# Slow troponin C gene expression in chicken heart and liver is regulated by similar enhancers

J.S. Grewal, J. Bag*

*Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ont. N1G 2W1, Canada*

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**Abstract Two isoforms of troponin C (TnC) are encoded by distinct single copy genes. Expression of fast TnC is restricted to the skeletal muscle, whereas the slow isoform is expressed in both skeletal and cardiac muscle. Chicken slow TnC (cTnC) gene is also expressed in some non-muscle tissues like the liver and the brain. Expression of cTnC gene is regulated by two distinct enhancers in cardiac and skeletal muscles. The cardiac specific enhancer is located in the immediate 5′ flanking region (bp −124 to −79) of the murine cTnC gene whereas the skeletal enhancer is located within the first intron (bp 997 to 1141). In the present study we have examined how cTnC gene expression is regulated in the chicken liver. Transient transfection of liver cells with cTnC-CAT reporter constructs containing various regions of the murine cTnC gene showed that its expression in chicken liver is regulated by the cardiac specific enhancer. Furthermore, electrophoretic mobility shift assays using synthetic oligonucleotides corresponding to both CEF-1 and CEF-2 regions of the murine cardiac enhancer revealed formation of specific DNA–protein complexes. Ultraviolet light induced covalent linking of nuclear proteins to CEF-1 and CEF-2 oligomers were used to examine the nature of the cardiac enhancer binding polypeptides; one polypeptide of 48 kDa appeared to bind to both CEF-1 and CEF-2 sequences.**

*Key words:* Slow troponin C; Gene expression; Cardiac myocytes; Hepatocytes; Troponin enhancer

## 1 Introduction

Contractile proteins are usually expressed in a tissue specific manner [1]. However, separate isoforms of a number of contractile proteins including actin, dystrophin, myosin and tropomyosin occur in non-muscle tissues [2–6]. The non-muscle isoforms of these proteins arise from separate gene families and have distinct regulatory mechanisms [7–10]. In most animals the Troponin family (Troponin I, Troponin T and Troponin C) proteins are expressed only in striated muscles [11,12]. These members encode functionally interacting subunits of the calcium regulatory troponin complex. Troponin C (TnC) is the calcium binding subunit of the troponin complex. Its two isoforms are separate gene products [13]. The fast TnC (fTnC) gene is exclusively expressed in the fast twitch skeletal muscles while the slow TnC (cTnC) gene is expressed extensively in cardiac and slow twitch skeletal muscles, and only transiently in embryonic fast skeletal muscles [2,14,15]. These two isoforms have distinct differential expression mechanisms [11]. A core promoter and a transcriptional enhancer are located within the immediate flanking region (bp −124 to +32) of the gene and function in a cardiac specific fashion [7]. In skeletal myotubes cTnC gene expression is under the control of an independent transcriptional enhancer positioned within the first intron (bp 997–1141). The cTnC protein has recently been detected in non-muscle tissues, e.g. human HeLa cells [16], chicken liver and brain [17]. However, the function and regulation of expression of this gene in non-muscle cells is still unknown. In the studies presented here we have examined how the cardiac cTnC gene is expressed in chicken liver. Since the chicken cTnC gene has not been characterized we have examined the mouse cardiac enhancer for its ability to express a reporter CAT gene in chicken liver hepatocytes in culture and to form specific nucleoprotein complexes with chicken liver and brain nuclear extracts.

## 2. Materials and methods

### 2.1. Cell cultures

Primary cardiac myocyte cultures were prepared using 14-day embryonic chicken hearts as previously described [18,19]. In short, hearts from several embryos were minced into small pieces and digested with 0.1% collagenase and 0.1% hyaluronidase. Fibroblasts from all these digestions were removed by differential centrifugation, while cardiac myocytes were pelleted at $3000\times g$ for 5 min. $2\times10^5\cdot ml^{-1}$ cardiac myocytes were plated in each well (2 $cm^2$) of a 12-well tissue culture plate (Nunc). Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco/BRL) containing glutamine, 10% equine serum, 3% fetal bovine serum, and 100 units/ml penicillin/100 μg/ml streptomycin. The medium was changed daily. Primary hepatocyte cultures were derived from 14-day-old embryonic chick liver [2], by dissociation of cells with 0.1% trypsin in Hank's Balanced Salts (HBS) solution (Gibco) at 37°C for 15–30 min. Hepatocytes were grown as monolayer in gelatin-coated 12-well plates (2 $cm^2$) to a density of about $2–5\times10^5$ cells·$ml^{-1}$ in minimum essential Eagle's medium (MOD.) supplemented with 10% fetal bovine serum and antibiotics. The medium was changed daily.

