

# Analysis of the Upstream Regions Governing Expression of the Chicken Cardiac Troponin T Gene in Embryonic Cardiac and Skeletal Muscle Cells

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**Abstract.** The chicken gene encoding cardiac troponin T (cTNT) is expressed in both cardiac and skeletal muscle during early embryonic development, but is specifically repressed in skeletal muscle during fetal development. To determine if the *cis*-acting sequences governing transcription of a single gene in these two related cell types are the same, we have transfected promoter/upstream segments of the cTNT gene coupled to the bacterial chloramphenicol acetyltransferase gene into primary cultures of early embryonic cardiac and skeletal muscle cells. Using this assay system, chloramphenicol acetyltransferase activity directed by the cTNT promoter/upstream region was between two and three orders of magnitude higher in cardiac or skeletal muscle cells than in fibroblast cells, indicating that *cis* elements responsible for cell-specific expression reside in this region of the cTNT gene. Deletion experiments showed that a 67-nucleotide DNA seg-

ment residing between 268 and 201 nucleotides upstream of the cTNT transcription initiation site is required for cTNT promoter activity in embryonic cardiac cells. This region is not required in embryonic skeletal muscle cells because a cTNT promoter construction containing only 129 upstream nucleotides is transcriptionally active in these cells. These results demonstrate that different *cis*-acting sequences are required for cTNT expression in early embryonic cardiac and skeletal muscle cells. Nonessential regions residing farther upstream, on the other hand, affected the level of expression of these minimum regions in a similar manner in both cell types. The data from these experiments indicate, therefore, that transcription of the cTNT promoter in early embryonic cardiac and skeletal muscle cells is governed both by common and divergent regulatory elements in *cis* and in *trans*.

CARDIAC and skeletal muscle cells are the two types of striated muscle cells. Both share a remarkable degree of similarity in terms of their sarcomere and myofibrillar structure, strongly suggesting that they arose during evolution from a common ancestral cell type. Despite their underlying similarity, cardiac and skeletal muscle are morphologically and physiologically distinct from one another. In addition, the genes encoding cardiac and skeletal muscle contractile proteins, while clearly evolutionarily related, are distinct. Vandekerckhove and Weber (62) indicate that the cardiac and skeletal actin isoproteins diverged from a common cardiac-actin-like ancestral gene before the appearance of reptiles. Therefore, the divergence of these two related cell lineages must have been an ancient one.

During embryonic development, however, skeletal muscle cells exhibit many characteristics which are similar to those of cardiac cells. For example, like cardiac cells, embryonic skeletal muscle cells are intrinsically and rhythmically contractile, and individual cells are electrically coupled to their neighbors via gap junctions (37). Most intriguing, perhaps, are the recent observations that embryonic skeletal muscles express "cardiac-specific" isoforms of actin (3, 30, 34, 50, 56), myomesin (2), and troponins C and T (11, 12, 60). Thus, embryonic development of skeletal muscle cells en-

tails the expression of genes and phenotypic traits which are characteristic of cardiac cells. It is not yet clear whether expression of cardiac genes in early embryonic skeletal muscle represents a vestigial gene regulatory mechanism due to the common ancestry of these two cell types, or a specific developmental requirement met by the cardiac gene products.

To begin to understand the mechanisms which regulate genes in embryonic cardiac and skeletal muscle cells, we have begun to analyze the *cis* elements governing transcription of individual genes transfected into both cell types. The *cis* elements which govern expression of contractile protein genes in skeletal muscle cells are beginning to be identified using this approach. For example, transcription of sarcomeric actin genes transfected into skeletal muscle cells appears to depend on DNA sequences residing within, or upstream of, their transcriptional promoters (28, 47, 50, 51). On the other hand intragenic DNA sequences have been implicated in the cell specific regulation of the troponin I gene (40) and in the muscle specific repression of the thymidine kinase gene (48). The mechanism(s) by which these DNA segments govern skeletal muscle specific transcription is not yet known. Cell specific transcription of many eukaryotic genes has been shown to be governed by cell-specific enhancer elements (9, 15, 16, 21, 29, 32), and in a few instances, by cell specific

promoter elements (15, 18, 46). A number of conserved sequence elements in contractile protein genes have been implicated in cell specific regulation due to their location within or near promoter regions (5, 36, 40, 50, 53, 55). Kedes and coworkers have shown that a conserved CCArGG motif is required for efficient expression of the human cardiac actin gene in skeletal muscle cells (50, 51). Conserved sequence elements have been shown to act as binding sites for common regulatory factors. In the analysis of contractile protein gene regulation, conserved sequence elements are of particular interest because they may explain the apparent co-expression of diverse genes within a single muscle type as well as between muscle types.

There is no information to indicate whether the *cis* elements governing gene expression in skeletal muscle cells are similar to or different from those operating in cardiac muscle cells. Indeed, there has been little experimental work on cardiac specific gene expression, partly due to the lack of a myocardial cell line. For the present, therefore, analysis of cardiac specific gene regulatory elements can only be conducted in primary cultures. Morkin and colleagues have identified upstream regions which are required for the hormone responsiveness of the rat cardiac myosin heavy chain gene using transfection into primary cultures of rat neonatal myocardial cells (31). Here we have used transfection into primary cultures of chicken embryonic cardiac and skeletal muscle cells to identify the *cis* elements required for expression of one gene in both cell types.

For these experiments we have chosen to study the chicken gene encoding the cardiac form of troponin T (cTNT).<sup>1</sup> The cTNT gene serves as a good model because it is transcriptionally activated at early stages of both embryonic cardiac and skeletal muscle development, and is expressed at similar levels in both cell types throughout early development (11). However, at about day 14 of gestation the cTNT gene becomes transcriptionally repressed in developing skeletal muscle, while simultaneously being strongly upregulated in developing cardiac muscle (44). All cTNT mRNAs share common 5' termini, demonstrating that all arise from transcripts initiated at the same two sites which are separated by three nucleotides, regardless of cell type or developmental stage (12). This makes the cTNT gene an attractive candidate to address the question of whether cardiac and skeletal myocytes share common or distinct transcriptional regulatory programs to activate transcription of a single gene. If the transcriptional regulatory programs are shared, we would expect the same *cis* control elements to govern cTNT gene transcription in both cell types. On the other hand, if the transcriptional regulatory programs are different, we would expect the requirements for cTNT promoter expression in the two cell types to differ.

To differentiate between these hypotheses we have analyzed the ability of the cTNT promoter and upstream regions to govern chloramphenicol acetyltransferase (CAT) expression in transiently transfected embryonic cardiac and skeletal muscle cells. When placed in culture, both cell types constitutively express the endogenous cTNT gene (61, and our unpublished observations). However, because both cell types

take up exogenous DNA with widely differing efficiencies, we analyzed CAT gene expression both enzymatically, in cell extracts, and on an individual cell basis, by immunocytochemistry. Using this approach, we then determined that the cTNT promoter/upstream region directs 2–3 orders of magnitude higher levels of CAT expression in cardiac and skeletal muscle cells than in fibroblast cells. Transfection of 5' deletion mutants showed further that different regions in *cis* are required for the activity of the cTNT promoter in these two striated muscle cell types. The requirement for different regions in *cis* is consistent with the presence of different positive-acting regulatory factors in *trans* within both cell types. Therefore, despite their molecular, morphological, and physiological similarities, embryonic and cardiac skeletal myocytes use different regulatory programs to positively regulate expression of the cTNT gene.

## Materials and Methods

### Materials

Restriction endonucleases and S1 nuclease were purchased through New England Biolabs (Beverly, MA) or Boehringer-Mannheim (Indianapolis, IN) and were used according to the instructions from the manufacturers. Reverse transcriptase was obtained from Life Sciences, Inc. (St. Petersburg, FL). Media and media components were obtained from the Cell Culture Facility at the University of California (San Francisco, CA) or from Gibco Laboratories, (Grand Island, NY). Fetal bovine serum was obtained through HyClone Laboratories (Logan, UT). Radioactive compounds were obtained from Amersham Corp. (Arlington, Heights, IL). Other materials were obtained as indicated below.

### Methods

**Recombinant DNA.** Protocols for restriction endonuclease digestion, gel electrophoresis, plasmid DNA preparations, DNA fragment isolation, and ligations were essentially as described in standard cloning manuals (45). Transformations of *Escherichia coli* DH-5 were performed by the procedure described by Hanahan (33).

