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Interaction of Nuclear Proteins with Repeat Sequences in the 5′ Flanking Region of Mouse Muscle Creatine Kinase Gene during Aging

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The 5′ flanking region of the mouse muscle creatine kinase (MCK) gene contains two repeat sequences—a mononucleotide repeat, $(A)_{22}$ (−2694 to −2673), and a tetranucleotide repeat, $(GTTT)_8$ (−2962 to −2931). We show here that these repeats in the mouse MCK gene bind to specific nuclear protein factors. Some of the factors interacting with these sequences are tissue-specific and show age-related decrease in the binding activity. Nonspecific competitor and heterologous DNA probes failed to compete out the complexes showing that the interaction is specific to the repeat sequences. These proteins may have a role in the expression of the gene during aging.

Repetitive DNA sequences constitute a substantial portion of the genome of eukaryotes. Microsatellites constitute approximately 30% of these repeat sequences. They have a simple internal repeat structure with repeat units ranging from one to five basepairs and are referred to as simple tandem repeats (STR). The microsatellites are stably inherited, are highly polymorphic, and are used for population genetic analysis. Several studies have implicated repeat expansion in neuromuscular disorders (1, 2). Repeats can alter the conformation of DNA locally (3, 4), affecting the replication of DNA (5, 6) and transcription of a gene (7). Some of the repeat sequences have been shown to bind to protein factors (7, 8), and in a few cases even play a role in gene regulation.

The enzyme, muscle creatine kinase (MCK), plays an important role in the energy metabolism of muscle. This enzyme leaks out of the heart after myocardial infarction and is used for diagnosis. The mouse MCK gene spans 11 kb and has 7 introns. Expression of the MCK gene is restricted to skeletal muscle and heart in mice (9). It is regulated by several *cis*-acting elements such as MEF-2, E boxes, A/T-rich and CArG elements located in the enhancer region (−1256 to −1050) (10). We have shown that the expression of the MCK gene decreases as a function of age in the heart of mice, and also that the binding activity of the nuclear *trans*-acting factors of the heart that interact with some of the elements in the enhancer region of the gene decreases with age (data communicated).

The 5′ flanking region of the mouse MCK gene contains two repeat sequences, a mononucleotide repeat, $(A)_{22}$ (−2694 to −2673), and a tetranucleotide repeat, $(GTTT)_8$ (−2962 to −2931). The present work was carried out to study the interaction of the nuclear factors with these repeat sequences, and to examine whether there is any tissue-specific and age-related variation in their binding activity.

MATERIALS AND METHODS

Animals. Male AKR mice of three different ages used for the experiments were young (4 weeks), adult (25 ± 2 weeks), and old (70 ± 5 weeks).

Oligonucleotides used. Oligonucleotides containing the repeat sequences and their complementary strands (based on the sequence of the mouse MCK gene compiled in the laboratory of J. Chamberlain, University of Michigan, from clones obtained in the laboratory of S. Hauschka) were obtained from Cybersyn Inc., Aston, U.S.A. $(A)_{22}$ repeat of MCK (38 mer): 5′ TTTTATACAT**AAAAAAAAAAAAAAAAAAAAAA**CCAAAC 3′ (upper strand) and 3′ AAATATGTA**TTTTTTTTTTTTTTTTTTTTTT**GGTTTGT 5′ (lower strand). $(GTTT)_8$ repeat of MCK (32 mer): 5′ TT**GTTTGTTTGTTTGTTTGTTTGTTTGTTTGT** 3′ (upper strand) and 3′ **AAACAAACAAACAAACAAACAAACAAACA**T 5′ (lower strand). The complementary oligos were annealed by incubating equimolar concentrations of both the oligos in 1× TNE buffer (10 mM Tris–Cl, pH 7.8; 100 mM NaCl; 1 mM EDTA) at 95°C for 15 min and allowing it to cool down to room temperature gradually. The double stranded oligos were labelled for EMSA experiments by end-filling using [α-^{32}P]-dATP and other dNTPs in the presence of Klenow fragment.