### 2.2. Nuclear extracts

Nuclear extracts were prepared as previously described [20]. Various tissues were dissected from 14-day-old chicken embryos, were rinsed and minced into small pieces in cold phosphate-buffered saline (PBS). Tissues were suspended in five volumes of hypotonic buffer {10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT (dithiothreitol)} and kept for 20 min on ice. Tissues were homogenized with a Dounce-homogenizer until greater than 90% lysis of cells was achieved. The cell lysate was centrifuged at $3000\times g$ for 5 min at 4°C to obtain nuclei. The nuclear pellet was washed once with PBS, resuspended in one volume of extraction buffer {20 mM HEPES, pH 7.9; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA (disodium ethylenediaminetetraacetate); 0.5 mM phenyl-methylsulfonyl fluoride; 0.5 mM DTT and 20% glycerol} and incubated on ice for 30 min with intermittent mixing. Nuclear debris was removed by centrifugation at $12\,000\times g$ for 10 min. The PBS and extraction buffers were supplemented with protease inhibitors, Leupeptin (2 μg/ml), Aprotinin (2 μg/ml) and Pefabloc (10 μg/ml). Supernatants were stored at −80°C until used.

*Corresponding author.


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*2.3. Plasmids*

Plasmids containing the pCAT (chloramphenicol acetyltransferase gene) driven by various regions of murine cTnC gene were obtained from Dr. M.S. Parmacek, University of Chicago. Plasmid p2.2CAT had 2.2 kb *Bam*HI-*Alu*I cTnC genome fragment (bp −2200 to +32) subcloned into *Hin*dIII site of the pCAT-Basic (Promega) in a 5′ to 3′ orientation with respect to chloramphenicol acetyltransferase (CAT) gene; p-124CAT contained 156 bp *Bal*I-*Alu*I cTnC genomic fragment (bp −124 to +32) subcloned into *Hin*dIII-*Xba*I digested pCAT-Basic; p-79CAT had *Hin*dIII-*Xba*I-linked cTnC subfragment (bp −79 to +32) subcloned into *Hin*dIII-*Xba*I digested pCAT-Basic, pSPCA-T308ENH contained 308 bp cTnC *Pvu*II genomic subfragment (bp 881 to 1188) with cTnC skeletal muscle specific enhancer into *Bam*HI site of the pSPCAT vector [7]. The pSMVβgal [21] reference plasmid contained the β-galactosidase gene under the control of a long terminal repeat from the Moloney murine sarcoma virus.

*2.4. Transfections and CAT assays*

Primary culture of neonatal murine cardiac myocytes, chicken embryonic (14 day) hepatocytes and cardiac myocytes were co- transfected with plasmids (containing various cTnC-CAT constructs) and pMSVβgal reference plasmid using the cationic liposome (DOTAP) according to the manufacturer's (Boehringer Mannheim, Canada) instructions. In short, 70% confluent cultures were used for transfection assays. The medium was changed 2–4 h prior to the transfection experiment. The transfection mixture was prepared by combining 15 μg pCAT plasmid DNA and 5 μg pMSVβgal reference plasmid with 15 μg of DOTAP in 150 μl of 20 mM HEPES (pH 7.4) buffer then incubated at room temperature for 10 min before diluting to 350 μl with culture medium containing 2% fetal bovine serum. Cells were treated with the transfection mixture for 16 h at 37°C in a humidified atmosphere of 5% $CO_2$. Transfection mixture was then replaced with fresh culture medium which was changed daily. The cells were harvested after 48 h. Cell extracts were prepared by repeated freeze/thawing and CAT activity was measured by enzyme linked immunosorbent assay (CAT-ELISA) using a kit according to the manufacturer's (Boehringer Mannheim, Canada) instructions. Variations in transfection efficiencies were corrected by assaying cell extracts for β-galactosidase activities as previously described [7].

