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Ca^{2+}-mediated Site-specific DNA Cleavage and Suppression of Promiscuous Activity of KpnI Restriction Endonuclease*[S]

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The characteristic feature of type II restriction endonucleases (REases) is their exquisite sequence specificity and obligate Mg^{2+} requirement for catalysis. Efficient cleavage of DNA only in the presence of Ca^{2+} ions, comparable with that of Mg^{2+}, is previously not described. Most intriguingly, KpnI REase exhibits Ca^{2+}-dependent specific DNA cleavage. Moreover, the enzyme is highly promiscuous in its cleavage pattern on plasmid DNAs in the presence of Mn^{2+} or Mg^{2+}, with the complete suppression of promiscuous activity in the presence of Ca^{2+}. KpnI methyltransferase does not exhibit promiscuous activity unlike its cognate REase. The REase binds to oligonucleotides containing canonical and mapped noncanonical sites with comparable affinities. However, the extent of cleavage is varied depending on the metal ion and the sequence. The ability of the enzyme to be promiscuous or specific may reflect an evolutionary design. Based on the results, we suggest that the enzyme KpnI represents an REase evolving to attain higher sequence specificity from an ancient nonspecific nuclease.

Type II REases recognize specific DNA sequences, which vary from 4 to 8 bp and cleave both the strands in Mg^{2+}-dependent reaction. They exhibit remarkable features in discriminating between specific and nonspecific DNA, not only in DNA binding but also during DNA cleavage (1). Based on the divalent metal ion requirement for DNA binding, REases have been classified into two groups (2). For example, EcoRI, BamHI, RsrI, and a few other enzymes bind to DNA preferentially at their recognition sites in the absence of divalent metal ions (3–5). In contrast, EcoRV, PvuII, TaqI, and a few other enzymes bind to cognate sequences in a divalent metal ion-dependent manner (6–8). However, both the groups of enzymes need Mg^{2+} for catalysis. The role of metal ions in DNA binding and the subsequent hydrolysis of the phosphodiester bond at the recognition site have been studied in detail for several REases.[1] Although the natural cofactor for all type II REases is Mg^{2+}, Ca^{2+} can replace Mg^{2+} for specific DNA binding, without leading to the formation of productive complex (9). Some type II REases form stable protein-DNA complexes in the presence of Ca^{2+} but do not support the DNA cleavage even though Ca^{2+} is a nearly perfect analogue for Mg^{2+} (10). Ca^{2+} also increases the binding specificity of several REases such as MunI, TaqI, and Cfr10I toward their cognate sites (9, 11).

Several type II REases show reduced sequence specificity at suboptimal reaction conditions such as low ionic strength, elevated pH, in the presence of water-miscible solvents, and when Mg^{2+} is substituted with Mn^{2+}. This relaxed specificity was first observed for EcoRI REase and termed as star activity (12). Subsequently, relaxed specificity has been characterized for several REases such as BamHI (13), DdeI (14), EcoRV (15), PvuII (16), and RsrI (17).

KpnI REase isolated from *Klebsiella pneumoniae* recognizes the palindromic double-stranded DNA sequence 5′-GGTAC↓C-3′ and cleaves DNA by generating a 3′, 4-base overhang (18). The enzyme binds to specific DNA in the absence of divalent metal ions (19). In this paper, we describe the highly promiscuous cleavage of DNA in the presence of Mg^{2+} and Mn^{2+}. The promiscuous activity is completely suppressed in the presence of Ca^{2+}, which induces site-specific DNA cleavage.

EXPERIMENTAL PROCEDURES

Enzymes and DNA—KpnI REase was purified as described previously (18). The enzyme was diluted in binding buffer (20 mM Tris-HCl (pH 7.4), 25 mM NaCl, and 5 mM 2-mercaptoethanol) for all the studies. The concentration of the enzyme was estimated by the method of Bradford (20). One unit of KpnI REase is defined as the amount of enzyme required for complete digestion of 1 μg of λDNA at 37 °C for 1 h by using assay buffer containing 5 mM Mg^{2+}. T4 polynucleotide kinase and Klenow polymerase were purchased from New England Biolabs. Oligonucleotides were from Microsynth Inc., Switzerland, and purified on 18% urea-polyacrylamide gel (21). The purified oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol). *Pfu* and *Taq* DNA polymerases, pUC18 and pBR322, were obtained from Bangalore Genei Pvt. Ltd., India. The plasmid clones used for primer extension studies were from the laboratory collections.