Nuclear extract preparation. Nuclear extract was prepared from skeletal muscle, heart (ventricles), and brain (cerebral hemispheres) of mice (11, 12). Briefly, 10% homogenate of the tissues was prepared

Abbreviations used: MCK, muscle creatine kinase; EMSA, electrophoretic mobility shift assay; NE, nuclear extract; PK, proteinase K; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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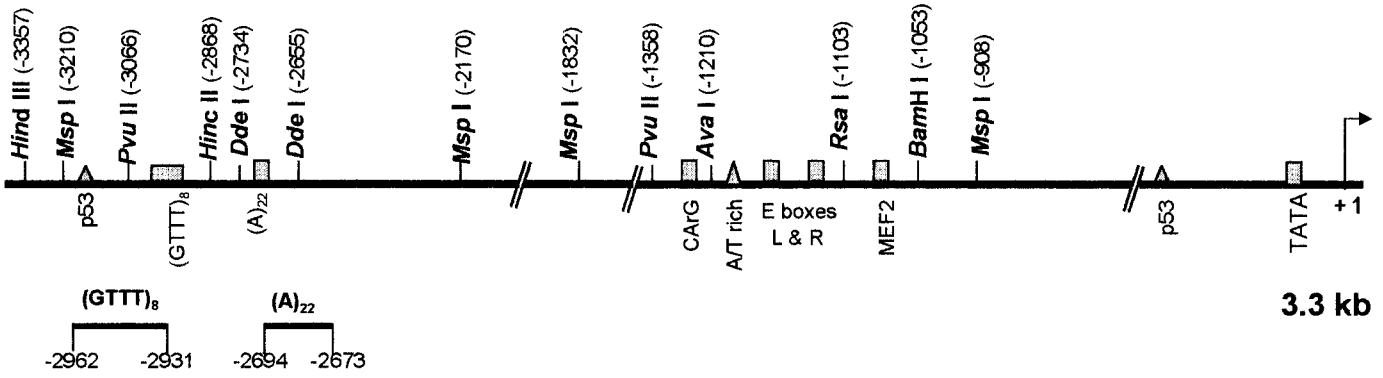

FIG. 1. Restriction map of 3.3 kb 5′ flanking region of mouse MCK gene showing the location of the repeat sequences, $(A)_{22}$ and $(GTTT)_8$.

in buffer A (10 mM Hepes–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF) containing 0.3 M sucrose. The homogenate was centrifuged at 1000*g* for 15 min at 4°C after passing through 4 layers of cheese cloth. The pellet was resuspended in half the original volume of buffer A containing 0.3 M sucrose and 0.2% Triton X-100. An equal volume of buffer A containing 1.8 M sucrose was added and mixed thoroughly followed by centrifugation at 4000*g* for 15 min at 4°C. The crude nuclear pellet was washed thrice in buffer A containing 0.3 M sucrose and once in buffer A. The nuclear pellet was finally suspended in buffer C (20 mM Hepes–KOH, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml each of leupeptin, aprotinin, antipain and pepstatin). The suspension was stirred on ice for 30 min and centrifuged at 25,000*g* for 40 min at 4°C. The supernatant was aliquoted, frozen in liquid N_2 and stored at −70°C. The nuclear proteins were quantitated before use (13).

Electrophoretic mobility shift assay. In a 20-μl reaction volume, 0.1–0.25 ng (~5500 cpm) labelled DNA was incubated with 2–5 μg of nuclear extract in the presence of binding buffer (15 mM Hepes–KOH, pH 7.9, 40 mM KCl, 1 mM EDTA, 0.5 mM DTT, 5% glycerol) and 1 μg *E. coli* DNA for 15 min at room temperature. 5 μl of loading buffer (6% sucrose, 2 mM Tris–Cl, pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to the reaction mixture. The complexes were resolved on a 5% polyacrylamide gel (acrylamide:bis 30:1) in 0.5× TBE at 200 V with constant circulation of buffer between the chambers at room temperature. The gel was preelectrophoresed at 100 V for 1 h prior to loading. After electrophoresis the gel was fixed, dried and subjected to autoradiography.

Densitometric scanning. Densitometric scanning of the autoradiograms was carried out to quantitate the variations in the intensities of the signal.

