Purification and Preliminary Characterization of a Cardiac Kv1.5 Repressor Element Binding Factor

Paloma Valverde, Gideon Koren

Abstract—We have previously demonstrated that the cell-specific expression of Kv1.5 promoter is regulated by a silencer (Kv1.5 repressor element; KRE) containing a dinucleotide-repetitive element, \((GT)_{10}(GA)_{1}(CA)_{10}(GA)_{16}\). Electromobility gel shift assays (EMSAs) of KRE with GH3 nuclear extracts detected a unique DNA-protein complex, which was not detectable in Chinese hamster ovary or COS-7 cells. We further delineated KRE and determined that a 52-bp fragment that contained a \((GT)_{10}(GA)_{1}(CA)_{10}\) dinucleotide-repetitive element was sufficient for silencer activity. EMSAs using nuclear extracts isolated from the heart and from GH3 cells demonstrated that the 52-bp element formed specific and identical gel shift effects. These complexes were not detectable in EMSA experiments with liver nuclear extracts. Magnetic DNA affinity purification and UV cross-linking experiments identified a 27-kDa KRE binding factor (KBF) in GH3 cell nuclear extracts. Purified KBF reacted specifically with double-stranded KRE, abolishing the formation of multimeric KRE-DNA complexes. Thus, the interaction between KRE and KBF may play an important role in regulating the GH3- and cardiac-specific expression of Kv1.5. (Circ Res. 1999;84:937-944.)

Key Words: Kv1.5 ■ transcription ■ silencer ■ heart ■ K+ channel

Silencers are cis-acting regulatory DNA elements that downregulate gene transcription. They generally exhibit their activity in an orientation- and position-independent manner. In some cases, these silencer elements may serve to modulate the extent of expression of their respective genes in different cells or tissues. Such silencers would function as governors restricting fluctuations in gene activity, thereby preventing deleterious consequences of overexpression. Other silencer elements control cell type-specific gene expression, such as in the cases of the cardiac myosin light chain 2, type II Na+ channel, M4 muscarinic acetylcholine receptor, osteocalcin, and Kv1.5 genes. Although some silencer elements do not interact with DNA binding proteins, others appear to function through DNA-protein interactions.

DNA in general and dinucleotide-repetitive elements in particular are conformationally active molecules that are capable of adopting several types of conformations as dictated by their sequence, DNA binding proteins, and various ions. The DNA motif \((CA)_{n}\) is the most frequent tandem dinucleotide repeat in the mammalian genome. This repeat can respond to ionic conditions or negative supercoiling with a transition from the conventional B-DNA to a non-B-DNA forms. Some types of non-B-DNA conformation include left-handed Z-DNA, which occurs principally at segments of alternating C and G (or A) residues; cruciform DNA, which occurs at inverted repeat sequences; and bent DNA, which occurs at repeating A tracts. The mechanisms that account for transcriptional regulation in mammalian voltage-gated potassium channels are poorly understood. Several DNA elements that are important in the transcriptional regulation of Kv1.5, Kv1.4, and Kv3.1 genes have been identified. Of special interest for this report is the Kv1.5 repressor element (KRE) located in the 5’ flanking region of the cardiac Kv1.5 potassium channel gene. Deletion of KRE resulted in the loss of the cell-specific expression of a reporter gene containing the Kv1.5 promoter linked to chloramphenicol acetyltransferase (CAT). KRE acts as a silencer in cell lines that do not express Kv1.5 and has no effect on the reporter gene expression assessed in transfected GH3 cells (a pituitary cell line that expresses Kv1.5). KRE contains a dinucleotide-repetitive element that is necessary but not sufficient for mediating silencer effects in cells that do not express Kv1.5. Double-stranded KRE self-associates in vitro to form DNA-DNA complexes with slow electrophoretic mobility. Furthermore, incubation of KRE with GH3 cell nuclear extracts results in a specific gel shift effect. Here, we report a more detailed characterization of KRE and the detection of the binding activity in heart cell nuclear extracts. A fragment of 52 bp that contains 42 bp of the repetitive elements and 10 bp of the 5’ flanking sequence is sufficient to confer the silencing effect to a heterologous promoter and binding to GH3 cell nuclear extracts in electromobility gel shift assays (EMSAs). Magnetic DNA affinity purification and UV cross-linking experiments resulted in the identification of a 27-kDa KRE binding factor (KBF) present in GH3.
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cell nuclear extracts. EMSA competition experiments showed that KBF inhibits self-association and that an excess of KBF promotes self-association even in the presence of KRE. Collectively, these experiments suggest that KBF may regulate the cell-specific expression of Kv1.5 by abolishing the silencer effects of KRE.