*2.5. Electrophoretic mobility shift assay*

Oligonucleotides (Fig. 1) corresponding to CEF-1, mutant CEF-1 (mCEF-1), CEF-2, mutant CEF-2 (mCEF-2), and a control oligonucleotide corresponding to muscle creatin kinase (MCK) enhancers were synthesized by DNAgency, Aston, USA. The CEF1 double-stranded oligonucleotide was labelled with [α-$^{32}$P]GTP by fill-in reactions using large Klenow fragment. Both strands of the CEF2 oligomer were separately 5′-end-labelled with [γ-$^{32}$P]ATP using $T_4$ polynucleotide kinase, prior to annealing. Binding reactions, containing 0.5–1 ng radiolabelled probe (~$10^3$–$10^4$ cpm), 10–15 μg nuclear proteins, 300 ng of poly(dI:dC), 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 4% Ficoll, were carried out on ice for 15 min. Competitions with unlabelled oligomers were performed by adding the competitor prior to incubation of the cell extract with the radiolabelled probe. The reaction mixture was separated on a 5% non-denaturing polyacrylamide gel in 0.25×TBE (1×TBE = 100 mM Tris pH 7.9, 100 mM boric acid and 2 mM EDTA) at 4°C. The gel was dried and the autoradiogram developed using Kodak X-omat RP film.

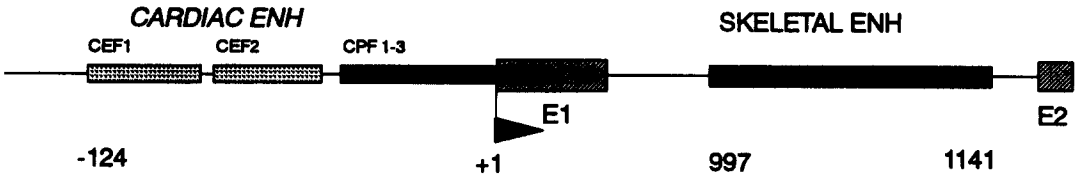
*2.6. Ultraviolet light-induced crosslinking of nuclear proteins to oligomers*

Double-stranded oligomers were labelled with $^{32}$P and incubated with nuclear extracts as previously described. The mixture was transferred to a Parafilm sheet and exposed to a UV transilluminator (3000 μW/cm$^2$) for 20 min. The samples were analyzed by electrophoresis in a 12% polyacrylamide gel in the presence of sodium dodecylsulphate (SDS) as previously described [22].

## 3. Results and discussion

*3.1. cTnC gene expression in chicken hepatocytes*

Studies from this laboratory have previously shown that the cTnC gene is expressed in the chicken liver and brain [17]. To examine the nature of the *cis*-regulatory sequences involved in regulating cTnC gene expression in chick liver, transient transfections of cells in culture were performed using cTnC-CAT reporter constructs (Fig. 2). Primary cultures of chicken hepatocytes, cardiac myocytes and mouse cardiac myocytes were transfected with various reporter plasmids. Results show that CAT expression was maximum in both chick liver and cardiac myocytes when −124pCAT was used for transfection. The level of CAT expression was also similar to that observed in murine cardiac myocyte culture (Fig. 2). In contrast the construct containing the skeletal muscle specific enhancer (pSPCAT308ENH) was unable to support CAT expression in these cells. The cardiac enhancer produced approximately 60–90-fold increase of CAT expression compared to that of




| | |
|---|---|
| CEF-1 | 5′ -CCA GCC TGA GAT TAC AG -3′ |
| mCEF-1 | 5′ -CCA GCC AGA GAT CTC AGG GA -3′ |
| CEF-2 | 5′ -GGT GGA GGA TAT TCC ACG -3′ |
| mCEF-2 | 5′ -GGT GCA GAT CTT TCC AGG -3′ |
| MCK enh | 5′ -CCC CCC CCC AAC ACC TGC TGC CTG A -3′ |

Fig. 1. Structure and location of a cardiac and skeletal specific cTnC promoter/enhancer of the murine cTnC gene and the nucleotide sequence of the various oligomers used in these studies are shown.

the enhancerless CAT constructs in all three cell cultures studied here. Results also show that presence of −79 to +32 region of murine cTnC gene (p-79CAT) was insufficient to support CAT expression. It is interesting to note that the p-2.2CAT containing −2200 to +32 region of the murine cTnC gene supported CAT expression only to an approximately 10% level of that observed with p-124CAT. This observation suggests the presence of negative regulatory elements within this region. The results, therefore, indicate that the cardiac enhancer may be responsible for cTnC gene expression in chicken liver.