RESULTS AND DISCUSSION

The two types of repeats, namely, a mononucleotide repeat, $(A)_{22}$, and a tetranucleotide repeat, $(GTTT)_8$, located in the 5′ flanking region of mouse MCK gene are of special interest (Fig. 1). It is noteworthy that the $(GTTT)_8$ repeat along with its complementary strand $(CAAA)_8$ is largely T/A-rich. Synthetic oligonucleotides containing the repeats and their complementary strands were annealed, and the resulting dsDNA was used for EMSA studies. They are the 32 bp MCK-$(GTTT)_8$ and the 38 bp MCK-$(A)_{22}$. Most of the work was carried out using nuclear extracts prepared from the heart.

Nuclear proteins that bind to the MCK-$(GTTT)_8$ fragment. The MCK-$(GTTT)_8$ spans from −2962 to −2931 bp relative to the transcription start site (Fig. 1). Mobility shift assay to study its binding to nuclear proteins in different tissues revealed the presence of factors which may be tissue-specific. Several complexes of different mobilities were observed with the nuclear extracts prepared from heart ventricles, skeletal muscle and brain. Two complexes were specific to the heart, and two to the skeletal muscle. One complex of similar mobility was seen in the heart and skeletal muscle and another complex of similar mobility was observed in the brain and skeletal muscle (Fig. 2A). Such binding may be due to the presence of both specific and ubiquitous factors in the nuclear extracts of various tissues

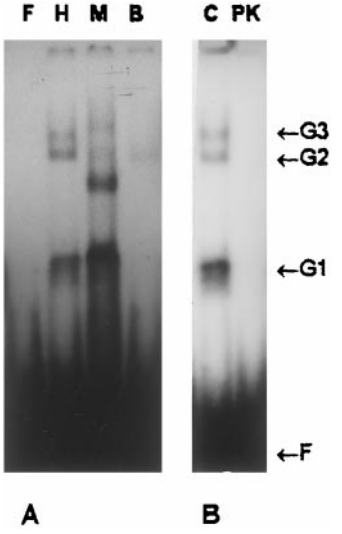

FIG. 2. EMSA with MCK-$(GTTT)_8$ to study tissue specificity and its nature of interaction. (A) Nuclear extract prepared from heart (H), skeletal muscle (M) and brain (B) of male AKR mice was incubated with ^{32}P-labeled MCK-$(GTTT)_8$. (B) Nuclear extract of heart was treated with proteinase K (PK) for 30 min at 37°C prior to incubation with labeled MCK-$(GTTT)_8$ or mock treated (C) without PK. DNA–protein complexes were resolved on 5% native polyacrylamide gel. F, free DNA.

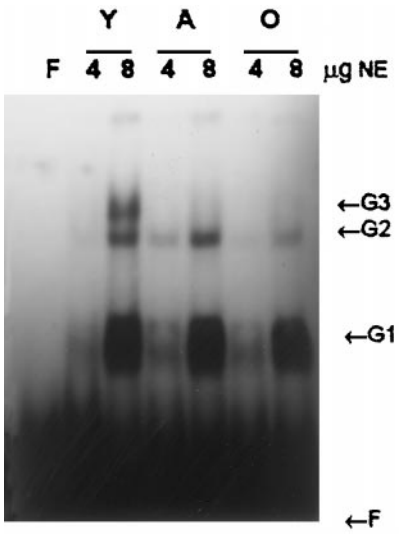

FIG. 3. EMSA with MCK-$(GTTT)_8$ to study age-related binding activity of nuclear factors of heart. 4 and 8 μg of nuclear extracts prepared from heart of young (Y, 4 weeks), adult (25 $\pm$ 2 weeks), and old (70 $\pm$ 5 weeks) male mice were incubated with ^{32}P-labeled MCK-$(GTTT)_8$. DNA–protein complexes were resolved on 5% native polyacrylamide gel. F, free DNA.

that bind to this sequence of DNA to regulate the expression of the MCK gene. The three complexes formed with the heart extracts (G1, G2 and G3) were due to DNA-protein interaction as revealed by the treatment of the extracts with proteinase K (Fig. 2B).