### Plasmid Constructions

**cTNT-CAT Plasmids.** The cloned cTNT gene (12) was truncated at nucleotide position +38 in exon 1 (all nucleotide positions are relative to transcription initiation site) by partial cleavage with Ava II followed by complete cleavage at an artificial Eco RI site located 3,000 nucleotides upstream of the transcription initiation site. During the process of subcloning this segment into a pUC 8 polylinker the Ava II site was destroyed. The cTNT fragment was subsequently excised from this plasmid by Hind III digestion and cloned into the Hind III site of pBR-CAT (63) to fuse the truncated cTNT untranslated region with the untranslated region of CAT via a polylinker. This plasmid is designated cTNT-3,000 and served as the starting plasmid for creation of a nested set of 5' deletions as shown in Fig. 1. The 5' deletions were created by partial or complete digestion of cTNT-3,000 at Pst I, Bgl II, Pvu II, Dde I, Hae III, and Sma I sites located at nucleotide positions -1,100, -550, -268, -201, -129, and -49, respectively. Restriction sites leaving overhanging ends were filled in with reverse transcriptase or digested with S-1 nuclease, and Hind III linkers added. After digestion with Hind III the fragments were gel isolated and cloned into the Hind III site of pBR-CAT. The structure of each plasmid was confirmed by restriction endonuclease mapping or nucleotide sequencing, or both. These plasmids, designated cTNT-1,100, -550, -268, -201, -129, -49, respectively, therefore share a common 3' end with cTNT-3,000, but have varying amounts of upstream DNA deleted as indicated in Fig. 1. Since each deletion mutant was inserted into the same position of pBR-CAT, the upstream plasmid DNA sequences were identical for all constructions. To remove the cTNT TATA box and transcription initiation site, cTNT-129 and cTNT-268 were digested with Sma I, which cleaves at nucleotide position -49 and at a polylinker site downstream of position +38, and recircularized with T4 ligase. These clones are designated as cTNT-129[Δ-49/+38] and cTNT-268[Δ-49/+38] (see Fig. 1 b).

1. **Abbreviations used in this paper:** CAT, chloramphenicol acetyltransferase; cTNT, cardiac troponin T; HSV, Herpes simplex virus; RSV-LTR, Rous sarcoma virus-long terminal repeat; STV, standard trypsin versene; tk, thymidine kinase.

**Enhancer Test Constructions.** The cTNT fragment from position -268 to -550 was isolated from cTNT-1,100 by simultaneous digestion with Pvu II and Bgl II. Hind III linkers were ligated to both ends after filling in the Bgl II site with reverse transcriptase. The fragment was cloned into the Hind III site of the pUC-based enhancer test plasmids pTE-2ΔS/N or pTE-2 which places the cTNT fragment either directly in front of the HSV tk promoter or at a position 600-bp upstream of the tk promoter, respectively (15). Orientation of the cTNT fragment with respect to the tk promoter was ascertained by restriction mapping.

**bACT-275-CAT.** The promoter region of the chicken beta-actin gene (41) was isolated by partial digestion with Hpa II at position +61 within the first, untranslated exon, followed by complete cleavage at an Xho I site located at position -275. After addition of Hind III linkers as described above, the promoter fragment was gel isolated and inserted into the Hind III site of pBR-CAT. The structure of the fusion between beta-actin exon I and the CAT gene was confirmed by DNA sequencing and restriction endonuclease mapping.

## Preparation of Cultured Cells

**Embryonic Skeletal Muscle Cultures.** Chicken day 11 embryo breast muscle tissue was dissected free of skin and cartilage and then dissociated either by mechanical means (8, 39), or by trypsinization (1), and then plated on gelatinized tissue culture plates at  $1.5 \times 10^6$  cells per 100-mm Corning tissue culture plate (American Scientific Products, McGaw Park, IL). Inclusion of 0.05% trypsin in 0.02% EDTA standard trypsin versene (STV) in the dissociation step did not affect the results presented here. The medium, consisting of Dulbecco's minimal essential medium (DME) supplemented with 10% horse serum (heat inactivated), 2.5% chicken embryo extract and 100 U/ml each of penicillin and streptomycin was changed the day after plating, 3 h before initiation of transfection.

**Embryonic Heart Cell Cultures.** Heart cell cultures were prepared by methods modified from established procedures (13, 14, 38, 59, 64). Briefly, hearts were excised from day 6 chicken embryos, dissected free of pericardium and great vessels under sterile conditions and then minced into small fragments. After rinsing in clean PBS, the tissue fragments were incubated in 10 ml of STV for 10 min at room temperature with gentle mixing. The supernatant from this was discarded and 7 ml fresh STV added. After an additional 8 min of digestion, the supernatant was transferred into a 50-ml Falcon tube containing 10 ml of ice-cold nutrient medium (5% fetal bovine serum, 100 U/ml of penicillin and streptomycin in M199 medium with Earle's balanced salt solution [EBSS]). The remaining tissue was redigested as above for 3 to 4 additional cycles and the supernatants pooled. Liberated cardiac cells were collected by centrifugation at 2,000 rpm for 10 min at 4°C, resuspended in nutrient medium, and passed through a 20-μm mesh nylon screen to remove aggregates. Dissociated heart cells were plated at a density of  $2.5 \times 10^6$  cells per 60-mm tissue culture plate. For histological studies, cells were cultured on rat tail collagen-coated aclar coverslips in 35-mm tissue culture plates. Cells were maintained in a 37°C humidified incubator with a 5% CO<sub>2</sub> atmosphere. Medium was changed the day after plating, 3 h before initiating transfections.

**Chicken Embryo Fibroblast Cultures.** Fibroblast cells were prepared from day 11 chicken embryos from which the head, viscera and appendages had been removed (58). After dissociation with STV, fibroblasts were grown in M199 medium supplemented with 10% tryptose phosphate, 10% FCS and 2% chicken serum. Primary fibroblast cultures were initially plated at  $1.5 \times 10^6$  cells per 100-mm culture plate and were passaged at least three times (1:3 split) to remove myogenic cells before being used in transfection experiments. For transfection experiments, fibroblast cells were plated at  $1.5 \times 10^6$  cells per 100-mm culture plate and the medium changed the day after plating, 3 h before transfection.

## Immunofluorescence Microscopy

The properties of the mouse monoclonal antibody prepared against bacterial CAT have been described (25). This antibody is highly specific for bacterial CAT and does not cross react with vertebrate cellular proteins. The rabbit antisera prepared against desmin binds to intermediate filaments in skeletal, cardiac, and smooth muscle but not to the vimentin intermediate filaments in fibroblasts (6, 17).

For double-label immunofluorescence analysis of transfected cells, cultures were rinsed with PBS and fixed for 5 min at room temperature in 4% formaldehyde in PBS. The cells were then rinsed twice briefly with PBS and coverslips placed in ice cold methanol for 4 min followed with ice-cold acetone for 2 min. Cells were then immediately rehydrated in PBS and washed three times for a total of 15 min with PBS. To stain cells simultaneously

with the rabbit polyclonal anti-desmin (gift of H. Holtzer, University of Pennsylvania [Philadelphia, PA] and ICN Nutritional Biochemicals, Cleveland, OH) and mouse monoclonal anti-CAT (gift of C. Gorman, Genentech Corp., South San Francisco, CA), coverslips were incubated simultaneously with the appropriate dilution of each antibody in a humid chamber at 37°C for 1 h, washed three times for a total of 30 min with PBS, and subsequently incubated with a 1:200 dilution each of rhodamine-labeled goat anti-mouse IgG and fluorescein-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) in a humid chamber at 37°C for 1 h. For some experiments, after primary antibody incubation and washing, CAT antibody was visualized by incubating cells with a 1:200 dilution of biotinylated horse anti-mouse IgG (Amersham Corp.) for 1 h at 37°C, followed by a 1:200 dilution of Texas Red-conjugated streptavidin (Amersham Corp.) at room temperature for 30 min. Cells were then washed three times for a total of 30 min with PBS, dehydrated in a graded ethanol series and mounted in Mowiol. In some preparations, the DNA binding fluorochrome DAPI (Sigma Chemical Co., St. Louis, MO) was used to visualize nuclei. Cells were examined with either a Leitz or a Zeiss epifluorescence microscope using the appropriate filters to selectively visualize fluorescein, rhodamine or Texas Red fluorescence.

Purity of the muscle cultures was monitored immunochemically to establish the fraction of muscle and nonmuscle cells at various times after plating. For skeletal muscle cells, 75 to 90% of the nuclei in skeletal muscle cultures resided in desmin-positive cytoplasm even after 72 h in culture. Heart cell cultures are 70-80% cardiac myocytes 24 h after plating; however, after 72 h in culture, the proportion of cardiac myocytes dropped to 50-60%. Based on incorporation of [<sup>3</sup>H]thymidine, this shift was apparently due to the lower mitotic rate of myocardial cells than nonmyocardial cells in culture because, over a 48 h period, only 17% of myocardial cells showed nuclear incorporation of [<sup>3</sup>H]thymidine, as compared with 66% for nonmyocardial cells.