### 3.3. CEF1 and CEF2 binding nuclear protein complexes in liver cells

To detect the presence of nuclear proteins in liver cells that show specific binding to the cTnC transcriptional enhancer, synthetic oligonucleotides corresponding to the CEF-1 and CEF-2 regions of the murine cTnC gene [7] were used in EMSAs (Fig. 1). Results show that the CEF-1 (Fig. 3A) oligomer forms two major complexes with chicken liver nuclear extract. One of these complexes displays mobility shifts corresponding to the major band formed with chicken heart nuclear extract. The faster migrating second band was only a minor component in the sample containing chicken heart extract. Similar complexes were observed with the chicken brain nuclear extract. A few additional bands were also shared between the liver and brain extracts. These liver and cardiac muscle specific bands also exhibited dose-dependent competition with the unlabelled CEF-1 oligomer. In contrast the mutant mCEF-1 oligomer was less efficient in the competition assays (Fig. 3B). In these studies nuclear extracts from newborn mouse heart showed only weak enhancer binding activities (Fig. 3A, lane 5). However, the major nucleoprotein complexes formed using chicken and mouse cardiac and chicken liver and brain nuclear extracts were similar and distinct from those formed with the chicken skeletal muscle nuclear extract (Fig. 3A, lane 7). Electrophoretic mobility shift experiments were also performed with the radiolabelled CEF-2 oligomer. Results (Fig. 3C and D) show that three distinct nucleoprotein complexes were formed with both chicken heart and liver extracts. One of these complexes appeared to be the most prominent and was also formed with chicken brain and murine heart nuclear extracts. Murine cardiac nuclear extracts also produced an additional presumably non-specific complex common to both $C_2C_{12}$ myotube and chicken skeletal muscle nuclear extract preparations. Formation of the major nucleoprotein complexes with heart, liver and brain extracts was similar to that reported previously [7]. To further assess the specificity of these interactions competition analyses were performed using unlabelled oligomers in excess. Results (Fig. 3D) show that the formation of these complexes was inhibited by the unlabelled CEF-2 oligomer. The mutant (mCEF-2) oligomer was unable to abolish formation of these complexes even when a 50-fold molar excess of unlabelled oligomer was used. The results of these studies using both CEF-1 and CEF-2 oligomers, therefore, suggest that chicken liver, brain and heart contain similar nuclear proteins that bind to the cardiac specific enhancer sequence of the cTnC gene.

