroid animals treated with thyroid hormone, and euthyroid controls. Serum thyroxine was undetectable in the propylthiouracil-treated animals after the initial 3-week treatment interval. Further, these animals also had a flat growth curve as compared with controls, consistent with hypothyroidism. Hypothyroid animals treated with T3 had significant increases in heart weights consistent with induced hyperthyroidism. As a control, Northern blot hybridization of total RNA purified from the hearts of these animals (Fig. 3) demonstrated the expected changes in the expression of the endogenous MHC genes: a transition from α- to β-MHC mRNA in the hearts of hypothyroid animals compared with controls and a switch from β- to α-MHC expression in those of hypothyroid animals treated with T3 compared with animals remaining hypothyroid (10). These parameters confirmed that the various treatments resulted in the altered cardiac states associated with thyroid hormone manipulations.

RSV-driven CAT activity was depressed by factors of between 8 and 3 in two experiments in hypothyroid animals treated with T3 versus both hypothyroid and control groups (Fig. 4*A*; data not shown). Although the explanation for this phenomenon has not yet been elucidated, mixing studies excluded the presence of a specific inhibitor of CAT activity in T3-treated heart tissue (data not shown). When luciferase activity was normalized to CAT activity (Fig. 4*B*), the positive responsiveness of the α-MHC promoter to thyroid hormone was evident. The mean luciferase/CAT ratio of hypothyroid animals treated with T3 was approximately 3 times greater than that of the hypothyroid animals not treated with T3 (P < 0.005) and two times greater than that of euthyroid controls (P < 0.05).

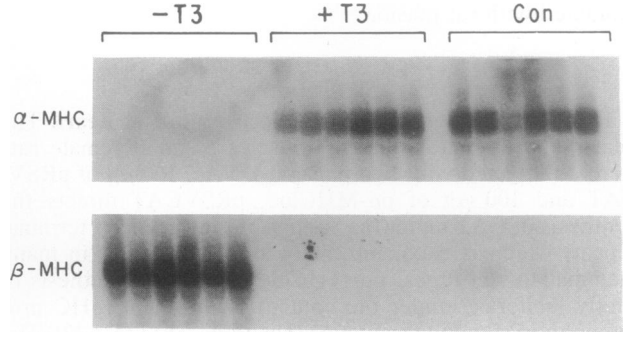

FIG. 3. Endogenous α- and β-MHC mRNA levels in hearts of thyroid-hormone-manipulated animals. Lanes −T3, +T3, and Con contain hypothyroid animals (n = 6), hypothyroid animals treated with T3 (n = 6), and euthyroid controls (n = 6), respectively. The above autoradiograms for α- and β-MHC resulted from 72- and 16-hr exposures, respectively, at −70°C with an intensifying screen.

