Kinetic analysis of DNA binding by the c-Myb DNA-binding domain using surface plasmon resonance

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Abstract Kinetics of the interaction of the c-Myb DNA-binding domain (R2R3) with its target DNA have been analyzed by surface plasmon resonance measurements. The association and dissociation rate constants between the standard R2R3, the Cys130 mutant substituted with Ile, and the cognate DNA are 2.3×10^5 M^{-1} s^{-1} and 2.6×10^{-3} s^{-1} at pH 7.5 and 20°C, respectively. Kinetic analyses of the binding of the standard R2R3 to the non-cognate DNAs and those of the R2R3 mutant proteins to the cognate DNA showed that the reduction of the binding affinity was mainly due to an increase in the dissociation rate.

Key words: Binding kinetics; c-Myb; DNA binding; Protein engineering; Surface plasmon resonance

1. Introduction

Protein-nucleic acid interactions are the origin of the control of gene expression. In order to elucidate the basis of this genetic regulation, the determinants of the specificity and the stability of protein-nucleic acid complexes must be understood. Various methods, such as structural, thermodynamic, and kinetic analyses, have been used for investigations of recognition mechanisms [1–3]. With the recent improvements in both isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR), these methods have been successfully employed for quantification of the molecular interactions. We have used these methods to analyze the mechanism of DNA recognition by the c-*myb* protooncogene product (c-Myb).

c-Myb is a transcriptional activator that binds with a dissociation constant of about 10^{-9} M to the specific DNA sequence $PyAAC^G/_TG$, where Py indicates a pyrimidine [4–6]. The DNA-binding domain (DBD) of c-Myb consists of three imperfect 51 or 52 residue repeats (designated R1, R2, and R3 from the N-terminus), and the last two repeats, R2 and R3, are the minimum unit for specific DNA binding [7–10]. The NMR structure of the complex of the minimum DBD (R2R3) with the Myb-binding DNA sequence (MBS-I) revealed that each third helix in R2 and R3 is engaged in direct and specific base recognition [11,12].

We have recently characterized the thermodynamics of the binding of the c-Myb R2R3 to DNA, using ITC to measure both the enthalpy change associated with the formation of the complex and the corresponding binding affinity [13]. In this study, we describe the kinetics of c-Myb R2R3 binding to DNA, using SPR methodology to measure both the association rate constant (k_{on}) and the dissociation rate constant (k_{off}). The binding affinity for the same interaction calculated from the SPR data can be compared with two other independent measurements, a filter-binding assay and ITC [13–15].

In every c-Myb R2R3 and mutant protein used in this study, the Cys130 in R2 was replaced with Ile, to facilitate protein purification and the DNA-binding assay. As shown previously, the affinity and the specificity of the C130I mutant are almost equal to those of the wild-type protein [14,16], so this mutant protein was used as the standard R2R3 in this study and is denoted R2R3*.

2. Materials and methods

2.1. Preparation of c-Myb R2R3 mutant proteins and oligonucleotides

The expression and purification methods of the mouse c-Myb R2R3* and all of the R2R3* mutant proteins were described previously [13]. The purified proteins were dialyzed against 100 mM potassium phosphate buffer (pH 7.5) containing 20 mM KCl (buffer A).

Six kinds of double-stranded oligonucleotides, with one strand biotinylated at the 5′-end of the sequence, were obtained from BEX (Tokyo). The sequences of these fragments are shown in Fig. 1. The complementary strands were annealed, and were dialyzed against buffer A, as described previously [13].

2.2. Surface plasmon resonance measurement

The BIAcore biosensor system, BIAcore 2000 (Biacore AB), was used to measure the real-time interaction between c-Myb R2R3 and DNA. The detection system uses SPR, a quantum mechanical phenomenon that detects changes in the refractive index at the surface of a sensor chip [17]. In order to immobilize the DNA, BIAcore sensor chip SA surfaces with streptavidin pre-immobilized to dextran were used. The biotinylated DNA fragments were diluted to 0.2 μM in 100 mM potassium phosphate buffer (pH 7.5) containing 20 mM KCl and 0.005% Tween 20 (buffer B), and were applied to the sensor chip surface at a rate of 5 μl/min during 2 min, which resulted in the capture of between 1000 and 1200 response units (RU). In order to measure the association, the c-Myb R2R3 mutant proteins, diluted at various concentrations in buffer B, were injected over the immobilized DNA at a rate of 20 μl/min during 3 min. The dissociation was measured by injecting buffer B alone. At the end of each experiment, the surface was regenerated with one 30 μl injection of a 1 M NaCl solution. All experiments were performed at 20°C.