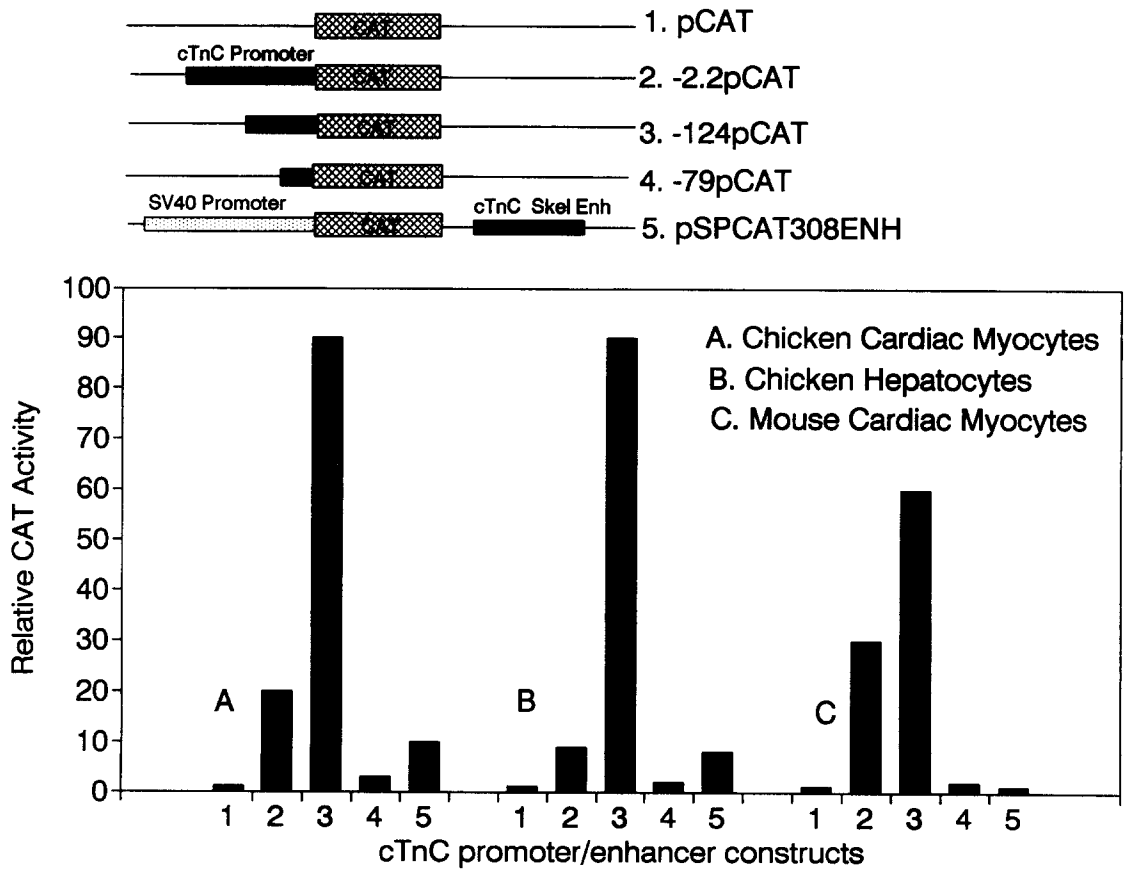



Fig. 2. Relative transcriptional activities of cTnC promoter/enhancer constructs in transfected cells. CAT-ELISA of cellular extracts were performed after transient transfection with DNA constructs listed as described in Materials and methods. CAT-activity values were corrected for the difference in transfection efficiencies using β-gal expression levels as controls.

Since electrophoretic mobility shift experiments reveal formation of specific DNA–protein complexes with both CEF-1 and CEF-2 oligomers and the chick liver extract we attempted to further characterize the polypeptides of these complexes by covalently joining the DNA and proteins using UV-crosslinking. Radiolabelled oligomer linked polypeptides were then examined by gel electrophoresis and autoradiography. These analyses show (Fig. 4A) the presence of one major polypeptide of approximately 62 kDa and an additional minor band representing a polypeptide of 65 kDa in crosslinked preparations using the CEF-1 oligomer. Appearance of these bands was also dependent upon UV-treatment of the reaction mixture (compare lanes 1 and 2). Formation of the labelled 62 and 65 kDa bands was efficiently blocked by the presence of unlabelled CEF-1 oligomer but not by mutant or MCK oligomers. Since the oligomer of approximately 14 kDa was covalently linked to the polypeptides, the approximate molecular mass of the CEF-1 binding polypeptide was determined to be 48 kDa. It is interesting to note that recent studies have shown that a GATA-4 transcription factor of 48 kDa which binds to the CEF-1 region of the mouse cTnC gene is required for its activation [23,24]. Members of the GATA-4 family of factors are also expressed in chicken heart and liver; therefore, it is possible that expression of chicken cTnC gene is similarly regulated.

Similar crosslinking studies using CEF-2 oligomer and chicken liver nuclear extract illustrate (Fig. 4B) the presence of two prominent and a number of minor bands representing different polypeptides covalently linked to the CEF-2 sequence. Covalent linking of these polypeptides was specific to the CEF-2 sequence as this interaction was not blocked by either mutant CEF-2 or the MCK oligomer. Substraction of the molecular weight of the oligomer from these bands suggests that the major band (arrow) also represents a polypeptide of 48 kDa. It should be noted that this molecular weight of one of the polypeptides is similar to that of GATA-4. Further studies are required, however, to characterize the chick liver CEF-1 and CEF-2 *cis*-regulatory sequence