## Cell Transfection

Twenty-four h after cell plating the medium was changed and 3 h later the cells were transfected with DNA-calcium phosphate precipitates as described by Graham and van der Eb (27) and Gorman, Moffit and Howard (25) using 10 μg of plasmid DNA which has been purified by banding twice on cesium chloride-ethidium bromide density gradients. After 4 h at 37°C, the medium containing the DNA precipitate was removed; cells were washed with 10 ml of serum-free medium and then treated with 1 ml of 15% glycerol in a Hepes (N-2-hydroxylpiperazin-N'-2-ethanesulfonic acid)-buffered sodium phosphate solution for 10 s (muscle cells) or 30 s (fibroblast cells) at room temperature (25). The glycerol solution was removed and the cells were washed with 10 ml of serum-free medium and then incubated with 10 ml of complete medium at 37°C for 48 h. Transfection of DNA into cardiac cell cultures was carried out as described above except that the transfection cocktail/medium mixture was left on the cells for 6 h, after which it was removed and the cells rinsed once with fresh nutrient medium and then glycerol shocked for 30 s.

Several measures were taken to ensure that CAT activity differences among promoters are not due to transfection variability. First, all comparisons are based on data collated from at least six separate experiments, except in a few cases where noted. Second, all constructions to be compared were transfected simultaneously in each experiment onto duplicate or triplicate plates. The values observed were reproducible from experiment to experiment. Third, the same DNA-calcium phosphate transfection cocktail was applied both to muscle and fibroblast cultures within each experiment. Fourth, different preparations of each plasmid construction were used in parallel experiments. These last two measures were taken to rule out either transfection cocktail or plasmid DNA preparation as potential sources of the differences observed. Using these procedures, the SEM, in most cases, is less than 10% of the observed signal. Experiments using co-transfection of a second control plasmid (63) showed competitive interference between the control promoters and the cTNT promoters. This was due to a property inherent in the cTNT promoter because co-transfection of RSV-β-galactosidase (63) did not interfere with expression of the HSV tk promoter in the cell systems reported here. In experiments using the RSV-β-galactosidase as the control plasmid, the mean corrected level of CAT activity directed by the HSV tk promoter and the SEM were essentially identical to that obtained when the above precautions were taken. Both methods gave activity values with SEM of less than 10% indicating that both procedures gave equivalent results.

The activity differences among constructions were also analyzed by statistical methods. The data were subjected to a two-way analysis of variance, or an analysis of variance for repeated samples, using Crunch statisti-

cal software (Crunch Softwares, Oakland, CA) to determine whether the CAT activity values determined for any two constructions were statistically different. Promoter activities were considered different only when the confidence level is within  $P = 0.01-0.05$ .

### CAT Assay

Cell extracts were prepared by sonication and centrifugation (25) and CAT activity was assayed using [ $^{14}$ C]chloramphenicol (25, 63). The amount of extract employed was varied to ensure that CAT activity was measured within the linear range. The linearity of the CAT assay reaction was determined empirically. Reaction products were separated by ascending chromatography on silica gel plates using chloroform-methanol (90:10 vol/vol). After autoradiography, acetylated and unreacted [ $^{14}$ C]chloramphenicol were quantified by liquid scintillation counting.

## Results

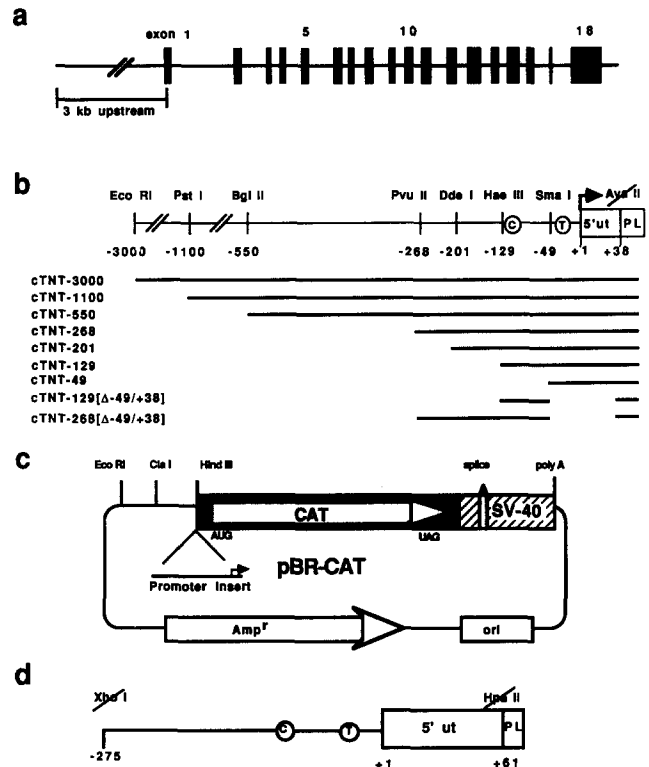
### Construction of cTNT-CAT Fusion Genes

The overall structure of the chicken cTNT gene, as shown in Fig. 1 *a*, was determined by nucleotide sequencing of the cloned gene (12). The promoter/upstream region of the cloned cTNT gene was isolated by partial digestion at an *Ava* II restriction endonuclease cleavage site located 38 nucleotides into exon 1, and an artificial *Eco* RI site located 3 Kb upstream (Fig. 1 *b*). This cTNT promoter/upstream segment was then cloned into the *Hind* III site of pBR-CAT (Fig. 1 *c*). This construction (cTNT-3,000) fuses the cTNT untranslated first exon to the untranslated region of the CAT gene, and places the transcription of the CAT gene under control of the cTNT promoter and transcription initiation site. Progressive deletions from the 5' end of cTNT-3,000 were made by partial or complete digestion at convenient restriction endonuclease cleavage sites (Fig. 1 *b*). Each deletion segment was then cloned into the *Hind* III site located upstream of the CAT gene in pBR-CAT (see Fig. 1 *c* and Materials and Methods). This created a nested set of cTNT promoter/upstream deletion mutants which had identical 3' endpoints in exon 1 and identical 5' flanking vector sequences, but which lacked varying amounts of upstream DNA as diagrammed in Fig. 1 *b*.

### Transfection of cTNT-CAT Fusion Genes into Embryonic Skeletal Muscle Cultures

Mononucleated myoblasts from day 11 chicken embryos were plated at  $1.5 \times 10^6$  cells per 100-mm plate and cultured using standard procedures. After  $\sim 48$  h in culture, the mononucleated myoblasts fuse to form differentiated, multinucleated myotubes. For transfection experiments, a calcium phosphate precipitate containing 10  $\mu$ g plasmid DNA was introduced into cultures 24 h after plating, when the level of myocyte differentiation is very low. 48 h after transfection, the well-differentiated muscle cultures were harvested for determination of the level of CAT expression. Thus, the greatest proportion of cells taking up plasmid DNA at the time of transfection were undifferentiated myoblasts, but by the time of harvest, the majority of these transfected cells had differentiated. Therefore, the CAT activity detected was attributable to expression within differentiated myotubes. This conclusion is further supported by immunocytochemical analyses described below.

Each of the cTNT deletion mutants shown in Fig. 1 *b* was transfected into embryonic skeletal muscle cultures and the resulting CAT activity determined. Table I shows the collated



**Figure 1.** Construction of expression constructs containing the chicken cTNT promoter/upstream region and the chicken beta-actin promoter region linked to the CAT coding sequence. (*a*) The structure of the 9.2-kb chicken cTNT gene. Closed boxes represent the 18 exons of the cTNT gene; thin lines represent intron and flanking sequences. (*b*) The cTNT promoter/upstream region. Upper portion is an expanded representation of the 3-kb upstream segment of the cTNT gene shown in (*a*). T and C represent the putative TATA and CAAT homologies; 5' ut, 5' untranslated region of the cTNT gene; (PL) polylinker region; arrow indicates the transcription initiation site. The *Ava* II site was destroyed in the process of cloning. Lower portion shows the cTNT promoter/upstream deletion segments generated by digestion of the 3-kb segment at the restriction enzyme sites shown. These segments were cloned into pBR-CAT as described in the text. (*c*) Structure of pBR-CAT (63). (Shaded box) coding sequence of the CAT gene with arrow showing the direction of transcription and the AUG codon is indicated; (hatched box) SV-40 splice signal and polyadenylation sequence. Test promoter segments were cloned into the *Hind* III site. Thin line represents pBR322 sequence; ori in open box, origin of replication of pBR322; Amp<sup>r</sup>, ampicillin resistance gene with arrow showing the direction of transcription. (*d*)  $\beta$ ACT-275. The beta-actin promoter segment was excised from the beta-actin gene by partial digestion with *Hpa* II and by complete digestion with *Xho* I and cloned into pBR-CAT as described in the text. Both the *Xho* I and *Hpa* II sites were destroyed in the cloning step. C, T, and PL indicate CAAT and TATA homologies and polylinker region, respectively.