Materials and Methods

Polymerase Chain Reaction (PCR) and Reporter Gene Constructs

The KRE sequence was divided into 3 fragments (A, B, and C) as previously described. 9 Deletion mutants of KRE shown in Figure 1 (except A9B1 and A9B1 in which 29 and 10 refer to length of fragment A in bp) were obtained by PCR with forward primers containing a HindIII restriction site (underlined), 5′-TTCAGGGGTTGCAAGCCTTGCTCTAGTC-3′ (for ABC), 5′-CCTGCCCTTCTGATAAGTGGAGCAA-3′ (for AB, A9B, A9B1B2, and KRE99), 5′-GGCAATTAGGAAGCTTTTCACTTCCA-TGTGGTGTA-3′ (for AB, A9B, and A9B1B2), and 5′-GGTAGAGTTTCAAAGCTTTGTGTGTG-3′ (for A9B1B2, A9B1B2, and KRE99). In all cases, p3100KvCAT 9 was used as a template. A neutralizing monoclonal antibody to the DNA polymerase of Thermus thermophilus (Clontech) was used to reduce nonspecific amplification products and primer-dimer artifacts. The PCR products were gel purified using a glass-based purification system (Pharmacia), digested with HindIII and BamHI restriction sites (underlined), and subcloned 5′ to thymidine kinase (TK)–CAT. The clone KRE99 (Figure 1A) was obtained when the PCR reaction was carried out in the absence of the antibody.

The clone KRE99 was made by PCR with the forward primer, 5′-GGCAAAAAGCCTAGTTTCTATGT9, containing a HindIII site (underlined) and the reverse primer, 5′-TCTCTCTGTGGATCCGTGTG-3′, containing a single base substitution in the B1 domain (G, in italics) and a BamHI site (underlined). The PCR product was then digested with HindIII and BamH1 and subcloned 5′ to TK-CAT.

The clones A9B1, A9B1, KRE772, KRE925, KRE925, and KRE928 were generated by annealing oligonucleotides containing HindIII and BamHI restriction sites (underlined) and then subcloning 5′ to TK-CAT. For example, M42a was made with the forward primer 5′-AGCTTCTAGATTTCATGT9, containing a HindIII site (underlined) and the reverse primer 5′-TCTCTCTGATCTCCGTGTG-3′, containing a single base substitution in the B1 domain (G, in italics) and a BamHI site (underlined). The PCR product was then digested with HindIII and BamH1 and subcloned 5′ to TK-CAT.

Cell Lines and Transfection Experiments

All cells used in this study were maintained in DMEM containing 10% FCS. Transient transfections were carried out with a liposome-based method (Life Technologies, Inc) following the manufacturer’s instructions. Forty-eight hours after transfection, the cells were harvested and assayed for protein concentration, β-galactosidase, and CAT activities as described by Mori et al. 10 CAT activity values were normalized for transfection efficiency by β-galactosidase activity and for cell density by protein concentration as described by Mori et al. 10 Relative CAT activity was calculated by comparing the activities of TK-CAT plasmids containing KRE deletion mutants with the activity of control TK-CAT plasmid (100%). Values are presented as mean±SD for a minimum of 3 determinations, each of which was performed in either duplicate or triplicate. Statistical significance was determined by 1-factor ANOVA, with P<0.05 considered significant.

Figure 1. Segments of KRE that are important for silencer activity. A, Schematic representation of KRE deletion mutants. The KRE sequence was divided into 3 fragments: fragment A, 58 bp 5′ to the repetitive sequence; fragment B, 102 bp containing the dinucleotide-repetitive element; and fragment C, 18 bp 3′ to the repetitive element. The dinucleotide-repetitive element was subdivided into 3 domains: B1, (GT)9(AGA)15; B2, (CA)15, and B3, (GA)16. The numbers 29 and 10 (left side) indicate length of fragment A in bp. B, Determination of silencer activity. Shown is a comparison of CAT expression of control TK-CAT containing KRE deletion mutants with the activity of control TK-CAT plasmid. Values are presented as mean±SD of at least 3 experiments. *Significant inhibition of CAT activity (P<0.05).