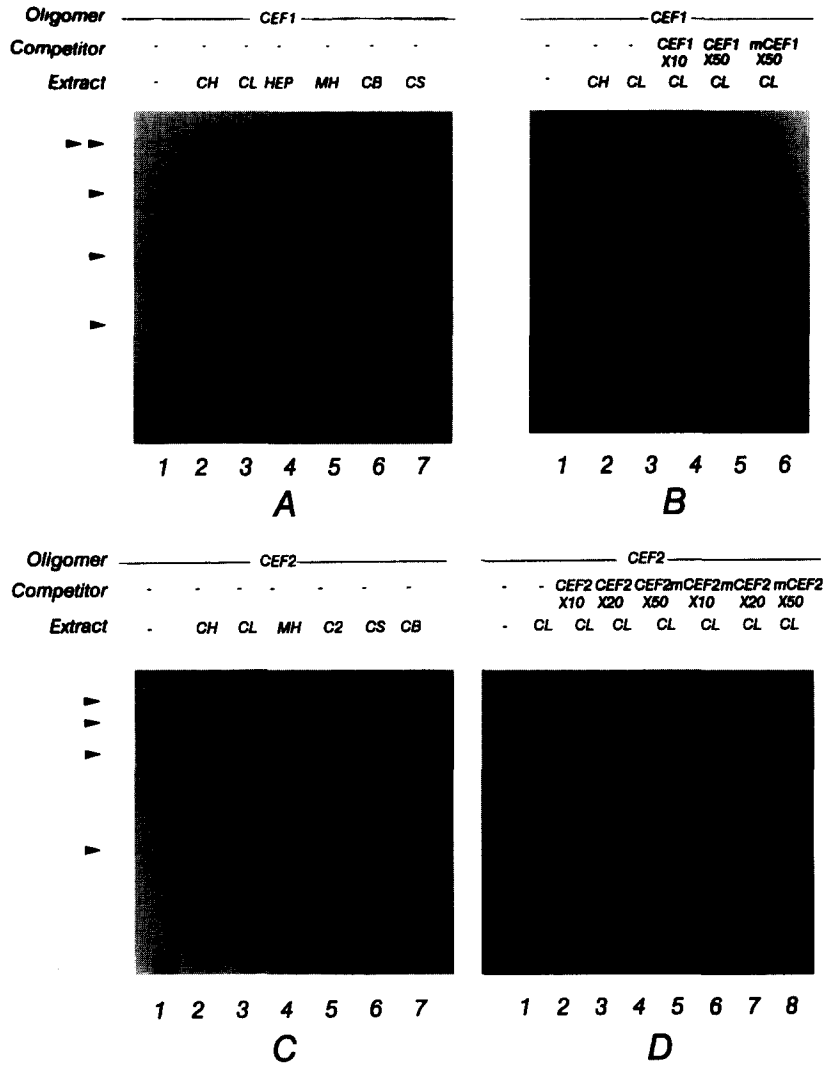



Fig. 3. EMSA analysis of the cardiac specific enhancer of murine cTnC gene. Examination of nuclear protein complexes binding to either CEF-1 (A and B) or to the CEF-2 (C and D) oligomers. Binding reactions and gel electrophoresis were performed as described in Materials and methods. Binding reaction was carried out with 0.5 ng of $^{32}$P-labelled oligomer and 15 μg of nuclear extract. Nuclear extracts from 14-day-old embryonic chicken liver (CL), chicken heart (CH), chicken brain (CB) and chicken skeletal muscle (CS) were used. Newborn mouse heart (MH) was used for the preparation of MH nuclear extracts. Hepatocytes culture (HEP) from 14-day chick embryo was used for preparing nuclear extract [18,19] as described in Materials and methods. In some experiments (C and D) the binding reaction mixture contained indicated excess of unlabelled competitor oligomers.