data from at least six independent experiments for each deletion mutant. Multiple experiments using triplicate samples and at least two different DNA preparations were used to control for transfection efficiency. Co-transfection of control markers with the cTNT-CAT constructions resulted in competition between the cTNT test promoter and control promoters. To ensure consistency between experiments, all the constructions were transfected simultaneously and control constructions (pBR-CAT, RSV-CAT and  $\beta$ ACT-275-CAT)

**Table I. Promoter Activity in Muscle and Nonmuscle Cells**

Promoter	CAT activity in		
	Skeletal myocytes $U \times 10^{-2}$	Cardiac myocytes $U \times 10^{-2}$	Fibroblast cells $U \times 10^{-2}$
cTNT-3000	15.0 ± 3.0	1.04 ± 0.28	0.28 ± 0.02
cTNT-1100	46.0 ± 6.0	1.49 ± 0.51	0.31 ± 0.03
cTNT-550	90.0 ± 7.0	4.07 ± 1.04	0.18 ± 0.02
cTNT-268	17.0 ± 2.0	1.41 ± 0.16	0.30 ± 0.03
cTNT-201	15.0 ± 3.0	0.24 ± 0.11	0.33 ± 0.03
cTNT-129	26.0 ± 3.0	0.17 ± 0.08	0.36 ± 0.04
cTNT-49	0.16 ± 0.05	0.14 ± 0.03	0.14 ± 0.05
cTNT-129[Δ-49/+38]	0.16 ± 0.05	0.09 ± 0.06	0.07 ± 0.03
cTNT-268[Δ-49/+38]	0.13 ± 0.05	0.16 ± 0.04	0.11 ± 0.02
βACT-275	90.0 ± 4.0	6.6 ± 0.98*	139 ± 14
RSV LTR	305 ± 108	20.9 ± 4.3*	693 ± 39

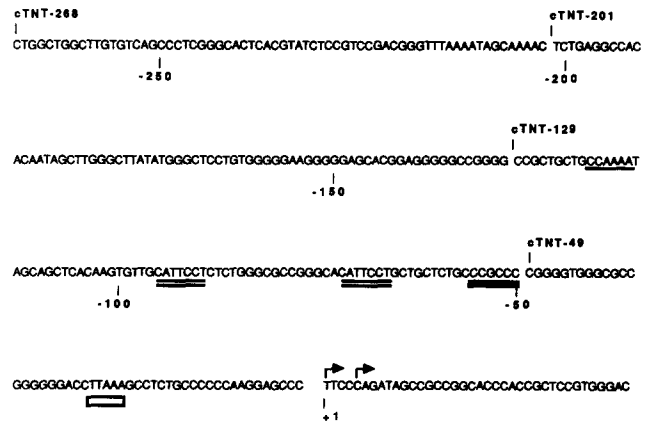
The CAT activity values shown are the average of a minimum of six determinations, except for that of cTNT-49, and cTNT-129[Δ-49/+38] for which only three determinations were made. The SEM is given in each case. The activity of pBR-CAT (0.44 ± 0.13; 0.28 ± 0.08 and 0.24 ± 0.04 for skeletal myocytes, cardiac myocytes and fibroblast cells, respectively) was subtracted from each value indicated.

\* As described in the text, the values shown for the beta actin (βACT-275) and RSV-LTR promoters in cardiac myocytes have been corrected to account for the fractional contribution of CAT activity derived from cardiac myocytes to the overall activity in these samples, determined as in Table II. The uncorrected values for the beta-actin and RSV-LTR promoters in heart cell cultures were 49.6 ± 7.4 and 158 ± 33, respectively.

were included in every experiment (for details, see Materials and Methods).

One of the primary goals of these experiments was to determine the minimum amount of upstream DNA required for activity of the cTNT promoter in embryonic skeletal muscle cells. Table I shows that cTNT-129 directs a high level of CAT expression in embryonic skeletal muscle cells. cTNT-129 contains many of sequence motifs expected for eukaryotic promoters, including putative TATA, SP-1, and CCAAT homologies, 38 nucleotides of exon 1, as well as two copies of the heptamer CATTCCCT which is found in many contractile protein gene promoter regions (see Fig. 2, and references 12 and 53). Deletion to position -49 (cTNT-49; Fig. 1), leaving only the proximal segment containing the putative TATA motif and exon 1 sequence (Fig. 2), reduces CAT activity to a basal level comparable to pBR-CAT which lacks a eukaryotic promoter (Table I). Similarly, the cTNT promoter is inactive in the absence of the proximal segment, between positions -49 and +38 (Table I). These internal deletion experiments indicate that both the proximal and distal segments of cTNT-129 appear to be required for activity in skeletal muscle cells.

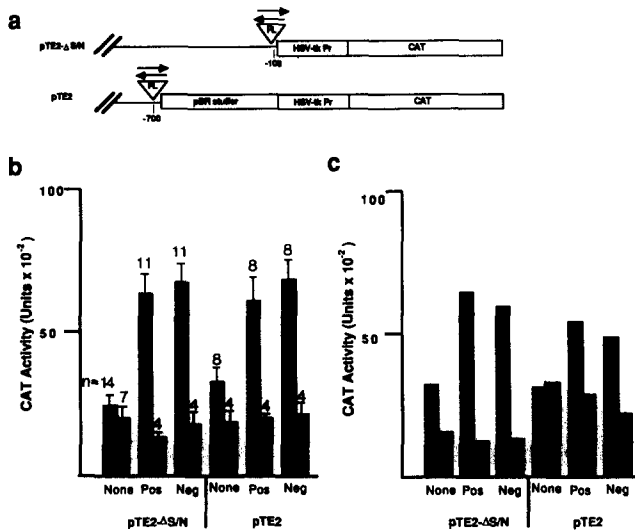
In addition to defining an approximate minimum promoter region, the results with the nested set of upstream deletions indicate that the presence of regions upstream of the -129 position can affect the overall level of activity of the cTNT minimum promoter. Inclusion of the region between positions -268 and -129 (cTNT-201, and cTNT-268, Table I) has little effect upon activity. However, additional inclusion of the -550 to -269 segment results in a threefold increase in the level of CAT expression (cTNT-550, Table I). Inclusion of additional upstream DNA (cTNT-1,100 and cTNT-3,000; Table I) results in diminution of the level of



**Figure 2.** Nucleotide sequence of the 5' flanking region of the cTNT gene. The nucleotide sequence of the 5' flanking 268 nucleotides and the first 38 transcribed nucleotides of the cTNT gene was determined as described elsewhere (12). The endpoints of several deletion mutants used in these studies are shown. The position of a putative CCAAT, SP-1 and TATA homologies are indicated by thin, thick and open bar underlining, respectively. Two copies of a conserved heptamer (53) are shown by double underlining. The signature double initiation site for the cTNT gene in embryonic skeletal and cardiac muscle (12) is shown by the double arrows.

CAT expression. These effects of upstream regions could be due to the presence of positive and negative regulatory elements or to effects of spacing between the cTNT promoter and plasmid vector sequences.