Of the three complexes, G1 did not show a significant age-related variation. However, there was a 2.5-fold decrease in the binding activity of proteins involved in the formation of complex G2 and, a 12.5-fold decrease in that of complex G3 (Fig. 3). This may be due to a decrease in the level of factors interacting with the repeat sequence. Non-specific *E. coli* DNA failed to compete out the complexes. At 100-fold molar excess homologous competitor DNA titrates out the complex G2 completely, and G1 and G3 to a lesser extent (Fig. 4). This may be due to a difference in the affinity of these factors for the DNA. Cross titration with MCK-$(A)_{22}$ competes out the complexes suggesting that the proteins have affinity for MCK-$(A)_{22}$. This may be because of the large extent of homology between the $(GTTT)_8$ and $(A)_{22}$ repeats as mentioned above. But cross titration using heterologous fragments like MCK-MEF-2 and MCK-E boxes + A/T-rich failed to compete for these protein factors suggesting that the proteins interacting with MCK-$(GTTT)_8$ are specific to the fragment (Fig. 4). Titration using ss-oligonucleotides for the repeat and the complementary strand did not affect the binding of the factors (Fig. 4) demonstrating that the factors are not single strand binding proteins.

Several lines of work have earlier shown the presence of minisatellite specific DNA binding protein (Msbp-1) in the nuclear extracts of various tissues of mouse, rabbit, *Xenopus* and *Drosophila* (14), and Msbp-2 and Msbp-3 in HeLa cells, human and mouse cell lines (15). Some of these proteins are tissue- and probe-specific (16). The GATA tetranucleotide repeat constituting the banded krait minor satellite DNA binds sex- and tissue-specific factors called Bkm-binding proteins (BPP) (17, 18). Further a role for BPP in the coordinated decondensation of the W chromosome of snakes, and Y chromosome of mouse and human germ cells has been suggested. The tetranucle-

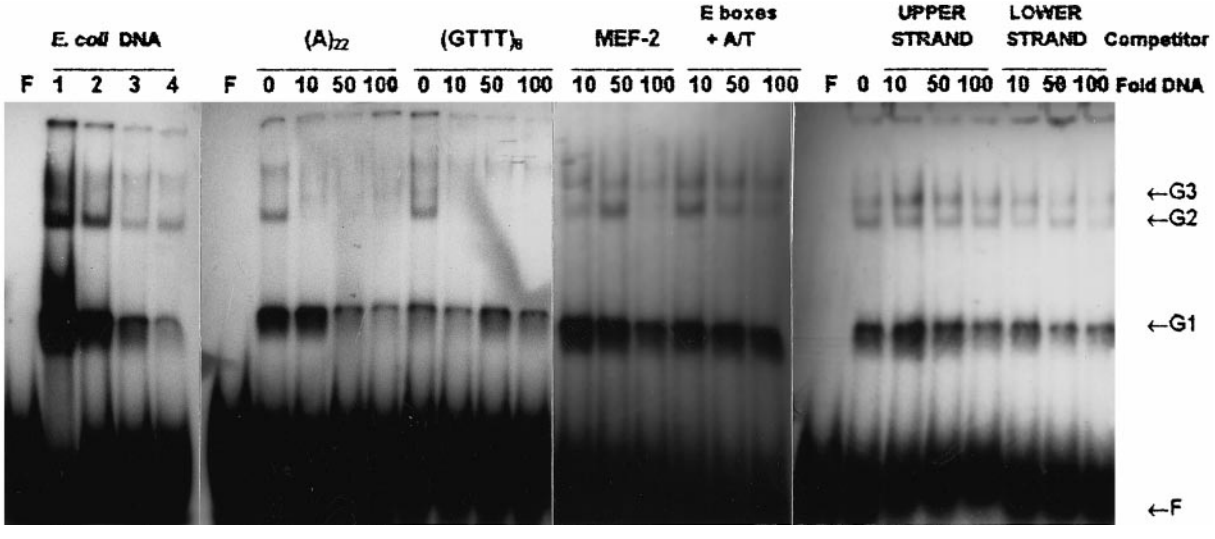

FIG. 4. EMSA with MCK-$(GTTT)_8$ to study specificity of interaction. 6 μg of nuclear extract prepared from heart of male mice was incubated with ^{32}P-labeled MCK-$(GTTT)_8$ in the absence (0-fold, lane 1) or presence (2500-, 5000-, and 10,000-fold excess, lanes 2–4) of *E. coli* DNA or in the presence of 0-, 10-, 50-, and 100-fold excess of cold competitor DNA-$(A)_{22}$, $(GTTT)_8$, MEF-2, E boxes + A/T, coding strand oligo or noncoding strand oligo. DNA–protein complexes were resolved on 5% native polyacrylamide gel. F, free DNA.