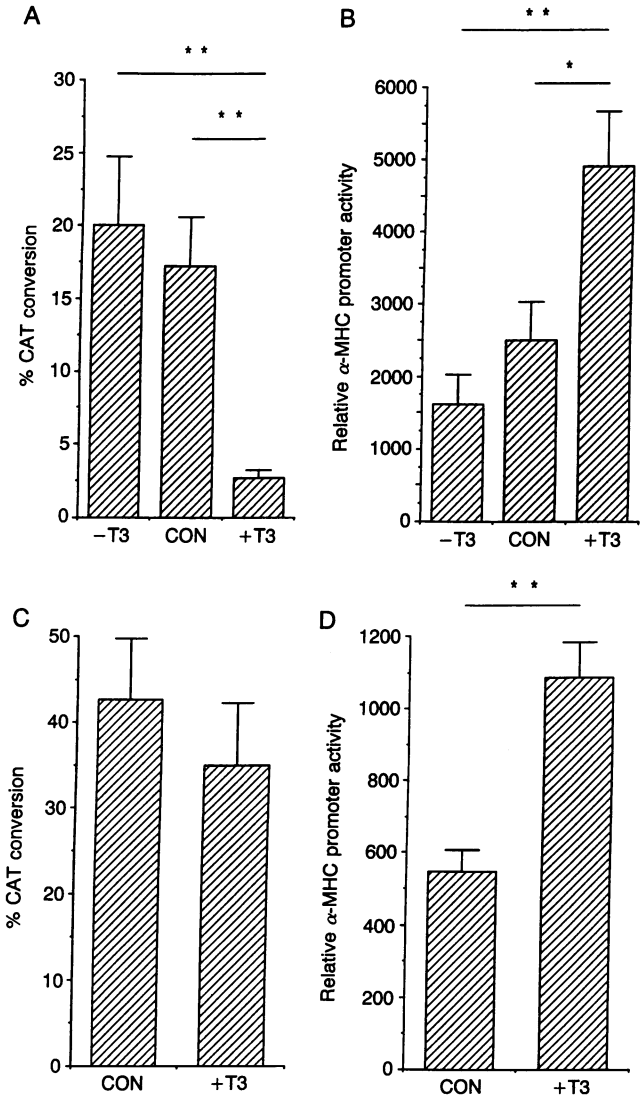

FIG. 4. Thyroid hormone modulation of the expression of a gene injected into heart muscle. Activities of CAT (% conversion) and luciferase [raw luminometer units (RLU)] in hearts co-injected with pα-MHCluc and pRSVCAT from thyroid-hormone-manipulated animals were measured. (*A* and *B*) CAT activity (*A*) (in 15% of the homogenate) and the relative α-MHC promoter activity (*B*) [luciferase/CAT ratio (in 5% of the homogenate/15% of the homogenate, respectively)] in hearts from hypothyroid animals (bars −T3; $n = 6$), hypothyroid animals treated with T3 (bars +T3; $n = 6$), and euthyroid controls (bars Con; $n = 5$) were measured. Homogenates were made from the same hearts as in Fig. 3. (*C* and *D*) CAT activity (*C*) (in 5% of the homogenate preincubated at 65°C for 10 min) and the relative α-MHC promoter activity (*D*) [luciferase/CAT ratio (in 5% of the homogenate/5% of the homogenate heated as above, respectively)] in hearts from euthyroid animals (bars Con; $n = 8$) and euthyroid animals treated with T3 (bars +T3; $n = 6$). Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.005$.

In the second protocol, hearts of euthyroid animals were co-injected with pα-MHCluc and pRSVCAT, after which half of the animals were treated with T3 as described above. Four days later, luciferase and CAT activities were determined in cardiac homogenates. As shown in Fig. 4*C*, RSV-driven CAT activities in the euthyroid and hyperthyroid hearts were not significantly different. Luciferase activity, when normalized to CAT activity (Fig. 4*D*), was significantly higher in the T3-treated animals ($P < 0.005$). The lower T3 responsiveness in the second of the two protocols (1.7 versus 3.0) is not surprising since euthyroid hearts are α-MHC predominant whereas hypothyroid hearts contain mostly β-MHC (see Fig. 3). These results coupled with the tissue-restricted nature of the α-MHC promoter strongly suggest that the cell type targeted for expression by the cardiac injections is the myocyte.

DISCUSSION

Our results demonstrate that the heart can be transfected *in vivo* by direct injection of a plasmid containing a viral or cellular promoter coupled to the coding sequence of a gene. Further, these experiments demonstrate that the expression of an injected gene spliced to a cellular promoter is regulated in a tissue-restricted and thyroid-hormone-responsive manner.

The amount of RSV-directed CAT activity per μg of DNA injected into skeletal muscle in our experiments was similar to that observed previously (6). Given the 10- to 100-fold higher CAT activities detected in hearts as compared with skeletal muscles injected with pRSVCAT, the heart appears to be especially receptive to this type of manipulation. Why muscle in general and heart in particular expresses injected genes at significantly higher levels than do other tissues (6) is unclear. Although cell damage or inflammation may mediate gene transfer, the discordant responses in different tissues suggests a more fundamental mechanism. Both heart and skeletal muscle have rich transverse tubule systems allowing extensive contact between the myocytes and the interstitial space, and this may play a role as may the fact that both organs consist primarily of either bi- or multinucleated cells. It is also clear that the physiological status of the muscle may have profound effects on its ability to take up and/or express injected DNA (Fig. 4 *A* and *C*).

These studies demonstrate the feasibility of identifying the regulatory elements of genes expressed in cardiac muscle using the direct injection technique. This approach may obviate the need for the expensive and labor-intensive generation of transgenic animals for these purposes. Further, our results indicate that the 613 base pairs of the 5′ flanking region of the cardiac α-MHC gene behaves the same in the intact heart and in transiently transfected cultured fetal myocytes with respect to thyroid hormone responsiveness (12). It is noteworthy that the α-MHC promoter was not negatively regulated in the hypothyroid animals, despite the fact that steady-state mRNA levels of α-MHC in the same animals were reduced by at least a factor of 5 relative to control animals (Fig. 3). Although the lack of a statistically significant reduction in promoter activity might have reflected the relatively small number of animals studied or the timing of sacrifice, this result suggests that posttranscriptional controls, perhaps influencing α-MHC mRNA half-life, are important in moderating the genetic response to this altered hormonal condition *in vivo*. A precedent for this type of posttranscriptional regulation has been seen with other hormones (25–27).

In summary, this report demonstrates that the heart is an excellent target organ for direct gene transfer and that cellular promoters transfected *in vivo* in this fashion behave in a tissue-restricted and physiologically responsive manner. The implications of this result for the study of cardiac physiology and disease are significant. By using this technique, it should be possible to map regions of cellular promoters responsive to complex stimuli that can be modeled only *in vivo*, such as hemodynamic overload and pathological states such as hypertension, conditions that are known to result in alterations in gene expression (7). In addition, constructs coupling specific genes to cellular promoters may be useful in targeting the expression of those genes to the heart and altering the physiology of the intact organ.

Note Added in Proof. While this manuscript was under review, two groups reported the expression of virally promoted reporter genes in rat hearts after direct DNA injection (28, 29).

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