EMSAs

Nuclear extractions from cultured cells were carried out essentially as described by Therrien and Douin. 21 Nuclear extractions from rat heart or liver were performed as described by Thai et al. 22 EMSA experiments were performed as described by Mori et al. 9,20 EMSA competition experiments with unlabeled DNA were carried out following the same protocol, except that the appropriate amount of unlabeled competitor fragments was added 30 minutes before the
labeled probe. Complex bands were quantified by densitometry with the NIH Image software program.

**Label Transfer by UV Cross-Linking**

Probes were generated by PCR. The PCR mixtures contained bromodeoxyuridine triphosphate and dCTP at 40 μmol/L each; dATP, dGTP, (α-32P)dATP, and (α-32P)dGTP at 20 μmol/L each; and (α-32P)ddCTP at 10 μmol/L. Purified protein (10 ng) was preincubated with 10 ng of poly(dI-dC) for 15 minutes at room temperature. Then, 10<sup>3</sup> Cerenkov cpm of the 32<sup>P</sup>-labeled probe was added to the binding reaction mixture in either the presence or the absence of unlabeled competitor. After 30 minutes of incubation at room temperature, the mixtures were irradiated in a UV Stratalinker 1800 (Stratagene) with UV light to a total dose of 0.6 J. Probe DNA was then digested for 10 minutes at 37°C after the addition of 1 U of DNase I, 1 U of micrococcal nuclease, 33 mmol/L MgCl<sub>2</sub>, and 33 mmol/L CaCl<sub>2</sub>. After the addition of EDTA to 30 mmol/L, the mixtures were resolved by SDS-PAGE.

**Purification of KBF**

All buffers contained 0.5 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL pepstatin, 2 μg/mL aprotinin, and 1 mmol/L DTT. KBF was purified using the magnetic DNA affinity purification method developed by Gabrielsen and Huet. Briefly, double-stranded DNA was biotinylated by a dTTP analog with biotin covalently attached to the pyrimidine ring (Clontech) using a Klenow reaction. Then, the biotinylated probe was bound to streptavidin M-280 Dynabeads (Dynal) following the manufacturer’s recommendations. The protein binding was performed in 1× EMSA buffer (in mmol/L, HCl [pH 7.5] 10, NaCl 50, and EDTA 1, and 5% glycerol) supplemented with 0.5 to 1 μg of poly(dI-dC) per microgram of nuclear extract used as starting material. Then, the mixtures were washed 2 to 4 times in 1× EMSA buffer, and the bound protein was eluted with 1× EMSA buffer supplemented with 0.5 μmol/L NaCl. SDS-PAGE was performed according to the method of Laemmli. Gels were stained with the Silver Stain Plus kit (Bio-Rad) or Coomassie brilliant blue (Sigma). Purified samples were desalted and concentrated with microcon-10 or centricon-10 (Amicon) depending on the volume of sample. Protein concentration from purified samples was determined by a microbicinchoninic acid method (Pharmacia) and by absorbance at 280 nm.

**Results**

**KRE Core Structure Definition**

To define the sequences of KRE that are essential for its silencer activity, we created serial deletions at either the 3′ or 5′ end of KRE (Figure 1A). The KRE sequence was divided into 3 fragments as previously described. The repetitive element (fragment B; Figure 1A) was further analyzed by subdividing it into 3 domains: B1, B2, and B3 (Figure 1A). Each KRE deletion mutant was cloned 5′ to the TK-CAT promoter and tested for silencer activity using transient transfections of Chinese hamster ovary (CHO), COS-7, and GH3 cell lines. Serial deletion in fragment A revealed that 10 bp of fragment A (A<sub>10</sub>, abutting the B1 domain [Figure 1A]) were essential for silencer activity. Thus, all 3 deletions, including ABC, A<sup>5′</sup>B<sub>1</sub>C, and A<sup>5′</sup>B<sub>1</sub>C<sub>1</sub> functioned as effective silencers when cloned 5′ to the TK-CAT promoter (Figure 1B). However, deletion of the last 10 bp of fragment A (A<sub>10</sub> and cloning of BC in front of the TK-CAT promoter [BC]) did not reduce expression of CAT activity in COS-7 and CHO cell lines, thus abolishing silencer activity (Figure 1B). In contrast, deletion of fragments C (A<sup>5′</sup>B<sub>2</sub> and A<sup>3′</sup>B<sub>2</sub>) and B3 (A<sup>5′</sup>B<sub>1</sub>B<sub>2</sub> and A<sup>3′</sup>B<sub>1</sub>B<sub>2</sub>) did not affect silencer activity. However, replacement of part of the B1 domain with (CA)<sub>17</sub> or deletion of B2 (A<sup>5′</sup>B<sub>1</sub>B<sub>1</sub> and A<sup>3′</sup>B<sub>1</sub>B<sub>1</sub>1) abolished silencer activity. Thus, both B1 and B2 seem to form part of the core element of the silencer. None of the KRE deletion mutants mediated silencer activity in GH3 cells (Figure 1B). We next correlated the silencer activity with the ability of these fragments to form DNA-protein complexes when incubated with GH3 nuclear extracts. EMSA using 32<sup>P</sup>-labeled (KRE<sup>M99</sup>) or deletion of B2 (A<sup>5′</sup>B<sub>1</sub>B<sub>1</sub> and A<sup>3′</sup>B<sub>1</sub>B<sub>1</sub>1) abolished silencer activity. Thus, both B1 and B2 seem to form part of the core element of the silencer. None of the KRE deletion mutants mediated silencer activity in GH3 cells (Figure 1B).