Data were analyzed using the BIAevaluation program 2.1, which

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Abbreviations: ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; DBD, DNA-binding domain; R2R3, minimum c-Myb DNA-binding domain composed of the second and the third repeat fragments; MBS, Myb-binding DNA sequence; k_{on}, association rate constant; k_{off}, dissociation rate constant; R2R3*, stable mutant of R2R3, in which Cys130 is substituted with Ile; RU, response unit

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	-3 1 5 10 15 19	
MBS-I	5′- CACCCTAACTGACACACATTCT	-3′
	3′- GTGGGATTGACTGTGTGTAAGA	-biotin -5′
[A4G]MBS-I	CACCCTGACTGACACACATTCT	
[A5G]MBS-I	CACCCTAGCTGACACACATTCT	
[C6T]MBS-I	CACCCTAATTGACACACATTCT	
[G8A]MBS-I	CACCCTAACTAACACACATTCT	
[NC-b]MBS-I	CACCTTGCTTGACACACATTCT	

Fig. 1. Sequences of DNAs used in the present study. The base numbering follows that suggested by Ogata et al. [11]. The consensus base sequence is boxed in MBS-I. In the non-cognate DNAs, the sequence of one strand is indicated, and the 5′-end of the complementary strand is biotinylated. The substituted bases are indicated in italics. The names of the base pair substituted DNAs follow those suggested by Oda et al. [13].

was supplied with the BIAcore. In this program, a non-linear least squares method was used for the determination of the rate binding constants. The k_{off} values were calculated from the dissociation phases of the sensorgrams at various concentrations of the analyte, using the following equation.

$$\ln (RU_1/RU_n) = k_{off} \times t \quad (1)$$

The k_{on} values were calculated from the association phases of the sensorgrams:

$$k_s = k_{on} \times C + k_{off} \quad (2)$$

The association constants (K_a) were calculated from the two rate constants:

$$K_a = k_{on}/k_{off} \quad (3)$$

The equilibrium association constants ($K_{a,eq}$) can also be determined from Scatchard analysis:

$$RU_{eq}/C = K_{a,eq} \times RU_{max} - K_{a,eq} \times RU_{eq} \quad (4)$$

where C is the free analyte concentration, RU_{eq} is the steady-state response, and RU_{max} is the total surface binding capacity.

3. Results

3.1. Interactions between the c-Myb R2R3* and the cognate and non-cognate DNAs

The kinetics of binding to cognate and non-cognate DNA fragments were analyzed using the BIAcore system. For the single base substituted DNAs, the base pairs at positions 4, 5, 6, and 8 in the 22-mer MBS-I were substituted by the other purine or pyrimidine base to maintain the DNA structure [18], and for the multiple base substituted DNA, [NC-b]MBS-I, the base substitutions were designed to have almost no trace of the consensus sequence (Fig. 1). The purified c-Myb R2R3* was passed over the surface of the sensor chip, on which the biotinylated DNA fragment had been immobilized. Portions of the sensorgrams of the R2R3* binding to MBS-I and [NC-b]MBS-I, and the corresponding Scatchard plots are shown in Fig. 2. The analyzed data are summarized in Table 1. All measurements were repeated with at least five different protein concentrations, and typical experimental errors for the k_{on} and k_{off} values are also shown.

In all of the binding to the non-cognate DNAs, the k_{off} values were larger than that of the binding to the MBS-I, while the k_{on} values were almost the same as that. The equilibrium association constants obtained from the Scatchard plots were similar to the kinetically determined association constants, within a factor of three or better. The current K_a values tended to decrease for each non-cognate DNA duplex, similar to the corresponding K_a values previously obtained using the filter-binding assay and the ITC measurement, although the K_a values obtained using the BIAcore were higher than those obtained using the previous two methods, with at most a 30-fold variation [6,13–15].

