A conserved CATTCCCT motif is required for skeletal muscle-specific activity of the cardiac troponin T gene promoter

(myogenesis/promoter element/gene transfer)

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Communicated by Norman H. Horowitz, April 25, 1988

ABSTRACT Transcription of the cardiac troponin T (cTNT) gene is restricted to cardiac and embryonic skeletal muscle tissue. A DNA segment containing 129 nucleotides upstream from the cTNT transcription initiation site (cTNT-129) directs expression of a heterologous marker gene in transfected embryonic skeletal muscle cells but is inactive in embryonic cardiac or fibroblast cells. By using chimeric promoter constructions, in which distal and proximal segments of cTNT-129 are fused to reciprocal segments of the herpes simplex virus thymidine kinase (HSV tk) gene promoter, the DNA segment responsible for this cell specificity can be localized to the cTNT distal promoter region, located between 50 and 129 nucleotides upstream of the transcription initiation site. The ability of the cTNT distal promoter region to confer skeletal muscle-specific activity upon a heterologous promoter is abolished when it is displaced 60 nucleotides upstream, indicating that its ability to direct skeletal muscle-specific transcription probably requires proximity to other components of the transcription initiation region. Two copies of the heptamer, CATTCCCT ("M-CAT" motif) or "M-CAT" motif), reside within the 80-nucleotide cTNT distal promoter region. A 3-nucleotide mutation in one of these copies inactivates the cTNT promoter in skeletal muscle cells. Therefore, the M-CAT motif is a distal promoter element required for expression of the cTNT promoter in embryonic skeletal muscle cells. Since the M-CAT motif is found in other contractile protein gene promoters, it may represent one example of a muscle-specific promoter element.

Recent evidence suggests that activation of a relatively small number of genetic elements is required to initiate the myogenic differentiation program (1–3). A major developmental problem, therefore, is understanding the mechanisms by which the large number of contractile protein genes is activated by such global signals as well as by the subsequent cell lineage- and developmental stage-specific signals that mediate the intricate gene switching patterns accompanying striated muscle development (4). One possibility is that many muscle genes have common cis regulatory elements that account for their coexpression. The presence of short conserved sequence motifs in regulatory regions of many contractile protein genes (5–11) is consistent with such a model but has yet to be directly tested.

We have been using the chicken cardiac troponin T (cTNT) gene as a model to study gene regulatory programs operating during the embryonic development of cardiac and skeletal muscle. The cTNT gene is suited for such an analysis because it is transcriptionally activated, along with a large cohort of other contractile protein genes, at the onset of cardiac and skeletal muscle development (12–15). At late fetal stages, transcription of the cTNT gene is specifically repressed in developing skeletal muscle tissue and strongly up-regulated in cardiac tissue (16). Repression of the cTNT gene does not occur in cultured embryonic skeletal muscle cells unless they are exposed to nerve or nerve extracts (17). Thus, the cTNT gene is subject to complex transcriptional regulation during cardiac and skeletal muscle development.

By using the cTNT transcriptional promoter and upstream region functionally linked to the marker gene chloramphenicol acetyltransferase (CAT; ref. 18), we have shown that efficient expression in embryonic skeletal muscle cells requires only 129 upstream nucleotides (19). Expression in embryonic cardiac cells, however, requires the presence of additional upstream segments (19). In this report, we have analyzed subsequences of the cTNT promoter for the presence of sequence elements capable of governing skeletal muscle-specific transcription. We show that the 80-nucleotide distal region of the cTNT promoter is sufficient to direct skeletal muscle-specific transcription from a heterologous proximal promoter segment. By using site-directed mutagenesis, we further demonstrate that this muscle-specific activity is dependent upon an intact CATTCCCT heptamer present in the cTNT distal promoter region. The CATTCCCT heptamer is present in the presumptive regulatory regions of other contractile protein genes (8) and may represent one example of a regulatory element shared among muscle-specific genes.