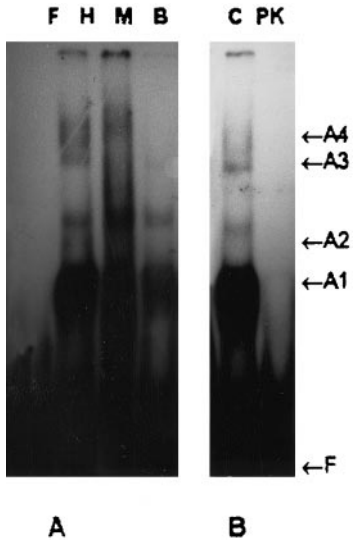

FIG. 5. EMSA with MCK-$(A)_{22}$ to study tissue specificity and nature of interaction with nuclear proteins. (A) Nuclear extract prepared from heart (H), skeletal muscle (M) and brain (B) of male AKR mice was incubated with ^{32}P-labeled MCK-$(A)_{22}$; (B) Nuclear extract of heart was treated with proteinase K (PK) for 30 min at 37°C prior to incubation with labeled MCK-$(A)_{22}$ or mock treated (C) without PK. DNA–protein complexes were resolved on 5% native polyacrylamide gel. F, free DNA.

otide repeat (TCAT) located in the first intron of the tyrosine hydroxylase gene acts as a transcriptional regulatory element *in vitro* in an orientation independent manner (7). Also, this element binds sequence specific nuclear proteins, one of which seems to be a member of Fos-Jun family.

Nuclear proteins that bind to MCK-$(A)_{22}$ fragment. The 38 bp homopolymeric stretch of A is located between −2694 to −2673 bp relative to the start site of the mouse MCK gene. The present study revealed the formation of four complexes with the NE prepared from heart of which two appear to be specific to heart and the other two have mobility similar to the complexes formed with the extracts of skeletal muscle and brain (Fig. 5A). Further, the complexes were due to DNA–protein interaction as revealed by proteinase K treatment (Fig. 5B). Out of the four complexes, only complex A4 formed with the nuclear extract of heart showed a 3.5-fold decrease from young to old (Fig. 6). The specificity of the interaction was confirmed by competition analysis. Homologous competitor, MCK-$(A)_{22}$, effectively titrates out the complexes, whereas neither the non-specific competitor (*E. coli* DNA) nor the heterologous competitors like MCK-MEF-2 and MCK-E boxes + A/T-rich elements were able to titrate out the complexes (Fig. 7). However, MCK-$(GTTT)_8$ competes for these complexes partially showing lesser affinity in comparison to the homologous fragment. When ss oligonucleotides were used as competitor, only the complex A2 was titrated out by both the strands (Fig. 7). The protein factor involved in this complex appears to be a single strand binding protein with no apparent specificity for the sequence.

Among the mononucleotide repeats, the homopolymeric stretches of A are the most abundant. Such stretches occur at a frequency of once in every 4 kb in the eukaryotic genome. Studies on *S. cerevisiae* suggested their possible involvement in transcriptional activation (19, 20). The oligo dA tracts in DNA can cause an alteration in the conformation of the DNA (21, 22). Such tracts have been reported to be unfavourable for nucleosomal assembly (23). Varshavsky and coworkers have shown preferential binding of proteins to this repeat in yeast and higher eukaryotes (24–26). An oligo dA · oligo dT tract binding protein called datin has been characterized in yeast (26). The presence of poly-dG tracts can alter the conformation of DNA and gene expression in a length dependent manner *in vitro* and *in vivo* (27).

It is of relevance to note that two p53-binding sequences have been reported for the mouse MCK gene, a distal element (−3182 to −3133) and a proximal element (−177 to −81). Promoter deletion analysis and electron microscopic studies have shown that the proximal and distal p53 elements synergistically activate the MCK gene, and that the interaction between the two causes looping out of the intervening DNA (28). The repeat sequences, especially homopolymeric tracts of A are known to cause bending of DNA, and other repeats are known to alter the conformation of the DNA locally. The presence of $(A)_{22}$ and $(GTTT)_8$ repeats downstream to the distal p53-binding site may cause