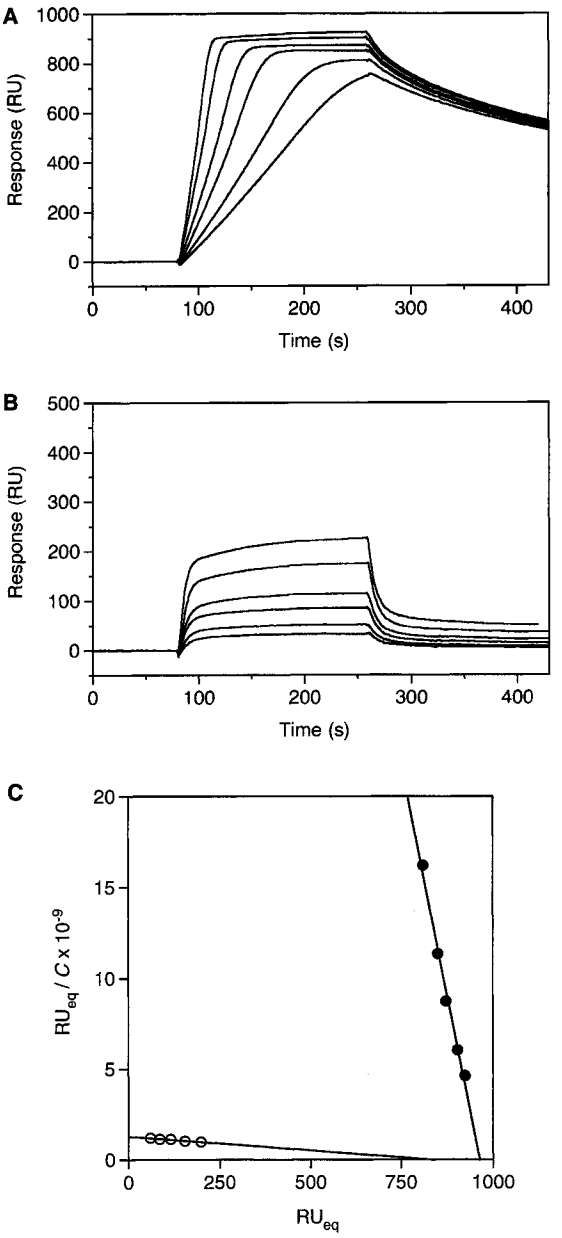

Fig. 2. Sensorgrams and Scatchard analysis of the interactions between c-Myb R2R3* and two kinds of DNA fragments. A, B: A 60 μl aliquot of a 37.5, 50, 75, 100, 150, or 200 nM solution of R2R3* in buffer B was passed over the immobilized MBS-I (A) or [NC-b]-MBS-I (B) at 20°C with subsequent washing with buffer B only. C: The RU_{eq}/C values were plotted versus the RU_{eq} values for the binding of R2R3* (50–200 nM) to immobilized MBS-I (closed circle) and [NC-b]MBS-I (open circle), from the data in A and B, respectively.

Table 1
Kinetic and association constants of R2R3* binding to DNAs

DNA	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	K_a (M^{-1})	$K_{a,eq}$ (M^{-1})
MBS-I	$2.6\pm0.1\times10^{-3}$	$2.3\pm0.5\times10^{5}$	8.9×10^{7}	1.0×10^{8}
[A4G]MBS-I	$4.0\pm0.3\times10^{-2}$	$2.4\pm0.4\times10^{5}$	6.1×10^{6}	2.8×10^{6}
[A5G]MBS-I	$2.5\pm0.2\times10^{-2}$	$2.5\pm0.2\times10^{5}$	9.9×10^{6}	4.7×10^{6}
[C6T]MBS-I	$4.9\pm0.1\times10^{-2}$	$2.3\pm0.2\times10^{5}$	4.6×10^{6}	2.1×10^{6}
[G8A]MBS-I	$2.0\pm0.2\times10^{-2}$	$2.2\pm0.2\times10^{5}$	1.1×10^{7}	6.6×10^{6}
[NC-b]MBS-I	$7.0\pm0.6\times10^{-2}$	$2.1\pm0.2\times10^{5}$	3.1×10^{6}	1.5×10^{6}

k_{off} and k_{on} are the average of six experiments for protein concentrations from 37.5 to 200 nM, and $K_{a,eq}$ is the average of five experiments for protein concentrations from 50 to 200 nM.