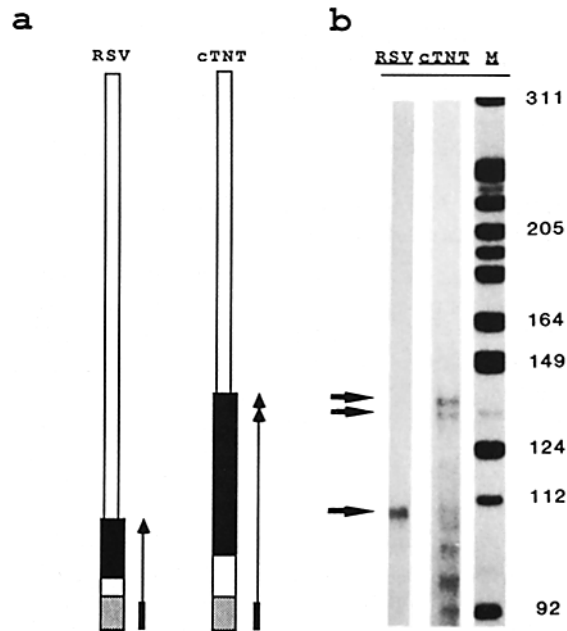
To determine if the apparent positive effect of the -550/-269 region could act independent of surrounding sequences, we tested its ability to affect CAT expression under the control of a heterologous promoter. For these experiments we used a series of pUC-based plasmids designed for testing potential enhancer-containing DNA segments. Test sequences may be inserted into a polylinker located either at a distal site 600 nucleotides upstream (pTE-2), or a proximal site immediately upstream (pTE-2ΔS/N) of the Herpes simplex virus thymidine kinase (HSV tk) gene promoter (15). The level of CAT activity expressed under control of the HSV tk promoter in the plasmids carrying a test segment was then compared with that of the parent plasmid. The -550/-269 segment was excised and cloned into these two plasmids via linkers in both orientations (Fig. 3 a). Fig. 3 b shows that the presence of the -550/-269 segment increased expression of CAT under control of the HSV tk promoter approximately two- to threefold in skeletal muscle cultures, in an orientation- and position-independent manner. Little or no potentiation was observed after transfection into fibroblast cultures (Fig. 3 b). Unlike the cTNT promoter, the HSV tk promoter was found to be minimally affected by the presence of a co-transfection marker. Using RSV-β-galactosidase as a co-transfected marker to control for transfection efficiency the preferential potentiation of CAT activity in muscle cells transfected with test plasmids containing the -550/-269 segment was also evident (Fig. 3 c). Thus, the level of potentiation of CAT expression from the HSV tk promoter by the -550/-269 segment is similar to that observed when it is present in its normal position upstream of the cTNT promoter.



**Figure 3.** Test of ability of  $-550/-269$  segment to enhance expression from a heterologous promoter. (a) The cTNT-550/-269 segment was inserted into a polylinker (PL) in either a proximal or distal position upstream of the HSV tk promoter in the plasmids pTE-2ΔS/N or pTE-2, respectively (15). Recombinant plasmids with the insert in either a positive or negative orientation (indicated by arrows) relative to its normal position in the cTNT gene were selected and transfected into skeletal muscle and fibroblast cultures. (b) The average CAT activity and SEM of each parental tk-CAT plasmid without an insert (designated none), and tk-CAT containing the  $-550/-269$  segment in the positive (pos) or negative (neg) orientation is shown. The number of independent determinations (n) for each experiment is shown above the corresponding bar. For details see Materials and Methods. (Black bars) CAT activity in skeletal muscle cells; (striped bars) CAT activity in chicken embryo fibroblasts. (c) As in b except that CAT activity was normalized to co-transfected RSV-β-galactosidase. Values are derived from duplicate samples.

### Transcriptional Strength of the cTNT Promoter in Skeletal Muscle Cells

The transcriptional strength of the cTNT promoter/upstream segments in skeletal muscle cells was estimated by comparing CAT activity governed by each of them with that directed by two control promoters, those of the Rous sarcoma virus long terminal repeat (RSV-LTR) and the chicken beta-actin gene ( $\beta$ ACT-275, see Fig. 1 d). Both of these promoters are strong chicken promoters which are active in a wide variety of cell types (19, 24). The construction of RSV-CAT has been described (24). The  $\beta$ ACT-275 plasmid contains the DNA segment between  $-275$  and  $+40$  relative to the site of transcription initiation in the chicken beta-actin gene (see Fig. 1 d). Although the endogenous beta-actin gene is down-regulated in differentiated muscle cells in vivo, we found the activity of  $\beta$ ACT-275-CAT was similar in transfected skeletal muscle and fibroblast cells (Table I). Thus, the *cis* sequences required for beta-actin gene down regulation do not appear to be present (or active) on the segment we have isolated, so  $\beta$ ACT-275-CAT serves as a good cell type non-specific control promoter in these experiments. The level of CAT expression under control of the  $\beta$ ACT-275 was equivalent to that under control of cTNT-550, the strongest cTNT promoter/upstream segment (Table I). Both of these cellular promoters were  $\sim 1/3$  as active as the RSV-LTR (Table I).



**Figure 4.** Primer extension analysis of CAT mRNA in transfected chick primary skeletal muscle cells. (a) Diagrammatic representation of the structure of the initiation site regions of RSV-CAT and cTNT-550-CAT. (Short open bar) 5' flanking DNA; (tall open bar) polylinker DNA; (long black bar) exon 1 sequence; (long stippled bar) CAT gene sequence. Thick and thin portions of vertical arrow represent oligonucleotide primer and extended cDNA, respectively. Arrowheads indicate the position of the single expected initiation site for RSV-CAT and the signature double initiation sites of the cTNT gene. (b) Autoradiograph of 6% sequence gel showing primer extension run-off products. Horizontal arrowheads show the position of the run-off products for 10 μg total RNA from; (RSV) cultures transfected with RSV-CAT at 109 nucleotides; and (cTNT) the double run-off bands at 145 and 141 nucleotides for cultures transfected with cTNT-550-CAT. Nonspecific terminations below 100 nucleotides were present in both samples but are more visible in the cTNT lane because it was exposed five times longer than the RSV lane. Only the full-length run-off products indicated by the arrows were scanned for quantitation. Size markers are derived from a Hpa II digest of pBR-322 endlabeled with [<sup>32</sup>P]dCTP using reverse transcriptase.

Since the RSV-LTR is one of the strongest known viral promoters (24, 63) and is stronger than any known cellular promoter, we conclude that both cTNT-550 and  $\beta$ ACT-275 are strong cellular promoters in skeletal muscle cells.

The site of transcription initiation from the transfected cTNT and RSV promoters was determined by primer extension using an oligonucleotide primer complementary to codons 6-11 of CAT mRNA (63). RNA from cultures transfected with RSV-CAT gave a primer extension run-off length of 109 nucleotides indicating that CAT mRNAs governed by the RSV promoter were initiated at the expected site (Fig. 4; reference 63). RNA from cultures transfected with cTNT-550-CAT yielded two predominant run-off lengths of 141 and 145 nucleotides (Fig. 4), indicating that initiation occurred at the first and fifth nucleotides of cTNT exon 1. This double initiation signature is characteristic of cTNT transcription initiation (see Fig. 2 and reference 12) and indicates that transcription initiation from the transfected gene mimics

that of the endogenous gene. The relative level of correctly initiated CAT mRNA under control of the cTNT and RSV promoters was estimated by densitometry scanning of the films to be 0.2, in good agreement with the relative level of CAT activity of 0.3 specified by the two promoters (Table I).

### ***Transfection of cTNT-CAT Fusion Genes into Embryonic Heart Cell Cultures***

Heart cell cultures were established using cells dissociated from day 6 and day 10 embryo hearts. Cultures from day 6 embryo hearts were chosen for the experiments reported below because they were found to be more easily transfected than those from day 10 embryos. Results with heart cell cultures from either age embryo were, however, qualitatively identical. The optimum schedule for transfection of myocardial cultures was found to be the same as that for skeletal muscle cultures; i.e., cells were transfected 24 h after plating and harvested for CAT activity 48 h later. Myocardial cell cultures were less homogeneous, in terms of the proportion of myogenic cells, than skeletal muscle cultures. Immunocytochemical analysis showed that ~80% of the nuclei resided in cytoplasm binding antibody against the muscle specific intermediate filament protein desmin after 24 h in culture. However, after 72 h in culture the proportion of desmin-positive cells had decreased to 50–60%. This change was attributable to a higher rate of cell division for nonmyocardial cells as demonstrated by a fourfold higher rate of thymidine incorporation (see Materials and Methods).

The transfection efficiency of myocardial and nonmyocardial cell types within embryonic heart cell cultures was also found to differ. Using a monoclonal antibody for CAT protein (23), transfected cells could be detected immunocytochemically. Table II shows that in heart cell cultures transfected with RSV-CAT, an average of 6.5 times more nonmyocardial (desmin-negative) cells were CAT-positive than myocardial cells (desmin-positive). Thus, the overall transfection efficiency of myocardial cells is much lower than that of nonmyocardial cells. These immunofluorescence data indicate, as a first approximation, that of the total CAT activity directed by the RSV-LTR or beta-actin promoters measured in transfected heart cell cultures, only 1 part in 7.5 is attributable to activity in myocardial cells (see legend to Table I). The lower levels of CAT activity for heart cultures vs. skeletal muscle cultures (Table I), therefore, reflect the lower percentages of myogenic cells transfected in heart cultures. Skeletal muscle and fibroblast cultures were transfected with similar efficiency (~2%, data not shown).