REase Digestion—Standard assay conditions involved lower enzyme units to DNA ratio (up to 10 units of enzyme), whereas in relaxed assay conditions more than 15 units of enzyme were used. Digestions were carried out by incubating different units of KpnI REase with plasmid DNA or 0.2 pmol of labeled oligonucleotides in assay buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM β-mercaptoethanol, and appropriate concentrations of divalent metal ions at 37 °C for 1 h. The reactions were terminated by adding stop dye containing 0.6% SDS and 25 mM EDTA. The cleavage products of plasmid DNA and oligonucleotides were analyzed on 1% agarose or 12% urea-polyacrylamide gel, respectively. The Ca^{2+} chase reactions were carried out at a fixed concentration of Mg^{2+} (2 mM) or Mn^{2+} (0.5 mM) by using increasing concentrations of Ca^{2+}.

Primer Extension Analysis—The different plasmids (2 μg) were incubated with 20 units of KpnI REase in a buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol, and 5 mM Mn^{2+} at 37 °C for

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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. 1 and 2.

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[1] The abbreviations used are: REase, restriction endonuclease; MTase, methyltransferase.

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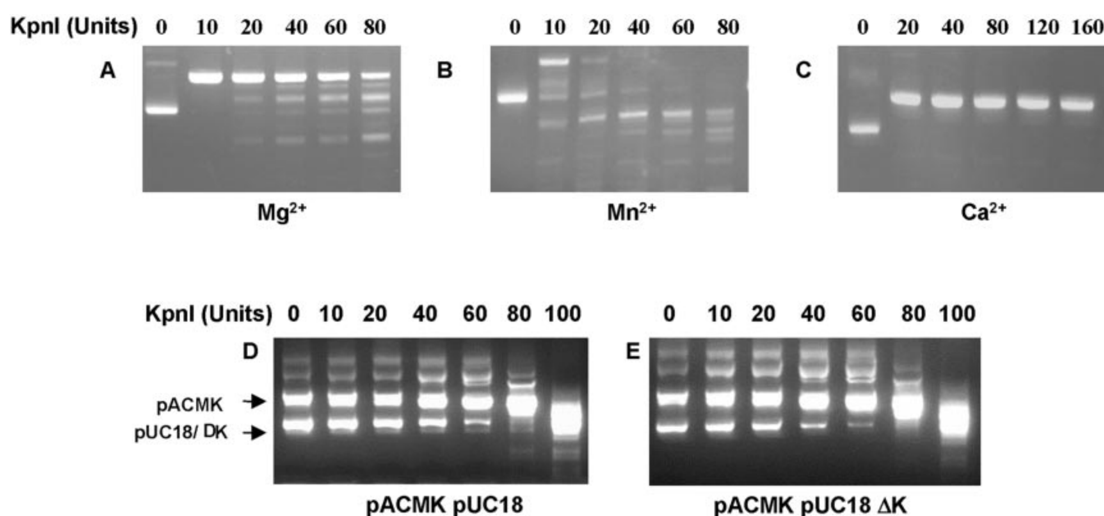