3.2. Interactions between the c-Myb R2R3* mutant proteins and the cognate and non-cognate DNAs

The interactions of the various R2R3* mutant proteins, in which the amino acids in the recognition helix (K128, S187), the hydrophobic core (V103, V107), and the linker connecting the two repeats (N139, P140, E141) were substituted, respectively, with the MBS-I were also investigated, using the BIAcore. The analyzed data are summarized in Table 2. The interactions of the R2R3* mutants with the [NC-b]MBS-I could not be determined accurately, since their sensorgrams were poorly defined, due to their weak interaction. In addition, interactions between the R2R3* mutants and the [G8A]MBS-I were observed, and the results are indicated in Table 3. Similar to the results of the R2R3* binding to the non-cognate DNAs, all of the reductions in the DNA-binding affinities of the mutant proteins were due to increases in the k_{off} values.

3.3. Salt dependence of the interactions between the c-Myb R2R3* and the cognate DNA

The salt dependence of the binding kinetics of R2R3* to the cognate MBS-I was analyzed using the BIAcore, and the analyzed data are summarized in Table 4. Similar to the ITC experiments [13], higher KCl concentrations affected the binding affinity. The decrease in the affinity was due to the increase in the k_{off} value. At concentrations higher than 210 mM, the interactions were too weak to be determined.

4. Discussion

In the present kinetic analyses of the interactions between c-Myb R2R3 mutants and various DNA fragments, we found that specific and non-specific DNA binding resulted from differences in the dissociation rate. Until now, SPR methodology has been used to study protein-DNA interactions [19–27]. Precise analyses of specific and non-specific DNA binding, using several non-cognate DNAs, have shown that increases in affinity and specificity are mostly attributable to decreases in the dissociation rate [23,24], which is in good agreement with the present results. Additionally, we showed that electrostatic forces appear to contribute to the interaction between c-Myb R2R3 and DNA, in which the dissociation rate increased by increasing the salt concentration. This phenomenon was also observed in other interactions, which have been mostly attributed to electrostatics [24,28]. Recently, Zargarian et al. [29] reported a kinetics study of the complex formation of R2R3 and the 16-mer DNA by monitoring tryptophan fluorescence, and observed two-step kinetics. The apparent rate of the fast step k_{on} was 7×10^{5} M^{-1} s^{-1}, which was three times our rate but in different experimental conditions.

Although the BIAcore system offers the benefit of measuring the binding kinetics, k_{on} and k_{off}, for interactions of biological macromolecules, it requires chemical modification and immobilization. On the other hand, the ITC method can measure not only the binding affinity but also the thermodynamics in the solution phase without modification, although a relatively large sample is needed. Thus far, there have been several reports that analyzed the same interaction using both SPR and ITC [30–32]. In the present work, the K_a value obtained by the SPR measurement was higher than that obtained by the filter binding assay and the ITC measurement [6,13–15]. There is no obvious explanation for this difference. On the whole, the difference between the data becomes larger as the binding becomes weaker.

The binding data may be influenced by the rebinding and mass transport effects that are often observed in BIAcore experiments [33–35]. When the dissociation curves of the various protein concentrations are normalized, they become almost identical and the rebinding effect can be ignored. In contrast, almost all of the k_{on} values are constant, independent of the