Table I shows the CAT activity levels specified by each member of the set of cTNT deletions after transfection into heart cell cultures. These results indicate that the shortest deletion mutant which is active in cardiac cells contains 268 upstream nucleotides (see Fig. 2 for nucleotide sequence). Deletion to position -201 essentially abolishes CAT activity under control of the cTNT promoter, reducing it to a level comparable to the promoterless pBR-CAT (see Table I). Similarly, cTNT-129, which was strongly active in skeletal muscle cells, is also inactive in cardiac cells. Thus, the upstream segment between positions -268 and -201 appears to be required for a significant level of cTNT promoter activity in cardiac cells.

The region between -550 and -269, while not required for activity, also increases the level of CAT expression under

**Table II. Immunocytochemical Analysis of Transfection Efficiency in Heart Cell Cultures**

Experiment no.	Number of CAT-positive cells staining		Ratio
	Desmin-positive	Desmin-negative	
1	28	202	11.3
2	36	121	4.3
3	80	337	4.6
			Av. 6.5

Heart cell cultures established on aclar coverslips in 35-mm dishes were transfected 24 h after plating, essentially as described in Materials and Methods. 48 h after transfection, the cells were processed for immunocytochemistry as described in Materials and Methods. Every CAT-positive cell on a 22 mm<sup>2</sup> aclar coverslip was determined and assayed for staining with anti-desmin. The percentage of desmin-positive cells was then determined for each experiment to allow normalization of the relative number of myocardial and nonmyocardial cells for determination of relative transfection efficiency (Ratio). The fraction of desmin-positive cells was 0.61, 0.56 and 0.52 for experiments 1, 2, and 3, respectively. Each transfection experiment was conducted independently by a separate operator and the immunocytochemical counting performed by a fourth operator.

control of the cTNT promoter in cardiac cells by approximately threefold, in a manner similar to its effect in skeletal muscle cells. In addition, the regions upstream of position -550 appear to suppress the maximal activity of the cTNT promoter similar to the effect of this region in skeletal muscle cells. Therefore, the effects of upstream regions upon activity of the minimum cTNT promoter in both cardiac and skeletal muscle appear to be similar.

The transcriptional strength of the cTNT promoter in cardiac myocytes was also evaluated by comparison to that of the RSV-LTR and beta-actin promoters. As indicated above, the beta-actin and RSV-LTR promoters are preferentially expressed in nonmyocardial cells owing to a difference in transfection efficiency. Since the cTNT promoter was only expressed in myocardial cells (see below), the measured level of CAT activity directed by each of the cTNT constructions was directly comparable to that of the corrected values of the beta-actin and RSV-LTR promoters as shown in Table I. Using this comparison, the activity of cTNT-550 is approximately equivalent to that of the beta-actin promoter in cardiac muscle cells. Thus, the relative strength of cTNT-550 appears to be approximately equivalent in embryonic cardiac and skeletal muscle cells. The low efficiency of transfection of myocardial cells precluded using primer extension to determine the transcription initiation site of the transfected cTNT promoter. However, the site of transcription initiation from the endogenous cTNT gene is identical in embryonic cardiac and skeletal muscle cells (12) so there is no a priori reason to suggest that the transfected cTNT gene would use a different site in cardiac muscle cultures than in skeletal muscle cultures. In addition, deletion of the -49 to +38 region of cTNT-268 (which contains the putative TATA motif, transcription initiation sites and exon 1; see Fig. 2) abolishes activity in cardiac cells (cTNT-268[Δ-49/+38]; Table I), which is also consistent with expression being dependent upon transcriptional initiation at the natural cTNT sites.

### ***Cell-specificity of the cTNT Promoter***

The cell specificity of the cTNT and control promoters was assessed by comparing the level of CAT expression in skele-



tal or heart muscle cells with that in fibroblast cells. Chicken embryo fibroblasts were prepared and cultured under standard conditions and passaged at least three times before use in transfection experiments to eliminate potentially myogenic cells. Fibroblast cultures were transfected 24 h after plating and harvested 48 h later. In most cases, the same transfection cocktail was simultaneously applied to fibroblast cultures and to myogenic cultures.

While both the beta-actin and RSV-LTR promoters were found to be highly active in fibroblast cells, the activity of the cTNT promoter/upstream segments was extremely low in these cells (Table I). The activity of the cTNT promoter/upstream segments was only approximately twofold higher than that of pBR-CAT, which lacks a eucaryotic transcriptional promoter (Table I). Thus, the cTNT promoter appears to be essentially inactive in fibroblast cells regardless of the amount of upstream DNA present. The degree of cell specificity of each cTNT deletion mutant was estimated by comparing its level of CAT expression to that of the noncell-specific  $\beta$ ACT-275-CAT in each cell type (Table III). These results indicate that the minimum cTNT promoter in cardiac and skeletal muscle cells is approximately two orders of magnitude more active than it is in fibroblasts (Table III). With addition of the -550/-269 region, the overall level of cell specificity increases to almost three orders of magnitude for both cell types. Thus, by these criteria, the cTNT promoter appears to carry *cis* elements directing a high degree of striated muscle specific transcription.

The cell-specificity of the cTNT promoter in skeletal and cardiac muscle cells was also analyzed by immunocytochemistry. Cardiac and skeletal muscle cultures, grown on coverslips, were transfected with either RSV-CAT or cTNT-CAT and, after 48 h, were fixed and incubated simultaneously with anti-desmin and anti-CAT, as in Table II. Representative micrographs from cardiac and skeletal muscle cultures transfected with either cTNT-CAT or beta-actin-CAT and stained with anti-CAT plus anti-desmin are shown in Fig. 5. As noted elsewhere (23), CAT staining was observed both within the cytoplasm and nuclei. Desmin staining was restricted to intermediate filaments in the cytoplasm of muscle cells.

The total number of CAT-positive cells which were either

desmin-positive (cardiac or skeletal myocytes) or desmin-negative (nonmyocyte) was then determined as in Table II. After transfection with RSV-CAT both desmin-positive and desmin-negative cells stained positively for CAT in cardiac and skeletal muscle cultures indicating that the RSV promoter is active in all three cell types (Table IV). The relative number of CAT-positive nonmyocytes is low in skeletal muscle cultures (Table IV, ‡), owing to the relative scarcity of these cells in these cultures (see Methods in Materials and Methods). The relative efficiency of transfection of myocytes and nonmyocytes appears to be approximately equivalent in embryonic skeletal muscle cultures (see legend, Table IV, ‡). However, in heart muscle cultures transfected with RSV-CAT (Table IV, \*), the proportion of CAT-positive nonmyocytes is higher owing to the higher relative number of nonmyocardial cells, and their higher degree of transfectability, in agreement with the data presented in Table II.

When transfected with cTNT-550-CAT, on the other hand, only desmin-positive cells were observed to be CAT positive either in embryonic heart cell cultures or in embryonic skeletal muscle cultures (Table IV). In the thousands of cells screened by this procedure, no CAT-positive/desmin-negative cells have ever been observed after transfection with cTNT-CAT. These results corroborate those based upon biochemical determination of CAT activity and indicate that the expression of the cTNT promoter/upstream segments tested here is highly specific for the same two striated muscle cell types to which it is restricted in vivo.

## Discussion

In these experiments, we have used the CAT marker gene (24, 25) to analyze the transcriptional activity of the cTNT promoter/upstream region after transient transfection into primary cultures of chicken embryo cardiac and skeletal muscle cells, and fibroblast cultures. This approach has been used successfully in characterization of muscle actin gene promoters (5, 28, 51). However, no direct comparison has been made of the activity of one muscle promoter in both cardiac and skeletal muscle cells. We found that direct comparisons of transcriptional activity of cTNT-CAT constructions in cardiac and skeletal muscle cells were complicated