MATERIALS AND METHODS

Enzymes were purchased from New England Biolabs or Boehringer Mannheim and used according to the manufacturers' instructions. Radiolabeled compounds were purchased from Amersham. Protocols for recombinant DNA, restriction endonuclease mapping, and DNA sequence analysis were conducted by standard procedures (20). The plasmid cTNT-129 (ref. 19) contains 129 nucleotides upstream and 38 nucleotides downstream of the cTNT transcription initiation site cloned into the HindIII site of pBR-CAT (21). cTNT-113 is identical to cTNT-129 except that the segment between position −129 and −114 has been deleted. Chimeric promoters were also constructed in pBR-CAT. The proximal herpes simplex virus thymidine kinase (HSV tk) promoter segment was obtained from the double linker scanner mutant LS-105-95/56-46 (ref. 22), and the HSV tk distal promoter segment was isolated from pTE2-ΔS/N (ref. 23). The structure of each promoter construction was confirmed by nucleotide sequencing.

Abbreviations: CAT, chloramphenicol acetyltransferase; cTNT, chicken cardiac troponin T; HSV tk, herpes simplex virus thymidine kinase; M-CAT, muscle-CAT heptamer CATTCCCT.

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Standard procedures (24, 25) were used for preparation of chicken embryonic muscle and fibroblast cultures. Cells were transfected, harvested, and analyzed for CAT activity as described elsewhere (18, 19, 26, 27). All CAT activity values are expressed in \(10^{-2}\) unit (1 CAT unit = 1 nmol of chloramphenicol acetylated per 30 min at 37°C) per \(1 \times 10^6\) cells after subtracting the background CAT activity directed by promoterless pBR-CAT in muscle and fibroblast cells (0.34 ± 0.02 and 0.28 ± 0.06, respectively, in unit \(\times 10^{-2}\)).

RESULTS AND DISCUSSION

The cTNT gene segment between nucleotide positions −129 and +38 (relative to the transcription initiation site; cTNT-129, see Fig. 1a) is sufficient to direct expression of a marker gene, CAT, 2 orders of magnitude higher in cultured embryonic skeletal muscle cells than in fibroblast cells (Fig. 2a).

The high degree of preferential skeletal muscle-specific CAT expression appears to be an inherent property of the cTNT promoter because CAT is equally expressed in skeletal muscle and fibroblast cells under control of the HSV tk promoter (Fig. 2a) and other viral and cellular promoters (ref. 19 and data not shown). To begin to characterize the cis element(s) responsible for the skeletal muscle-specific activity of the cTNT promoter, we subdivided cTNT-129 into proximal and distal regions by digestion with the restriction endonuclease Sma I, which cleaves between positions −50 and −49. The proximal region contains a nominal TATA motif, transcription initiation site, and part of exon 1, whereas the distal region contains a nominal CCAAT motif and the core recognition sequence for the transcription factor SP-1 (refs. 29–31; see Fig. 1a). As expected, neither the proximal nor the distal region of the cTNT promoter was able to direct CAT expression above background levels (Fig. 3),

![Diagram of cTNT and chimeric cTNT/HSV tk promoters.](image)

Fig. 1. Structure of cTNT and chimeric cTNT/HSV tk promoters. Diagramatic representation of the promoter constructions used to drive CAT gene expression in these experiments. The proximal and distal regions of a–d are indicated. (a) The regions distal and proximal to the Sma I cleavage site at position −49 are indicated as are the positions of the nominal CCAAT, SP-1, and TATA homologies. M represents the conserved heptanucleotide CATTCCCT (see text). (b) The plasmid pTE-2ΔS/N carries the HSV tk promoter driving CAT and is described elsewhere (23). The positions of the CCAAT, SP-1, and TATA homologies are shown and correspond to the second distal signal (DS-2), first distal signal (DS-1), and proximal signal (PS), respectively, as defined by McKnight and Kingsbury (28). (c) The tk/cTNT chimera contains the tk distal promoter segment fused to the cTNT proximal promoter segment. (d) The cTNT/tk chimera contains the cTNT distal promoter segment fused to the HSV tk proximal promoter segment. (e and f) The distal cTNT promoter segment was inserted into the polylinker region upstream of the tk promoter in either the positive (e) or the negative (f) orientation. (g) The distal conserved heptanucleotide CATTCCT in cTNT-129 was mutated by oligonucleotide-directed mutagenesis. (h) BAL-31 deletion of cTNT-129 to position −113 removed the CCAAT/CCArGG homology.
indicating that both a proximal and a distal region are required for cTNT promoter activity.