Table 2
Kinetic and association constants of R2R3* mutants binding to MBS-I

DNA	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	K_a (M^{-1})	$K_{a,eq}$ (M^{-1})
K128M[a]	$6.2\pm0.2\times10^{-2}$	$2.1\pm0.4\times10^{5}$	3.4×10^{6}	2.9×10^{6}
S187G[a]	$5.6\pm0.5\times10^{-3}$	$2.3\pm0.3\times10^{5}$	4.0×10^{7}	5.4×10^{7}
S187A[a]	$4.0\pm0.1\times10^{-3}$	$2.2\pm0.2\times10^{5}$	5.6×10^{7}	5.8×10^{7}
V103I/V107H[a]	$9.7\pm1.0\times10^{-3}$	$2.4\pm0.1\times10^{5}$	2.5×10^{7}	1.0×10^{7}
N139G[a]	$1.2\pm0.1\times10^{-2}$	$2.3\pm0.2\times10^{5}$	2.0×10^{7}	9.3×10^{6}
P140G[a]	$1.1\pm0.1\times10^{-2}$	$2.5\pm0.2\times10^{5}$	2.3×10^{7}	1.0×10^{7}
E141G[b]	$2.7\pm0.1\times10^{-3}$	$2.4\pm0.3\times10^{5}$	8.8×10^{7}	5.8×10^{7}
N139A[a]	$2.8\pm0.8\times10^{-3}$	$2.5\pm0.4\times10^{5}$	8.8×10^{7}	4.2×10^{7}
P140A[a]	$7.8\pm0.9\times10^{-3}$	$2.4\pm0.3\times10^{5}$	3.0×10^{7}	1.7×10^{7}
E141A[b]	$2.8\pm0.2\times10^{-3}$	$2.3\pm0.3\times10^{5}$	8.0×10^{7}	8.9×10^{7}

[a] k_{off}, k_{on}, and $K_{a,eq}$ are the average of three experiments for protein concentrations from 50 to 200 nM.
[b] k_{off}, k_{on}, and $K_{a,eq}$ are the average of five experiments for protein concentrations from 50 to 200 nM.

Table 3
Kinetic and association constants of R2R3* mutants binding to [G8A]MBS-I

DNA	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	K_a (M^{-1})	$K_{a,eq}$ (M^{-1})
K128M	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
S187G[b]	$2.5\pm0.2\times10^{-2}$	$2.7\pm0.4\times10^{5}$	1.1×10^{7}	2.9×10^{6}
S187A[b]	$2.0\pm0.4\times10^{-2}$	$2.4\pm0.3\times10^{5}$	1.2×10^{7}	2.7×10^{6}
V103I/V107H[c]	$3.0\pm0.6\times10^{-2}$	$2.5\pm0.5\times10^{5}$	8.3×10^{6}	1.2×10^{6}
N139G[c]	$3.0\pm0.1\times10^{-2}$	$2.2\pm0.4\times10^{5}$	7.3×10^{6}	n.d.[d]
P140G[c]	$3.1\pm0.2\times10^{-2}$	$2.3\pm0.3\times10^{5}$	7.4×10^{6}	n.d.[d]
E141G[b]	$1.5\pm0.4\times10^{-2}$	$2.2\pm0.2\times10^{5}$	1.5×10^{7}	3.5×10^{6}
N139A[b]	$2.5\pm0.4\times10^{-2}$	$2.3\pm0.2\times10^{5}$	9.3×10^{6}	3.7×10^{6}
P140A[c]	$3.4\pm0.1\times10^{-2}$	$2.4\pm0.3\times10^{5}$	7.0×10^{6}	n.d.[d]
E141A[b]	$1.4\pm0.4\times10^{-2}$	$2.2\pm0.2\times10^{5}$	1.6×10^{7}	3.2×10^{6}

[a]Not determined from the sensorgram, because the increase of the RU was too small.
[b]k_{off}, k_{on}, and $K_{a,eq}$ are the average of four experiments for protein concentrations from 25 to 200 nM.
[c]k_{off}, k_{on}, and $K_{a,eq}$ are the average of three experiments for protein concentrations from 50 to 200 nM.
[d]Not determined from the sensorgram, because the RU_{eq} was not observed during the experimental time.

ionic strength, for both specific and non-specific binding, and so the rate of mass transport to the sensor surface might appear as an intrinsic problem in the binding kinetics. However, this possibility can also be ignored. If it were the case, then (1) the upper limit of the detectable on-rate constant would be determined by the mass transport effect, and somewhat smaller k_{on} values would be observed [35,36]. In addition, (2) we were careful to analyze each response curve only after the characteristic time for the mass transport, where d(RU)/dt is constant [37], and (3) the equilibrium binding constants evaluated from the Scatchard plot analysis using RU_{eq} were in reasonably good agreement with the kinetically determined values [38]. Therefore, the current results are not considered to be experimental artifacts.