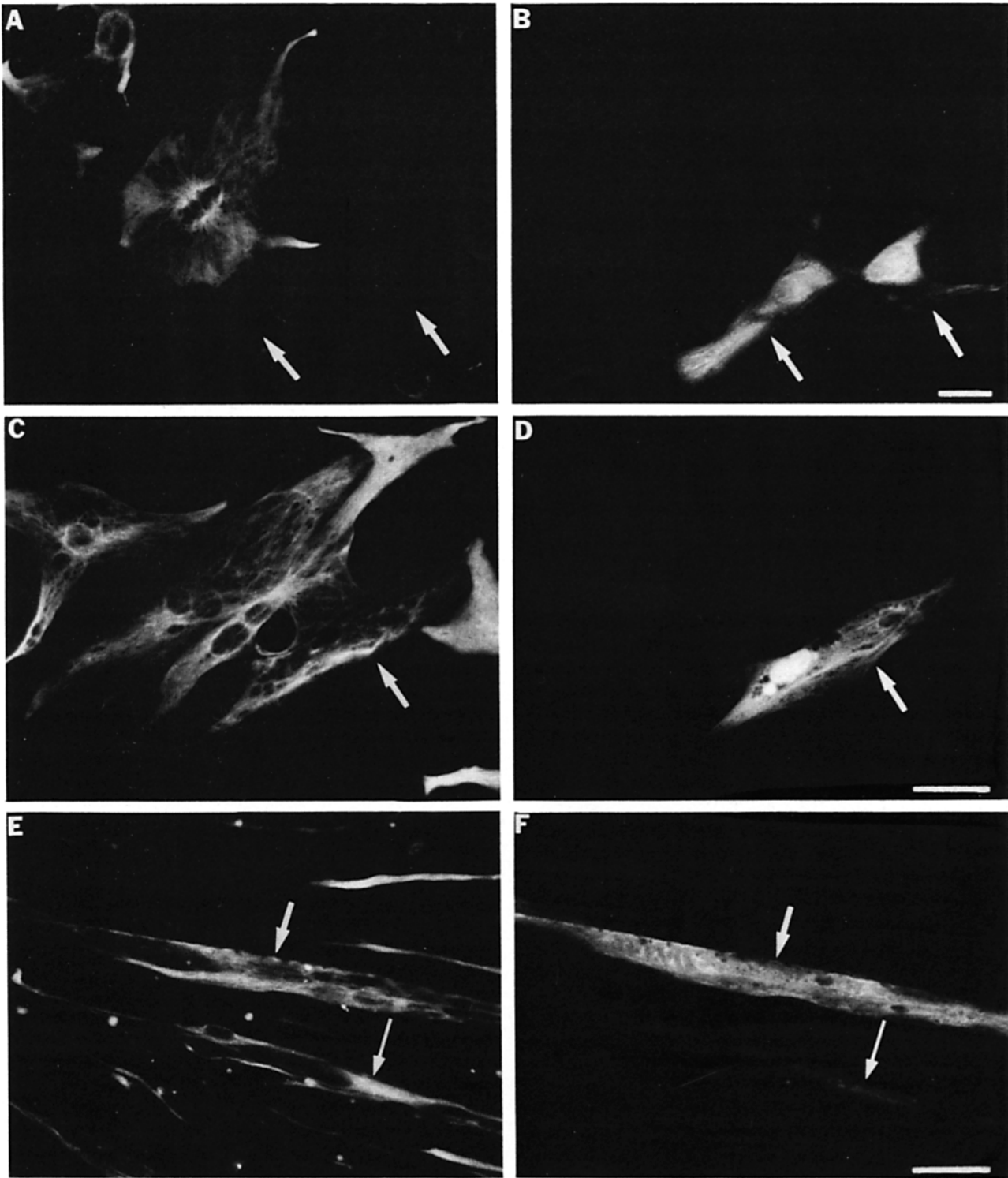
Table III. Relative Activity of cTNT Promoter Segments in Muscle and Nonmuscle Cells

Promoter	Activity relative to $\beta$ ACT-275 in			Activity ratio*	
	Skeletal myocytes	Cardiac myocytes	Fibroblast cells	Sk/F	Ca/F
cTNT-3,000	0.167	0.158	0.0020	83	79
cTNT-1,100	0.511	0.226	0.0022	229	102
cTNT-550	1	0.617	0.0013	772	478
cTNT-268	0.189	0.214	0.0022	88	99
cTNT-201	0.167	0.036	0.0024	70	—
cTNT-129	0.289	0.026	0.0026	112	—
cTNT-49	0.0018	0.021	0.0010	—	—
cTNT-129[ $\Delta$ -49/+38]	0.0018	0.014	0.0005	—	—
$\beta$ ACT-275	1	1	1	1	1
RSV-LTR	3.39	3.2	5.0	1	1

Using the data from Table I, the activity of the beta-actin promoter in each cell type was set at unity, and the relative activities for all other constructions calculated relative to that.

\* The ratio of activity for each construction in skeletal myocytes vs. fibroblast cells (Sk/F) and cardiac myocytes vs. fibroblast cells (Ca/F). Ratio values are rounded off to the nearest whole number, and are omitted where only nominal activity was detected in skeletal or cardiac myocytes.





**Figure 5.** Double-label immunofluorescence micrographs of transfected primary embryonic heart (A–D) and skeletal (E and F) muscle cultures. Cultures were transfected and fixed as indicated in the text, and stained simultaneously with a rabbit polyclonal antibody against desmin and mouse monoclonal antibody against CAT. Binding of the desmin antibody was visualized using fluorescein-conjugated anti-rabbit IgG (A, C, and E); binding of CAT antibody was visualized using either a rhodamine-conjugated anti-mouse IgG (D), or using a biotinylated anti-mouse IgG followed by Texas Red-conjugated streptavidin (B and F). (A and B) cultured heart cells 48 h after transfection with  $\beta$ ACT-275-CAT. The same microscopic field visualizing two CAT-positive cells (arrows in B) which are identified as nonmuscle cells by their failure to bind the antibody against desmin (A). Three desmin-positive cardiac myocytes are visible in this field. (C and D) Cultured heart cells 48 h after transfection with cTNT-268-CAT showing the same microscopic field visualizing a small cluster of desmin-positive heart cells (C) of which one binds the CAT antibody (arrows in C and D). (E and F) Skeletal muscle culture 48 h after transfection with cTNT-550-CAT. Two of the desmin-positive myotubes visible in (arrows in E) also bind the CAT antibody in F. Variations in the intensity of CAT staining, both between myotubes and along the length of a single myotube, were frequently observed. Bars, 20  $\mu$ m.

**Table IV. Immunocytochemical Analysis of Cardiac and Skeletal Muscle Specific Expression of the cTNT Promoter**

Cardiac muscle cells*		
Promoter	Number of CAT-positive cells staining	
	Desmin-positive	Desmin-negative
RSV	38	220
cTNT	40	0
Skeletal muscle cells†		
Promoter	Number of CAT-positive cells staining	
	Desmin-positive	Desmin-negative
RSV	429	33
cTNT	533	0

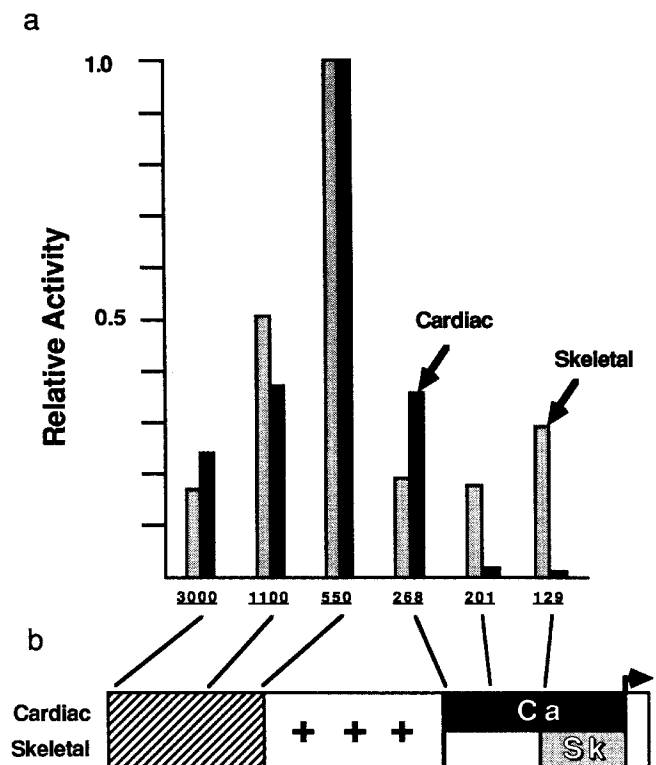
\* Immunocytochemical analysis of cardiac cell cultures which had been transfected with either cTNT-CAT or RSV-CAT was conducted essentially as described in Table II. The fraction of desmin-positive cells in cultures transfected with RSV-CAT and cTNT-CAT were 0.5 and 0.52, respectively.