Construction of Chimeric Promoters. To determine whether one or both regions carry the cis element(s) responsible for restricting transcription to skeletal muscle cells, chimeric promoters were constructed in which the cTNT proximal and distal promoter regions were functionally linked to the reciprocal region derived from the HSV tk gene promoter (see Fig. 1b). The HSV tk promoter has been highly characterized as to the functional elements present in its proximal and distal promoter regions, neither of which can function independently (22, 32). The construction of chimeric tk/cTNT and cTNT/tk promoters was made such that the position of the known and putative conserved promoter elements approximated that of the natural promoters (Fig. 1c and d).

The chimeric tk/cTNT promoter, containing the tk distal promoter region and the cTNT proximal promoter region, was equally active in skeletal muscle cells and fibroblast cells (Fig. 3), indicating that the cTNT proximal promoter region can functionally complement a heterologous distal promoter region. CAT activity directed by this chimeric promoter is ~1 order of magnitude lower than that of the parent HSV tk promoter (compare Figs. 2 and 3), which may reflect nonoptimum positioning of various elements within each region relative to one another (22, 33). Alternatively, the cTNT proximal promoter region may, for unknown reasons, function more efficiently with its homologous distal promoter region. In either case, it is clear that the tk/cTNT chimeric promoter supports transcription of the CAT gene at comparable levels in muscle and fibroblast cells. We conclude that the cTNT proximal promoter region does not carry cis elements capable of conferring skeletal muscle transcription to a heterologous distal promoter segment.

In contrast to the above result, the cTNT/tk chimera, in which the cTNT distal promoter region is fused to the tk proximal promoter region, directs CAT expression in muscle cells at a level comparable to that of the tk/cTNT chimera but is inactive in fibroblast cells (Fig. 3). Since the HSV tk promoter carries no skeletal muscle specificity (see above and Fig. 2), and its proximal region is transcriptionally inactive by itself (Fig. 3), the 27-fold higher activity of the cTNT/tk chimeric promoter in skeletal muscle versus fibroblast cells is entirely attributable to the presence of the cTNT distal promoter region. Therefore, the cTNT distal promoter region contains a cis element(s) capable of supporting transcription in skeletal muscle cells but that cannot support, or block, such activity in fibroblast cells.

Enhancer Tests of the cTNT Distal Promoter Region. Since cell-specific transcription of many genes has been shown to be dependent upon the presence of cell-specific enhancer sequences in cis (21, 23, 34–38), we wanted to determine if the distal segment of the cTNT promoter contained a skeletal muscle-specific enhancer. We used position and orientation independence as criteria for enhancer function because prototype enhancers exert their effects over long distances and in positive and negative orientations (39). The cTNT distal promoter segment was inserted in positive and negative orientations into the polylinker cloning site of an enhancer test plasmid, pTE2-ΔS/N, which contains the complete HSV tk promoter driving CAT (see Fig. 1e and f; ref. 23). Fig. 2b shows that in this upstream position the cTNT distal promoter region elevates HSV tk promoter activity ~3-fold, in an orientation-independent fashion, as expected for a weak transcriptional enhancer (39). This modest enhancement activity, however, is not skeletal muscle specific because the degree of enhancement is approximately equivalent in skeletal muscle and fibroblast cells (Fig. 2b).
Thus, displacement of the cTNT distal promoter region 60 nucleotides upstream completely abolishes its ability to confer preferential expression in skeletal muscle cells. This sharp distance dependence is not expected for a cell-specific enhancer and suggests that the cTNT distal promoter element(s) responsible for its muscle-specific activity may require short-range interaction with other components during transcription initiation.