A simple model for the current binding kinetics would be as follows.

$$\text{protein} + \text{DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{protein·DNA (non}-\text{specific complex)} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{protein·DNA (specific complex)} \quad (5)$$

When k_2 is much slower than k_1, the association phase for the specific and non-specific binding should apparently be the same. However, since the non-specific binding also depends greatly on the ionic strength, as observed in our previous ITC study, the observed on-rate constant would depend on the salt concentration, in contrast to the current results.

Following the observation by Shimamoto [39], another chemical species, I_0, can be introduced as a 'DNA domain', where a protein is located in the neighborhood of the DNA and changes the refractive index, but does not interact with the DNA strongly enough to extract the condensed counterions from the DNA chain. The sliding of a protein on DNA [40] may occur in this DNA domain. Then, a new kinetic model could be as follows.

$$\text{protein} + \text{DNA} \underset{k_{-0}}{\overset{k_0}{\rightleftarrows}} \text{I}_0 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{protein·DNA (non}-\text{specific complex)} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{protein·DNA (specific complex)} \quad (6)$$

Here, one should note that the SPR methodology should not discriminate the species I_0, protein·DNA (non-specific complex), and protein·DNA (specific complex), because the local refractive indices of the solution around the immobilized DNA on the sensor surface are not very different among the species.

Thus, when the association rate constant, k_0, is assumed to be much larger than the other two rates, k_1 and k_2, and the dissociation rate constant, k_{-0}, is assumed to be much larger than the other two rates, k_{-1} and k_{-2}, only the k_0 and the k_{-1} or k_{-2} are mainly observed as changes of the local refractive index of the solution. Thus far, the observed K_a value could be different from that of the ITC observation, which measures directly the enthalpy change of the thermodynamic reaction upon DNA binding. In this model, the observed on-rate constant ($\cong k_0$) should not depend much on the salt concentration, but the off-rate constant that is contributed much from k_{-1} or k_{-2} can depend on it.

Table 4
Salt dependence of R2R3* binding to MBS-I

KCl (mM)	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	K_a (M^{-1})	$K_{a,eq}$ (M^{-1})
20[a]	$2.6\pm0.1\times10^{-3}$	$2.3\pm0.5\times10^{5}$	8.9×10^{7}	1.0×10^{8}
37[b]	$6.7\pm0.4\times10^{-3}$	$2.3\pm0.1\times10^{5}$	3.4×10^{7}	5.9×10^{7}
58[c]	$1.1\pm0.1\times10^{-2}$	$2.4\pm0.2\times10^{5}$	2.2×10^{7}	3.2×10^{7}
115[c]	$2.8\pm0.2\times10^{-2}$	$2.3\pm0.2\times10^{5}$	8.2×10^{6}	7.1×10^{6}
153[c]	$6.8\pm0.2\times10^{-2}$	$2.3\pm0.2\times10^{5}$	3.3×10^{6}	1.7×10^{6}
210	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]

[a]Data were taken from Table 1.
[b]k_{off}, k_{on}, and $K_{a,eq}$ are the average of four experiments for protein concentrations from 50 to 150 nM.
[c]k_{off}, k_{on}, and $K_{a,eq}$ are the average of four experiments for protein concentrations from 75 to 200 nM.
[d]Not determined from the sensorgram, because the increase of the RU was too small.

In our previous thermodynamic analyses, we showed that non-specific binding in protein-DNA associations is mainly entropically driven by electrostatic interactions [13]. Combined with the previous thermodynamic analyses, the current kinetic study suggests that proteins involved in the gene regulation of biological phenomena may interact equally with any nucleic acid sequence in the DNA domain at the initial association phase. They may interact with DNA chains at many different local energy minima, by non-specific DNA binding with weak binding constants and fast dissociation rates. When they interact with their corresponding specific sequences after the sliding procedure, they bind to them much more tightly than the other sites with slow dissociation rates, and then they can work as genetic regulators.

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