FIG. 1. **Promiscuous cleavage by KpnI REase.** Plasmid DNA cleavage by KpnI in the presence Mg^{2+}(*A*), Mn^{2+}(*B*), and Ca^{2+}(*C*). Different amounts of KpnI REase were incubated with pUC18 DNA in assay buffer containing 5 mM Mg^{2+} or Mn^{2+} or Ca^{2+} and 10 mM Tris-HCl (pH 7.4) at 37 °C for 1 h. The cleavage products were analyzed by 1% agarose gel electrophoresis. *D* and *E*, cleavage of methylated plasmid DNA substrates by KpnI REase. The plasmids pUC18 (*D*) and pUCΔK (*E*) isolated from *E. coli* DH10B (pACMK) expressing KpnI MTase were incubated with different concentrations of KpnI REase (0–100 units) in the presence of 5 mM Mg^{2+}. The reactions were terminated by adding a mixture of 0.6% SDS and 25 mM EDTA. The samples were analyzed on 1% agarose gel. In each panel, *lane 0* represents buffer control with the respective metal ions as indicated.

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1 h. The digested plasmids were purified by phenol/chloroform extraction, and end-labeled primers were used for primer extension reaction as described by Balke *et al.* (22). The cleavage sites were mapped by standard dideoxy sequencing reactions using TaqDNA polymerase (23).

DNA Binding Assay—Different concentrations of KpnI REase (12–1200 nM for noncanonical sites; 3–500 nM KpnI for cognate sites) were incubated with 3.75 nM of end-labeled, double-stranded oligonucleotides containing cognate and noncognate sites in the buffer (20 mM Tris-HCl (pH 7.4), 25 mM NaCl, and 5 mM 2-mercaptoethanol) on ice for 15 min. The free DNA and enzyme-bound complexes were separated on 8% native polyacrylamide gel and autoradiographed. The amounts of DNA in free and bound form were quantitated using PhosphorImager, and the results were analyzed by Scatchard plot.

RESULTS

Metal Ion-dependent Cleavage Reactions—The effect of divalent metal ions on the activity of KpnI REase was studied by using plasmid DNA as substrates. KpnI REase exhibits relaxed specificity at high enzyme to substrate ratios. A high degree of promiscuous activity was observed when more than 15 units of the enzyme was used in the presence of Mg^{2+} (Fig. 1*A*). The promiscuous activity was further enhanced in the presence of Mn^{2+} as compared with Mg^{2+} under similar reaction conditions (Fig. 1*B*). This activity of the enzyme was observed at a range of Mg^{2+} (1–5 mM) or Mn^{2+} (0.5–5 mM) concentrations and was more pronounced than the conventional star activity exhibited by several type II REases. Most surprisingly, unlike other type II REases, KpnI REase exhibits specific DNA cleavage in presence of Ca^{2+} (Fig. 1*C*) showing greater specificity even at high enzyme to substrate ratios in contrast to the cleavage pattern with Mg^{2+} and Mn^{2+}. The Ca^{2+}-mediated high fidelity, efficient DNA cleavage is a unique property of KpnI REase. In the presence of Mg^{2+} or Mn^{2+}, the enzyme exhibited promiscuous cleavage with pBR322 that lacks the specific KpnI recognition sequence (supplemental Fig. 1).

KpnI MTase Does Not Exhibit Promiscuous Behavior—In order to assess the promiscuous activity of KpnI on methylated DNA and to evaluate whether the MTase exhibits promiscuous behavior, both pUC18 and pUCΔK (pUCΔK was generated by deleting the KpnI site from pUC18 by digesting with EcoRI and HindIII) were isolated from *Escherichia coli* DH10B harboring KpnI MTase expressing plasmid, pACMK (18). The methylated pUC18 DNA was not cleaved at a canonical site by the KpnI REase under standard assay conditions (1–10 units of enzyme). However, under relaxed conditions both pUC18 and pUCΔK DNA isolated from KpnI MTase expressing cells exhibited promiscuous cleavage patterns by KpnI REase in the presence of Mg^{2+} (Fig. 1, *D* and *E*). From these results, we infer that the MTase does not exhibit promiscuous activity as only the cognate site was methylated. Furthermore, the modification of the cognate site has no influence on the promiscuous activity of the REase. Relaxed cleavage specificity was observed only in the presence of either Mg^{2+} or Mn^{2+}, and the modified DNA substrates were refractile to cleavage by KpnI REase in the presence of Ca^{2+}.