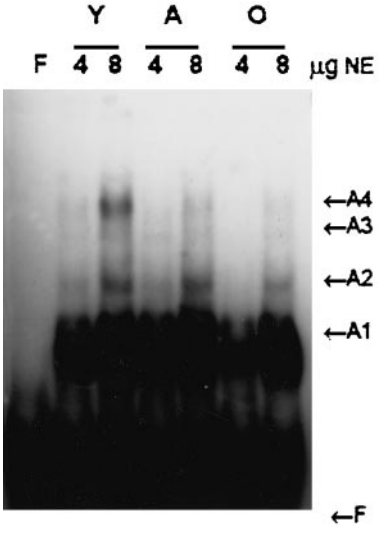

FIG. 6. EMSA with MCK-$(A)_{22}$ to study age-related binding activity of nuclear factors of heart. 4 and 8 μg of nuclear extracts prepared from heart of young (Y, 4 weeks), adult (25 ± 2 weeks), and old (70 ± 5 weeks) male mice were incubated with ^{32}P-labeled MCK-$(A)_{22}$. DNA–protein complexes were resolved on 5% native polyacrylamide gel. F, free DNA.

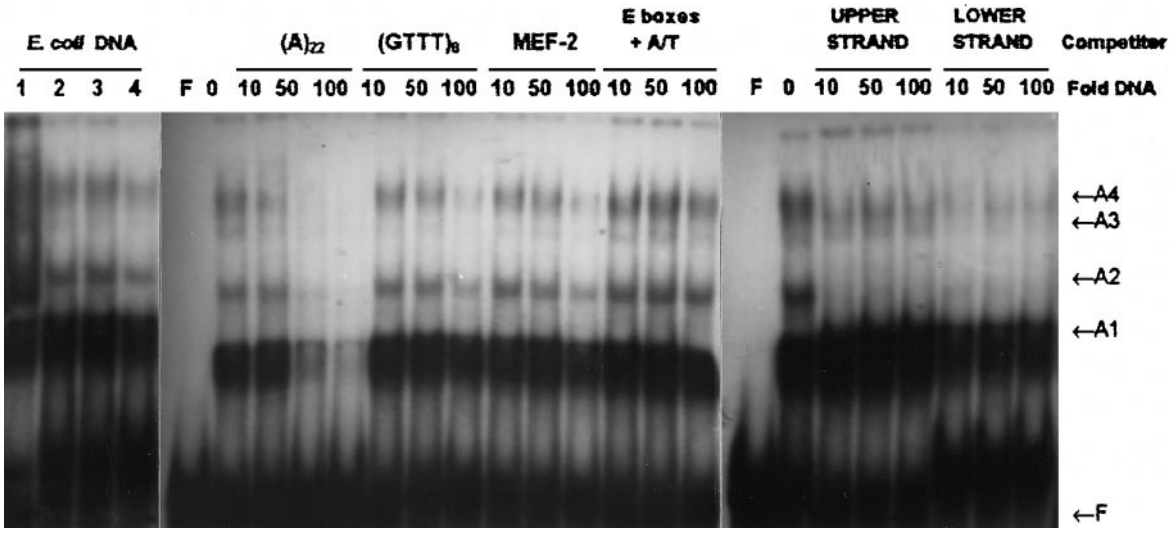

FIG. 7. EMSA with MCK-$(A)_{22}$ to study specificity of interaction with nuclear proteins. 6 μg of nuclear extract prepared from heart of male mice was incubated with ^{32}P-labeled MCK-$(A)_{22}$ in the absence (0-fold, lane 1) or presence (2500-, 5000-, and 10,000-fold excess, lanes 2–4) of *E. coli* DNA or in the presence of 0-, 10-, 50-, and 100-fold excess of cold competitor DNA-$(A)_{22}$, $(GTTT)_8$, MEF-2, E boxes + A/T, coding strand oligo or noncoding strand oligo. DNA–protein complexes were resolved on 5% native polyacrylamide gel. F, free DNA.

bending of DNA, and loop formation bringing the proximal and distal p53 sites closer.

Here we report for the first time an age-related decline in the binding activity of nuclear factors that interact with repeat sequences located in the 5′ flanking region of a gene. Further characterization of these protein factors and promoter deletion analysis will help in elucidating the role of these repeats in the expression of MCK gene in the heart during aging.

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