We next correlated the silencer activity with the ability of these fragments to form DNA-protein complexes when incubated with GH3 nuclear extracts. EMSA using 32<sup>P</sup>-labeled KRE deletion mutants indicated that all KRE deletion mutants that exhibited silencer activity in non-GH3 cells (Figure 1B) formed DNA-protein complexes with GH3 cell nuclear extracts (Figure 2). By contrast, the KRE deletion mutants that did not exhibit silencer effects (BC, KRE<sup>M99</sup>, A<sup>5′</sup>B<sub>1</sub>B<sub>1</sub>, and A<sup>3′</sup>B<sub>1</sub>B<sub>1</sub>) did not bind to GH3 nuclear extracts (Figure 2). These results indicated that the B1 and B2 domains of KRE and the 10 bp of fragment A are essential for mediating both the silencer effect in non-GH3 cells and the GH3 cell–specific binding activity. Interestingly, under the conditions optimized for EMSA, both ABC and A<sup>5′</sup>B<sub>1</sub>B<sub>2</sub> probes could form multimeric DNA complexes with slow electrophoretic mobility that migrated slower than the double-stranded probe but faster than DNA-protein complexes (Figure 2, lanes 1 and 5).

To further refine the core elements of KRE, series of short deletions and point mutations were created (Table 1). Point mutation of (GA)<sub>n</sub> to (CA)<sub>n</sub> (KRE<sup>M90</sup>) did not affect silencer activity or binding. We then created successive deletions of the (GT)<sub>n</sub> and (CA)<sub>n</sub> repeats and compared their silencer and binding activities with that of A<sup>5′</sup>B<sub>1</sub>B<sub>2</sub> deletion mutant (Table 1). The results showed that KRE deletion mutants with at least 10 (GT)<sub>n</sub> and 10 (CA)<sub>n</sub> repeats mediated silencer...
effects in non-GH3 cells and bound to GH3 cell nuclear extracts. However, the mutants KRE M42a and KRE M42b, which contained 5 (GT) n and 5 (CA) n, respectively, lost their silencing activity and gel shift effects (Table 1). Therefore, 10 repeats were sufficient for silencer activity. Taken together, these results suggest that the minimum number of (GT) n and (CA) n repeats necessary for maintaining silencer activity may range from 6 to 10 repeats.

To further test the core element defined in the transfection and gel-retardation assays, we used nuclear extracts derived from tissue that expresses Kv1.5 (rat heart) and control tissue (rat liver) that does not express the protein. EMSAs revealed that incubation of KREM52 with heart nuclear extracts resulted in a gel shift with a complex of a size indistinguishable from that obtained by reacting GH3 nuclear extracts with the same probe (Figure 3). By contrast, liver nuclear extracts did not contain any gel shift activity.

Identification and Purification of a KBF in GH3 Cells

To identify the KRE binding proteins present in GH3 cells, nuclear extracts from this cell line were submitted to a magnetic DNA affinity purification step as described by Gabrielsen and Huet.23 The experiments were carried out at 2 different ratios of poly(dI-dC) (to modulate the stringency of binding) and in the presence of protease inhibitors (to avoid the degradation of the purified protein). The same procedure was used with nuclear extracts derived from COS-7 cells to determine whether the silencer effects in non-GH3 cells involve any DNA-protein interaction that cannot be detected by EMSA. Silver staining of the proteins eluted from GH3 nuclear extracts and silencer activity were compared with the properties of the mutant A10B1B2.