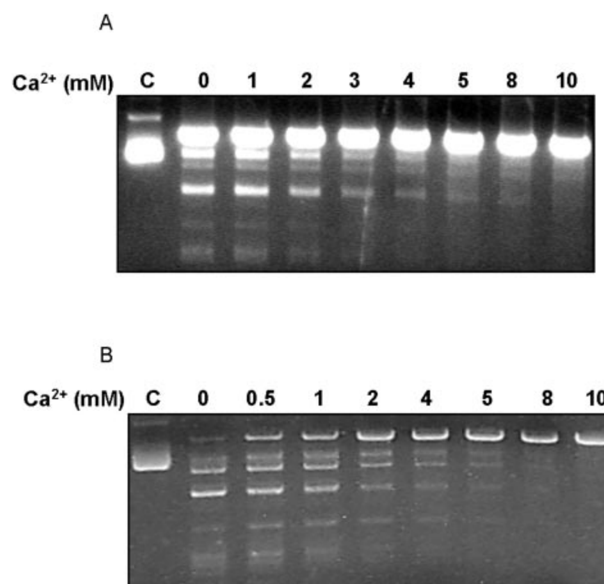

FIG. 2. **Effect of Ca^{2+} on Mg^{2+}- or Mn^{2+}-mediated promiscuous activity of KpnI REase.** 1 μg of pUC18 was incubated with 40 and 20 units of KpnI REase in the presence of 2 mM Mg^{2+}(*A*) 0.5 mM Mn^{2+} (*B*), respectively, and the reactions were chased with increasing concentrations of Ca^{2+} as indicated. The reactions were analyzed on 1% agarose gel. *Lane C* in both the panels represents supercoiled DNA substrate incubated with the enzyme in the absence of any divalent metal ions.

Suppression of Promiscuous Activity—It has been shown by a

TABLE I
The list of oligonucleotides used for binding and cleavage studies of KpnI

Canonical oligonucleotide
5′-ATTGCGTGGTACCCGCTCTT-3′
5′-AAGAGCGGGTACCACGCAAT-3′

Noncanonical oligonucleotides
5′-ATTGCGTGaTACCCGCTCTT-3′
5′-AAGAGCGGGTAtCACGCAAT-3′
5′-ATTGCGTGGaACCCGCTCTT-3′
5′-AAGAGCGGGTtCCACGCAAT-3′
5′-ATTGCGTGtTACCCGCTCTT-3′
5′-AAGAGCGGGTAaCACGCAAT-3′
5′-ATTGCGTGGTcCCCGCTCTT-3′
5′-AAGAGCGGGgACCACGCAAT-3′
5′-ATTGCGTGGTACgCGCTCTT-3′
5′-AAGAGCGcGTACCACGCAAT-3′
5′-ATTGCGTtGTACCCGCTCTT-3′
5′-AAGAGCGGGTACaACGCAAT-3′
5′-ATTGCGTGGatCCCGCTCTT-3′
5′-AAGAGCGGGatCCACGCAAT-3′

Nonspecific oligonucleotide
5′-GAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCT-3
5′-AGGAAGCGGAAGACGCCCAATACGCAAACCGCCTCTC-3′