Muscle-Specific Distal Promoter Motifs. Conserved regulatory sequence motifs have been hypothesized to control coordinate regulation of contractile protein genes. The cTNT distal promoter region contains two sequence motifs that are also found in comparable regions of other muscle-specific genes. One conserved sequence element is centered around the canonical CCAAT homology (Fig. 4a; positions -114 to -120). We designated this sequence motif as the canonical CCAAT homology because it resembles the canonical CCAAT homology typical of eukaryotic promoters in its position and sequence, except that it has more adenosine residues than usual (29, 30). Such adenosine-rich CCAAT motifs are a common feature of contractile protein gene promoter regions (see refs. 4, 5, and 10 for examples) and are often embedded within a somewhat longer conserved block dubbed the "CCAAT box" (where "t" represents repeated adenosine or thymidine residues) by Minty and Kedes (10).

To test the requirement for the CCAAT/CCArGG motif in the cTNT promoter, the -129/-113 segment was deleted from cTNT-129 to create cTNT-113 (see Fig. 1b). The activity of cTNT-113 is approximately one-third that of cTNT-129 in skeletal muscle cells (Fig. 4b) but remains inactive in fibroblast cells (data not shown). Therefore, although the CCAAT motif may play an important role in expression of actin genes (11), deletion of the CCAAT/CCArGG motif has only a modest effect upon the activity of the cTNT promoter in skeletal muscle cells. In addition, this result further delimits the cis region primarily responsible for specifying a high degree of skeletal muscle-specific transcription to the 64-nucleotide segment between positions -113 and -50.

Within that 64-nucleotide segment are two copies of another sequence element, CATTCTT, which is found in the promoter regions of many contractile protein genes (8). We refer to the CATTCTT heptamer as the "M-CAT" motif by analogy to the more common CCAAT homology found in many eukaryotic promoters (28, 29). There are two identical copies of the M-CAT motif residing between the nominal CCAAT and SP-1 homologies of the cTNT distal promoter region (M-CAT 1 and M-CAT 2; Fig. 4a). The conservation of the sequence and position of M-CAT motifs in some muscle-specific promoters suggests that it may play a role in their muscle-specific regulation.

To test the role of the distal-most CATTCTT motif of the cTNT promoter we changed the first, second, and fourth nucleotides of M-CAT 1 (Fig. 4a) by means of oligonucleotide-directed mutagenesis (41) to alter the most highly conserved portion of the heptamer (8). cTNT-129 carrying the M-CAT 1 mutation directed lower CAT activity (by a factor of 50) than wild-type cTNT-129 in skeletal muscle cells (Fig. 4b). Moreover, the M-CAT 1 mutation had no effect upon the inability of the cTNT promoter to be expressed in fibroblast cells (data not shown). It is not yet known whether M-CAT 1 requires the presence of an intact M-CAT 2 or CCAAT/CCArGG for full function. However, the fact that a 3-nucleotide change in M-CAT 1 virtually abolishes the activity of the cTNT promoter indicates that, at a minimum, an intact M-CAT 1 motif is essential for cTNT promoter function in embryonic skeletal muscle cells.

Summary and Conclusions. The results presented here demonstrate that the 80-nucleotide distal segment of the cTNT promoter carries a sequence element(s) responsible for directing skeletal muscle-specific transcription. Two sequence motifs within this distal region are shared by other contractile protein gene promoters. One of these, the CCAAT/CCArGG motif, plays an important role in the cardiac actin gene (11) but can be deleted from the cTNT promoter with only a modest effect upon its activity in skeletal muscle cells. In contrast, a 3-nucleotide mutation in a M-CAT motif (CATTCTT) abolishes activity of the cTNT promoter in skeletal muscle cells. Therefore, the M-CAT motif is essential for activity of the cTNT promoter in skeletal muscle cells and may play a similar role in the other muscle promoters in which it is found. It should now be possible to determine the molecular basis for that role.

We thank Steve McKnight and our colleagues at University of California, San Francisco, for making valuable plasmid constructions available. This research was supported by grants to C.P.O. from the National Institutes of Health. J.H.M. was supported by a National Institute of Child Health and Human Development post-doctoral fellowship.