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>Sequence*</th>
<th>EMSA†</th>
<th>% CAT Activity‡</th>
<th>GH3</th>
<th>COS-7</th>
<th>CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10B1B2</td>
<td>A10 (GT)19(GA)1(CA)15</td>
<td>+</td>
<td>104±12</td>
<td>50.5±3.2§</td>
<td>45±7§</td>
<td></td>
</tr>
<tr>
<td>KREM80</td>
<td>A10 (GT)19(CA)1(CA)15</td>
<td>+</td>
<td>94.5±10</td>
<td>56.9±6.6§</td>
<td>38.1±13§</td>
<td></td>
</tr>
<tr>
<td>KREM72</td>
<td>A10 (GT)19(GA)1(CA)15</td>
<td>+</td>
<td>110±13.3</td>
<td>55.4±4.5§</td>
<td>35.9±10§</td>
<td></td>
</tr>
<tr>
<td>KREM42a</td>
<td>A10 (GT)10(GA)1(CA)10</td>
<td>+</td>
<td>94.7±18.6</td>
<td>50.9±12§</td>
<td>31.2±7§</td>
<td></td>
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<tr>
<td>KREM42b</td>
<td>A10 (GT)10(GA)1(CA)5</td>
<td>+</td>
<td>91.5±17.4</td>
<td>97.9±13</td>
<td>84±10</td>
<td></td>
</tr>
<tr>
<td>KREM42c</td>
<td>A10 (GT)10(GA)1(CA)5</td>
<td>+</td>
<td>120±25</td>
<td>107±5.3</td>
<td>75.9±21</td>
<td></td>
</tr>
</tbody>
</table>

*Mutants KREMi (where “i” indicates the molecular mass) were created by PCR. Their binding to GH3 nuclear extracts and silencer activity were compared with the properties of the mutant A10B1B2.
†32P-labeled mutants were tested for binding to GH3 nuclear extracts by EMSA as described in Materials and Methods. + indicates the formation of a DNA-protein complex; −, undetectable DNA-protein complex.
‡A comparison of CAT expression of TK-CAT and TK-CAT reporter gene constructs containing the different mutants. Transfections and CAT assays were as in Figure 1B. Results are expressed as percentage change from the expression of control TK-CAT reporter gene. Values are presented as mean±SD of 3 independent experiments.
§Significant inhibition of CAT activity (P<0.05).

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and purified KBF resulted in an identical gel shift effect, which indicates that KBF most likely represents the binding activity present in GH3 cell nuclear extracts (Figure 4B). The DNA binding activity of the purified protein resulted in a 50% yield (Table 2). Thus, with a single purification step we obtained a 1400-fold purification. Taken together, these results suggest that double-stranded KRE interacts with a 27-kDa protein present in GH3 but not in COS-7 nuclear extracts.

We then proceeded with UV cross-linking experiments to confirm that the DNA binding activity detected by EMSA was related to the 27-kDa protein and not to minor protein contaminants. Purified KBF (10 ng) was incubated with A29 B1B2, and the reaction mixtures were irradiated with UV light, treated with DNase I and micrococcal nuclease, and fractionated by SDS-PAGE. The specificity of label transfer was tested by competition experiments with unlabeled A29 B1B2 or mutated nonfunctional KRE of identical size (KRE M99) (Figure 1A). The results show (Figure 5) that the labeled probe was crossed-linked to a polypeptide with an apparent molecular mass of ~27 kDa (lane 1). The labeling of the 27-kDa protein was completely inhibited by an excess of unlabeled A29 B1B2 (Figure 5, lane 3) but not by an excess of the unlabeled KRE M99 (Figure 5, lane 4). No protein was detected in UV cross-linking experiments in the absence of KBF (Figure 5, lane 2) or when A29 B1B2 was incubated with nuclear extracts from COS-7 cells (data not shown). Taken together, these results confirm that the 27-kDa polypeptide (KBF) is the protein that interacts with KRE.