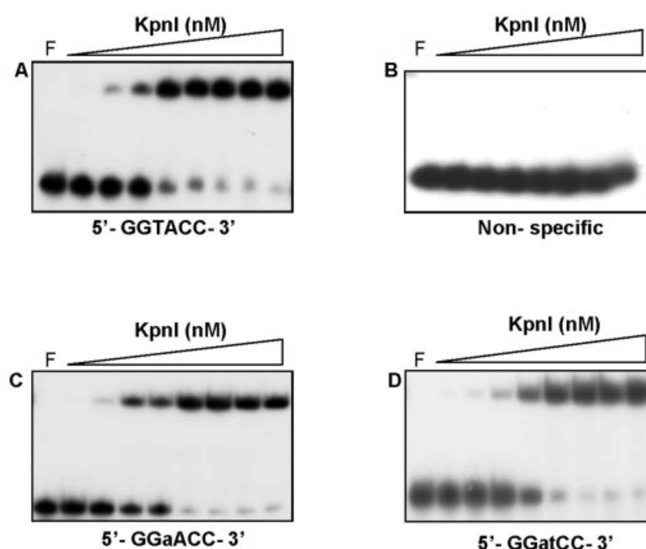

FIG. 3. **Electrophoretic mobility shift assay.** The DNA binding reactions were carried out by using 3.75 nM of different oligonucleotides (shown in Table I) containing canonical and noncanonical KpnI sites with increasing concentrations of the enzyme. *A*, canonical oligonucleotide. Concentrations of KpnI REase used were 2, 4, 7.5, 15, 30, 62, 125, and 250 nM. *B*, nonspecific oligonucleotide. Concentrations of KpnI REase used were 15, 30, 62, 125, 250, 500, 1000, and 1500 nM. *C* and *D*, noncanonical sites. These oligonucleotides were incubated with 5, 7.5, 15, 30, 62, 125, 250, and 500 nM of KpnI REase. The samples were analyzed using 8% native polyacrylamide gel as described under "Experimental Procedures." *Lane F* in each panel refers to oligonucleotide incubated with electrophoretic mobility shift assays buffer without enzyme.

large number of biochemical and structural studies with several type II REases that Mg^{2+} and Ca^{2+} occupy the same pocket in the active site (24–26). High fidelity cleavage in the presence of Ca^{2+}, irrespective of the enzyme concentrations and robust promiscuity at low concentrations of the enzyme in the presence of low amounts of Mg^{2+} (2 mM) and Mn^{2+} (0.5 mM), prompted us to carry out competition experiments between the different metal ions. In order to assess the effect of Ca^{2+} on Mg^{2+}- and Mn^{2+}-dependent promiscuous activity of KpnI REase, Ca^{2+} chase experiments were carried on Mg^{2+}- or Mn^{2+}-bound enzyme-DNA complex under relaxed conditions. The Mg^{2+}- or Mn^{2+}-mediated promiscuous activity was completely suppressed by an increase in Ca^{2+} ion concentrations (Fig. 2, *A* and *B*). In a converse experiment with fixed amounts of Ca^{2+} (2 mM) and increasing concentrations of Mg^{2+} or Mn^{2+}, the promiscuous activity of the enzyme was restored (data not shown). These observations indicate that Mg^{2+}, Mn^{2+}, and Ca^{2+} compete with each other to occupy the active site of the enzyme.

Binding and Cleavage Analysis of KpnI REase with Oligonucleotide Containing Noncanonical Sites—Primer extension strategy was used to map the noncanonical sites, and the representative data are shown in the supplemental Fig. 2. Analysis of the data obtained from primer extension products of different plasmid substrates revealed that many hexameric sequences with one nucleotide change from the canonical sequence are efficient cleavage sites. The sequences that have been mapped include tGTACC, GtTACC, GaTACC, GGaACC, GGTcCC, GGTAtC, GGTACg, and GGTACt, where the lowercase letter indicates noncanonical nucleotide. The cleavage position within each of these noncanonical recognition sequences was identical to that observed with the canonical sequence. The relaxation in the specificity of KpnI occurred at every nucleotide position within the hexameric recognition sequence.

To study the DNA binding properties of KpnI at noncanonical sites, several oligonucleotides were designed based on the results from the primer extension data (Table I). Double-stranded oligonucleotides containing different mapped sites with one of the strands labeled at the 5′ end were used as substrates for binding studies. Electrophoretic mobility shift assays were carried out in the absence of divalent metal ions, and the representative results are shown in Fig. 3. KpnI REase formed a single protein-DNA complex with oligonucleotide containing GGTACC (canonical site) and GGaACC and GGatCC (noncanonical sites) sequences (Fig. 3, *A*, *C* and *D*). All other noncanonical sequence containing oligonucleotides described above also formed similar complexes (data not shown). Scatchard analysis of the binding data revealed that the affinity of the enzyme to the various oligonucleotides varied from 8 to 34 nM (Table II). KpnI REase failed to show a stable complex with a completely nonspecific oligonucleotide (Fig. 3*B*) as observed earlier (19).