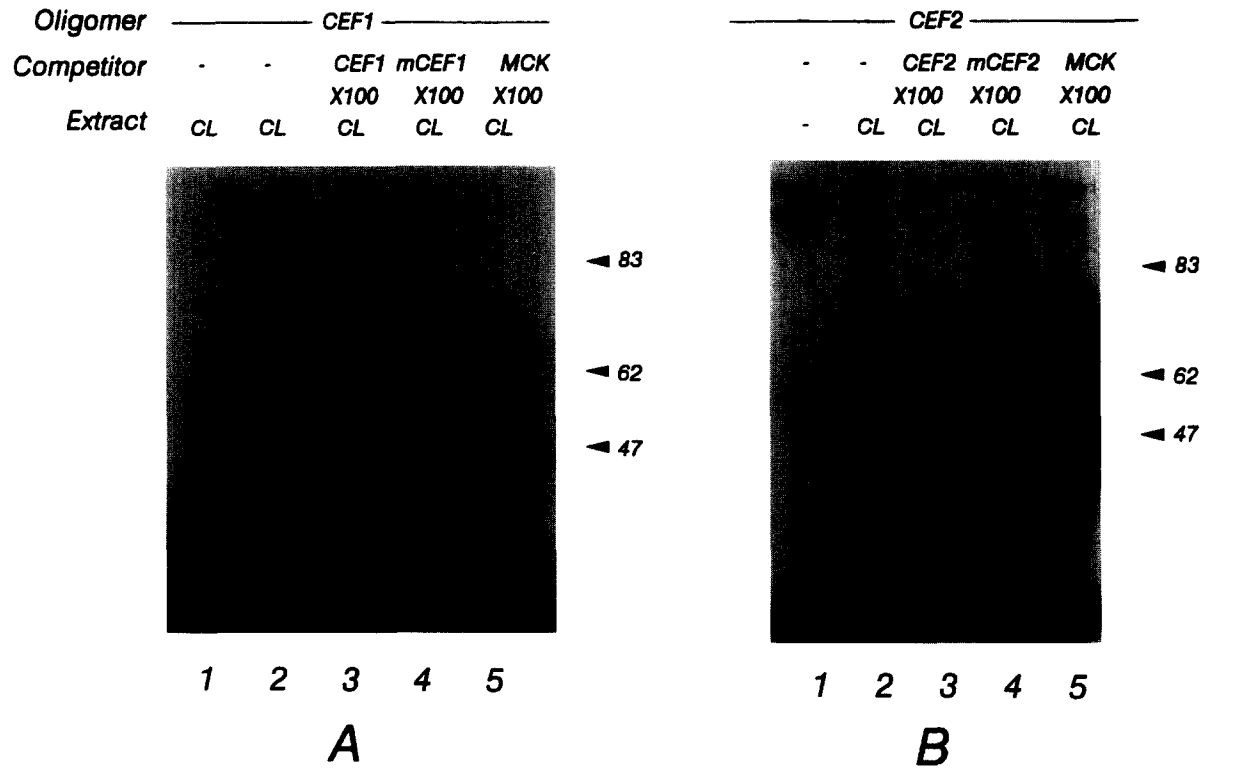



Fig. 4. UV-induced crosslinking of nuclear proteins to cardiac enhancer of murine cTnC gene. Radiolabelled double-stranded CEF-1 or CEF-2 oligonucleotide was mixed with the nuclear extract and subjected to UV treatment as described in Materials and methods. The samples were then analyzed by polyacrylamide gel electrophoresis in the presence of SDS [22]. CEF-1 oligomer (A), CEF-2 oligomer (B). The molecular weight standards are shown on the right hand side of each panel.

binding proteins. Studies using chicken heart and brain nuclear extracts also produced similar results (not shown).

Unlike other muscle proteins TnC, an important member of the contractile protein family, has not been found in all non-muscle cells. Of the two TnC isoforms, cTnC mRNA was previously reported in human fibroblast [16], while none of the murine non-muscle tissue revealed its existence [15]. Berezowsky and Bag (1992) reported the presence of cTnC mRNA in chicken liver and brain. Our results suggest that chicken embryonic hepatocytes may employ sequences similar to the murine cardiac myocytes *cis*-regulatory elements to bind to transcription factors for controlling cTnC gene expression. Since chicken cTnC gene has not been characterized, it will be essential to confirm these observations using the homologous gene when it becomes available. Furthermore, the function of cTnC in chicken liver is also unknown. One possibility is that it might act as a regulatory subunit of phosphorylase kinase [25–27], whose activity is $Ca^{2+}$ dependent, and TnC, alone or as part of the troponin complex, may serve as its δ-subunit [25,28,29].

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