**Binding of KRE and KBF Versus Self-Assembly of KRE**

We have previously shown that the double-stranded KRE self-associates spontaneously to form large DNA-DNA complexes with slow electrophoretic mobility and that an excess of cold fragment promotes these associations. As shown in Figure 6A, 0.5 ng of 32 P-labeled A29 B1B2 self-associates to form complexes with slow electrophoretic mobility in the presence of a 100-fold molar excess of cold fragment (Figure 6A, lanes 3 and 4). These complexes had an apparent mobility of 400 and 800 bp. By contrast, the addition of an excess of KREM99 did not promote the formation of these complexes even in the presence of a 100-fold molar excess of unlabeled KREM99 (Figure 6A, lanes 7 and 8).

We next tested whether the binding of purified KBF to KRE affects self-association of KRE. The self-association of KRE was favored by increasing the concentration of A29 B1B2 to 1 ng. In the absence of KBF (Figure 6B, lanes 1 and 7),

**TABLE 2. Purification Summary of KBF**

<table>
<thead>
<tr>
<th>Step*</th>
<th>Amount of Protein, μg†</th>
<th>Total Activity, Units‡</th>
<th>Specific Activity, U/μg</th>
<th>Recovery, %</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude nuclear extract</td>
<td>10 000</td>
<td>5000</td>
<td>0.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Magnetic DNA affinity chromatography</td>
<td>4.2</td>
<td>2959</td>
<td>704.5</td>
<td>59.2</td>
<td>1409</td>
</tr>
</tbody>
</table>

*Fifty plates (100 mm in diameter) of exponentially growing GH3 cells were used to obtain 10 mg of nuclear extract. The biotinylated A29 B1B2 was used to purify the 27-kDa KBF as described in Figure 4B.

†Protein concentration was determined by a microbicinchoninic acid method (Pharmacia) or by absorbance at 280 nm.

‡One unit of binding activity was defined as the amount of protein that converted 50% of the probe to DNA-protein complexes in EMSA.
labeled A<sup>29</sup>B<sub>1</sub>B<sub>2</sub> formed DNA complexes with slow electrophoretic mobility. Incubation of A<sup>29</sup>B<sub>1</sub>B<sub>2</sub> with KBF resulted in a gel shift effect forming 2 DNA-protein complexes similar to those observed with GH3 nuclear extracts (Figure 2A and 2B; Figure 6B, lanes 2 and 8). The addition of an excess of cold probe competed with the binding in a nonlinear fashion and promoted the reappearance of the complexes with slow electrophoretic mobility that are identical to those formed by spontaneous DNA-DNA interactions (Figure 6B, lanes 3 through 6). Importantly, the appearance of self-association bands coincided with the effective competition of the DNA-protein interactions. By contrast, an excess of KRE M99 did not promote the self-association of A<sup>29</sup>B<sub>1</sub>B<sub>2</sub> (Figure 6B, lanes 8 through 12). These observations indicate that binding of KBF to KRE may interfere with self-association of KRE and that an excess of KRE is necessary and sufficient to promote self-association of KRE, even in the presence of KBF.

**Discussion**

For most eukaryotic genes, the tissue specificity and the level of expression are determined by interactions between trans-acting factors, with cis-acting elements located within the 5' flanking sequences. We have previously identified a cis-regulatory element (Kv1.5 repressor element, KRE<sup>9</sup>) that participates in determining the cell-specific expression of Kv1.5 promoter in GH3 cells. KRE represses expression of Kv1.5-CAT and TK-CAT reporter gene constructs in transient transfections of cells that do not express Kv1.5. By contrast, expression of these constructs in GH3 cells, which express Kv1.5, is not affected.<sup>9</sup> In the present report, we further defined the minimal sequence that is necessary and sufficient for mediating the silencer activity. Our results indicate that a 52-bp DNA fragment containing 42 bp of alternating purines and pyridines and 10 bp of 5' flanking sequence is sufficient for mediating the silencing activity. EMSA experiments revealed that this core element is also sufficient for binding to nuclear factor(s) present in GH3 cell nuclear extracts. EMSA experiments revealed that this core element is also necessary for binding to nuclear factor(s) present in GH3 cell nuclear extracts. Moreover, indistinguishable gel shift activity is also present in the nuclear extracts derived from rat heart. By contrast, this gel shift activity is not present in the liver or multiple cell lines in which Kv1.5 transcript and polypeptide are undetectable.<sup>27</sup> The stability and specificity of interactions between KRE and the nuclear factor(s) present in GH3 cell extracts were sufficiently high to attempt a DNA affinity purification of this trans-acting factor. Indeed, a 27-kDa KBF present in the nuclei of GH3 cells was purified ~1000-fold by magnetic DNA affinity purification. The identification of KBF and the absence of KRE-silencer activity in GH3 cells suggest that KBF may abolish the silencing activity of KRE and therefore act as an "antisilencer." Our observation in this cell line may also apply to the regulation of Kv1.5 in vivo. Indeed, incubation of rat heart nuclear extract with KRE resulted in a gel shift effect indistinguishable from that of GH3 cell nuclear extract and KRE.