TABLE II
DNA binding affinities of KpnI REase for different oligonucleotides

Recognition sequence	K_d
	nM
-GGTACC-	8
-GaTACC-	13
-GtTACC-	13
-GGTcCC-	18
-tGTACC-	18
-GGaACC-	28
-GGTACg-	31
-GGatCC-	34

Cleavage reactions were carried out using double-stranded oligonucleotides in the presence of Mg^{2+} or Mn^{2+} as a cofactor. KpnI REase cleaves oligonucleotides containing various noncanonical sites having single nucleotide substitution, albeit less efficiently when compared with the cleavage with oligonucleotides containing canonical sequence (Fig. 4*A*). Noncanonical sites GtTACC, GaTACC, and GGTcCC were preferred over GGaACC, tGTACC, and GGTACg (Fig. 4*A*). No DNA cleavage was detected at the noncanonical sites in the presence of Ca^{2+} even at a high enzyme to substrate ratio (Fig. 4*B*). The Ca^{2+}-mediated DNA cleavage is confined to the canonical site irrespective of plasmid or oligonucleotide substrates.

DISCUSSION

DNA-binding enzymes involved in phosphoryl transfer reactions generally require divalent metal ions to promote their

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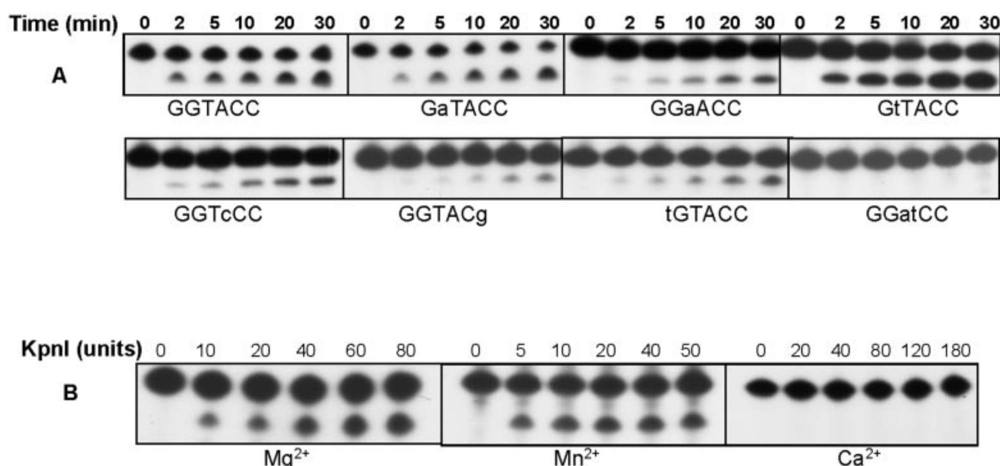

FIG. 4. **Cleavage at noncanonical sites by KpnI REase.** *A*, the DNA cleavage at different noncanonical oligonucleotides by KpnI REase in the presence of Mg^{2+}. The reactions were performed with 30 units of KpnI REase in DNA cleavage buffer containing 5 mM Mg^{2+} and 0.2 pmol of labeled double-stranded oligonucleotides containing cognate as well as noncognate sequences as indicated. The difference from the KpnI canonical sequence is shown by *lowercase letters* of noncanonical sequences. *B*, comparison of the cleavage profile of preferred noncanonical sequence (GtTACC)-containing oligonucleotide. The reactions were carried out with 0.2 pmol of labeled oligonucleotide and different concentrations of KpnI REase as indicated in the presence of 5 mM Mg^{2+} or Mn^{2+} or Ca^{2+}. In all the cases, the reactions were incubated at 37 °C for 30 min, and the products were analyzed on 12% urea-polyacrylamide gel.