† Skeletal muscle cell cultures were transfected 24 h after plating, and processed for immunocytochemistry as described in Table II and in Materials and Methods. The total number of CAT-positive cells on a 22 mm<sup>2</sup> aclar coverlip was counted and each assayed for staining with anti-desmin. Each desmin-positive/CAT-positive cell was counted as a single cell regardless of the number of nuclei present on the assumption that only one transfected nucleus was present per myotube. At the time point chosen ~75–90% of the cells in these cultures stain positively with anti-desmin, similar to control, nontransfected cultures. Since the relative transfection efficiency of muscle and nonmuscle cells appears to be approximately equal in these cultures, no correction for the relative number of desmin-positive and desmin-negative cells has been made.

by the fact that transfected DNA enters each cell type with very different efficiencies. While ~2% of skeletal muscle cells were CAT-positive by immunofluorescence, the percentage of CAT-positive myocardial cells was an order of magnitude lower (compare to Table IV). To calibrate the strength of each cTNT promoter/upstream segment in each cell type, therefore, we have compared the level of CAT activity under their control with that directed by the promoters of the chicken beta-actin gene and the RSV-LTR, which are strong cellular and viral promoters, respectively. In our hands, neither of these standard promoters showed any appreciable degree of cell specificity, as evidenced by the fact that they are highly active in all cell types tested and that their activity relative to each other remained relatively constant in the cell types tested (Tables I and III). The most active cTNT upstream segment in both cardiac and skeletal muscle cells (cTNT-550) directed levels of CAT activity equivalent to that directed by the beta-actin promoter, indicating that the cTNT promoter is a relatively strong promoter in cardiac and skeletal muscle cells. Moreover, the similarity in maximal activity of cTNT-550 in both cardiac and skeletal muscle cells served as a means for comparing the effects of other deletions in the two cell types. Fig. 6 summarized the data from Table I by comparing the relative activity of each upstream region of the cTNT gene in the two cell types. Our results show that while different minimum promoter/upstream regions are required for efficient activity in each cell type, other nonessential upstream regions may act in a similar manner in both cell types to modify the activity of those minimum regions.

### The Minimum Regions Required for Expression of the cTNT Promoter in Cardiac and Skeletal Muscle Cells

Embryonic cardiac and skeletal muscles differ in the mini-



**Figure 6.** Relative activity of the cTNT promoter segments in embryonic chicken cardiac and skeletal muscle cells. (a) The CAT activity value for each cTNT promoter segment shown in Table I have been normalized to that of cTNT-550 in each cell type. The value of cTNT-49 (see Table I), which is not active in either cell type, has been subtracted from the CAT activity value of each active cTNT promoter segment. (Solid bars) Relative CAT activity value in cardiac cells; (stippled bars) CAT activity value in skeletal muscle cells. (b) Diagram of the minimal cTNT promoter segments required for efficient transcription activity. Region which enhances the activity of the minimal promoter is shown with + + +; (hatched area) regions which had a negative effect on cTNT promoter activity in these experiments; arrow indicates transcription initiation site. Solid and stippled blocks indicate minimum regions required for expression in cardiac and skeletal muscle cells, respectively. The segment between -3,000 and -550 is not drawn to scale with that downstream of -550.

um 5' flanking regions required for cTNT gene expression. The minimum active segment in cardiac cells contains 268 upstream nucleotides (Fig. 6, a and b). The activity of the -268 segment is ~1/3 that of the most active segment which contains 550 upstream nucleotides (see Fig. 6 a, and below). Deletion of 67 nucleotides, to position -201, abolishes activity (Fig. 6 a, and Table I), to a level comparable to pBRCAT which lacks a eucaryotic promoter (63). This indicates that, as a minimum, the 67 nucleotide segment between positions -268 and -201 contains DNA sequences which are required for efficient expression of the cTNT promoter in embryonic cardiac cells. Obviously, sequences downstream of the -201 position may also be required (for example, see cTNT-268[Δ-49/+38], Table I), but these are not sufficient for expression in cardiac cells in the absence of the -268 to -201 segment.

In embryonic skeletal muscle cells, on the other hand, a segment containing only 129 nucleotides upstream of the

transcription initiation site is sufficient to direct approximately one-third of maximal expression (Fig. 5, *a* and *b*). Deletion to position  $-49$  abolishes activity, reducing it to a level comparable to that of pBR-CAT (Table I). This indicates that sequences within the segment between positions  $-129$  and  $-49$  are required for efficient expression in skeletal muscle cells. Addition of the segment required for efficient cardiac expression (up to  $-268$ ) has a neutral, or slightly negative, effect upon cTNT promoter expression in embryonic skeletal muscle cells. We conclude, therefore, that the minimum required segments for efficient expression of the cTNT promoter in embryonic cardiac and skeletal muscle cells are different.

### ***Nonessential Upstream Regions have Similar Effects in Both Cardiac and Skeletal Muscle Cells***

Our deletion experiments also indicate that inclusion of two upstream regions can affect the activity of the minimum cTNT promoter segments in the two cell types. While neither region is required for the activity of the minimum segments, the effect of each region is similar in both cell types. One of these, residing between positions  $-550$  and  $-269$ , increases the level of expression of the minimum cTNT promoter approximately threefold in both skeletal and cardiac muscle cells (Fig. 6). Inclusion of the upstream region between positions  $-3,000$  and  $-550$  appears to have a negative effect on CAT expression, essentially abolishing the apparent positive effect conferred by the  $-550/-269$  region. Negative-acting upstream sequences have been found in many genes (7, 22, 26, 42, 52, 54), although their role *in vivo* is not yet clear. The magnitude of the effects that these two upstream regions have upon CAT activity, although reproducible, is quite small which raises the possibility that these effects are artifactual interactions between cTNT upstream sequences and host vector sequences which can affect the activity of passenger promoters.

When the  $-550/-269$  region is placed upstream of the HSV tk promoter, in a pUC-based plasmid vector, it is also able to potentiate CAT expression approximately two- to threefold, in a position- and orientation-independent manner (Fig. 3). The magnitude of this effect is similar to that observed for this segment in its native position within the cTNT upstream region (Fig. 6). Moreover, the potentiating effect is observed in skeletal muscle cells, but not in fibroblast cells (Fig. 3, *b* and *c*). These results are consistent with the conclusion that the  $-550/-269$  region contains a muscle specific transcriptional enhancer. Preliminary experiments suggest a similar potentiating effect in cardiac cultures (data not shown) but are complicated in their interpretation by the preferential transfection of non-myocardial cells in these experiments. Nevertheless, the relatively modest effects of the putative enhancer ( $-550/-269$ ) and negative-acting ( $-3,000/-550$ ) regions indicate that further work will be required to unequivocally define their roles, if any, in the regulation of the cTNT promoter in cardiac and skeletal muscle cells.

### ***Sequences Controlling Tissue Specificity of the cTNT Promoter***

Transfection of the cTNT-CAT genes into embryonic cardiac and skeletal muscle cells demonstrated that the activities of minimal active promoter segments are about two orders of magnitude higher than in fibroblast cells (Table III). There-

fore, a high degree of cell specificity resides within the minimum promoter segments defined by these experiments. However, when the upstream DNA is extended to position  $-550$  (cTNT-550) expression in cardiac and skeletal muscle cells is over 400- and 700-fold higher than in fibroblast cells. Thus, the presence of the  $-550/-269$  segment has a modest quantitative effect upon the level of expression, as well as a modest qualitative effect upon the degree of cell specificity. As noted above, such modest effects from upstream segments can be difficult to discern from spacing effects. Nevertheless, in experiments with heterologous promoters, the potentiating effect of the  $-550/-269$  segment appears to be cell specific. The overall cell specificity of the cTNT promoter may therefore result from a combination of effects from separate domains. Such combinatorial activity of upstream elements has been shown to be important for cell specific expression of the vertebrate insulin, chymotrypsin (15, 63) and immunoglobulin (29, 46) genes. Moreover, the multiple cell specificities of the mouse alpha fetoprotein gene are determined by the combinatorial interaction of multiple upstream enhancers, each carrying a different cell tropism (21, 32). In *Drosophila*, the tissue and/or stage specific expression of the *white* (43, 57), *hsp26* (10), *fushi tarazu* (35), and yolk protein genes (20) has also been shown to be controlled by multiple upstream sequences. The cTNT gene differs from the above examples in that the contribution of upstream regions is neither strong nor required for cell specific function of the cTNT minimum promoter segments defined here. We conclude, therefore, that the cell specificity of the cTNT promoter is predominantly attributable to *cis* elements contained within its minimal promoter regions (Fig. 6 *b*), and that elements residing farther upstream play a possibly significant, but secondary role.

### ***Different Regulatory Programs Govern the cTNT Gene in Cardiac and Skeletal Muscle Cells***

The minimum upstream region required for transcription of the cTNT gene promoter in embryonic skeletal muscle cells is not sufficient for transcription in cardiac cells. Expression in cardiac cells requires the presence of additional upstream DNA which is not required for expression in skeletal muscle cells. This finding does not support the conclusion that co-expression of genes in embryonic cardiac and skeletal muscle cells reflects commonality in gene regulatory programs. Therefore, during their evolutionary divergence from a common ancestral cell type, one or both cell types has acquired novel regulatory programs for governing expression of the cTNT gene, and possibly other genes which are expressed in common during development.

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