Several trans-acting factors alter DNA conformation. A dinucleotide-repetitive element such as poly(GT/CA) can also change its conformation from the orthodox right-handed B-DNA to a non-B-DNA form.<sup>29</sup> This transition can be induced by increased superhelical density,<sup>29</sup> by 10 to 50 mmol/L of magnesium,<sup>14</sup> and by submillimolar levels of calcium.<sup>15</sup> Moreover, alternating purine-pyrimidine stretches can interact with each other through the formation of multi-stranded structures such as intermolecular triplexes or tetraplexes.<sup>17</sup> Indeed, double-stranded DNA fragments containing poly(CA)<sub>B</sub>(TG)<sub>B</sub> were shown to associate spontaneously in vitro to form stable 4-stranded structures that could be detected by gel electrophoresis and electron microscopy.<sup>30</sup> Similar complexes were also formed by the self-association of double-stranded KRE<sup>9</sup> or A<sup>29</sup>B<sub>1</sub>B<sub>2</sub>, which contain long (CA)<sub>B</sub> and (GT)<sub>B</sub> repeats. However, KRE<sup>M99</sup>, which contained a short (GT)<sub>B</sub> repeat, did not form such DNA-DNA complexes or retain the silencer effect.

Our deletion analyses, the EMSA competition assays, and the UV cross-linking experiments confirm that double-stranded KRE interacts with KBF in a sequence-specific and perhaps structure-specific manner and that the DNA-protein interaction may interfere with the formation of DNA-DNA interactions in vitro and perhaps also in vivo. It is tempting to speculate that the formation of DNA-DNA interactions by (CA)<sub>B</sub> and (GT)<sub>B</sub> repetitive sequences might be crucial for the silencer effect either directly or through interactions with ubiquitous nuclear proteins. Indeed, the ubiquitous high-mobility-group (HMG)-1 and HMG-2 proteins recognize
altered DNA conformations formed by (CA)$_n$ and (GT)$_n$ repetitive sequences. Moreover, recombinant HMG-1 protein binds to KRE in the absence of poly(dI-dC). KBF, by interfering with the self-association of KRE and/or the binding of HMG-1 and HMG-2, may abolish the silencer effect in GH3 cells (or heart). In cell lines (or tissues) in which KBF is not expressed, the default silencing effect would be dominant.

Several proteins, such as SWI/SNF in yeast, may facilitate transcriptional activation of specific genes by antagonizing chromatin-mediated transcriptional repression. The complex has been purified and shown to contain an ATP-dependent nucleosome-disruption activity. The DNA binding properties of SWI/SNF are similar to those of proteins containing an HMG box domain. Thus, the SWI/SNF complex interacts with the minor groove of the DNA helix, binds synthetic 4-way junction DNA (which is thought to mimic the topology of DNA as it enters or exits the nucleosome), and introduces positive supercoils into relaxed plasmid DNA. These properties are likely to be important in the remodeling of chromatin structure by the SWI complex and some HMG-box containing proteins. Members of the HMG-box family of proteins have been shown to interact functionally with other transcription factors or with other proteins that act as coactivators. It is also believed that members of the HMG-domain family of proteins regulate gene expression by coordinating the assembly of multiprotein complexes, thereby promoting interactions with the basic transcriptional machinery. The mammalian SWI/SNF-like complexes, which contain a subunit with a HMG box, may also interact with higher-order chromatin structure by 2 or more DNA binding domains, thereby mediating ATP-dependent nucleosome disruption.

Our results suggest that the interaction between KBF and KRE may be partly responsible for the cell-specific expression of Kv1.5 in GH3 cells. Therefore, the cloning of KBF may help us to elucidate the mechanisms that control the expression of Kv1.5 in the heart and other tissues.

Acknowledgments

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References

Purification and Preliminary Characterization of a Cardiac Kv1.5 Repressor Element Binding Factor
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