activity. In most cases Mg^{2+} is used as a cofactor. The preference for Mg^{2+} over other metal ions is probably due to its favorable physical and chemical properties, such as a small ionic radius with extensive hydration, high charge density, high transport numbers, and its abundance in the cell (27). In addition to DNA cleavage, Mg^{2+} is also required for specific binding of several type II REases to their recognition sequences. Divalent metal ion Ca^{2+} can substitute Mg^{2+} for DNA binding (9). The comparative DNA binding studies revealed that EcoRV exhibits a greater degree of specificity in the presence of Ca^{2+} for the canonical site (28). Binding of several other REases to their recognition sites is enhanced in the presence of Ca^{2+} (8, 9, 11). Thus, substitution of Ca^{2+} to Mg^{2+} in DNA binding studies indicates that these two metal ions occupy the same DNA-binding pocket. Tertiary structures of several REases reveal that Ca^{2+} indeed occupies the same position as that of Mg^{2+} in the active site (10, 24–26, 29, 30).

KpnI REase binds to cognate DNA sequence in the absence of metal ion cofactor (19). The exquisite cleavage specificity in the presence of Ca^{2+} and suppression of Mg^{2+}/Mn^{2+}-mediated promiscuous activity by Ca^{2+} are unique characteristics of KpnI REase. This suppression could probably be due to conformational changes at the active site of the protein leading to the formation of inactive or unstable complex at the noncanonical site ceasing DNA cleavage in the presence of Ca^{2+}. The metal ion competition experiments of EcoRI and EcoRV showed that the Mg^{2+}-dependent endonuclease activity was inhibited in the presence of Ca^{2+}. However, in a Mn^{2+}-dependent reaction, EcoRV showed enhanced activity by Ca^{2+} (9). In these two well studied enzymes, Ca^{2+} by itself does not induce DNA cleavage. The ionic radius of Ca^{2+} is larger compared with Mg^{2+} and Mn^{2+} which favors different coordination geometry. The bulk effect and different coordination chemistry of Ca^{2+} may cause steric interference and destabilize the bound oxygens that could perturb the transition state rather than the initial binding to the active site. The change in solvation at the interface of the enzyme-DNA complex is also known to play an important role in site-specific recognition. Influence of bound water molecules on site-specific recognition and cleavage of DNA was studied for several REases (31, 32). In the case of Mg^{2+}-mediated phosphodiester hydrolysis, an outer-sphere pathway that requires a solvated metal cofactor is favored as Mg^{2+} maintains a moderate to high hydration state. According to this mechanism the metal cofactor is involved in stabilizing the transition state, either electrostatically and/or through hydrogen bonding from the metal-bound water molecules. The solvation state of the metal cofactor is of critical importance in site-specific DNA recognition by these enzymes. Ca^{2+}-bound KpnI REase with fewer bound water molecules at the active site could be responsible for higher specificity in DNA recognition and cleavage than the Mg^{2+}/Mn^{2+}-bound enzyme.

The Ca^{2+}-dependent cleavage specificity of KpnI REase could probably be due to the involvement of additional residues neighboring or distal to the active site influencing the stabilization of the KpnI-Ca^{2+}-DNA transition state. Delineation of the KpnI active site architecture may open up the possibilities to engineer other type II REases for specific cleavage, resulting in high fidelity REases devoid of any promiscuous activity.

The ability of REases to recognize a wide range of sequences having one or two nucleotide changes in the recognition sequence is of evolutionary significance. The R-M systems appear to have evolved from two totally unrelated genes such as a nonspecific nuclease and a possible repair protein, which acquired an MTase activity (33, 34). While evolving to recognize the specific sequence, some of the REases would have been evolutionarily stuck at an earlier step to retain broader cleavage specificity. KpnI REase seems to represent one such system. Under most physiological conditions, the enzyme is unlikely to express the promiscuous behavior as it exhibits remarkable specificity in the presence of Ca^{2+}. However, the promiscuity of the enzyme could be advantageous to the bacteria if allowed to be expressed under certain circumstances especially when encountering the invasion by foreign genomes.

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