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DNA Annealing and DNA–Protein Interactions by Capillary Electrophoresis[1]

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This work deals with annealing of single-stranded DNA and the binding of a serum respond factor to a DNA probe containing specific binding site. Capillary electrophoresis (CE) method is explored and compared with the mobility-shift gel electrophoresis (GE) procedure. The results indicate the CE method offers direct and rapid annealing of the DNA strands. It requires no prior incubation with additives (polynucleotides, proteins) to reduce nonspecific DNA–protein interactions. Unwanted nonspecific interactions are not observed in the CE method. The presence of a fluorescein tag to the DNA probe yields identical results to those with the radioactive label. A fluorescein tag in the CE work can be used without any adverse effects. The dissociation constant (K_d) of this protein–DNA complex by the CE method was similar to those determined by the GE method ($\cong 10^{-6}$ M). The proposed method is extremely powerful, highly sensitive, quantitative, and fast. It can determine even very small conformational differences of the DNA probe.

Key Words: **binding constants; capillary electrophoresis; DNA conformation changes measured by CE; DNA–protein binding; DNA–protein interactions; MEKC; mobility shift gel electrophoresis assay; on-column annealing, binding constant; on-column DNA–protein complex formation; association constants.**

The study of sequence-specific binding of proteins to DNA is very important because these bindings are involved in DNA replication, recombination, and gene expression. Gene transcription for example, requires that regulatory proteins must recognize and interact with specific sites within the gene allowing specific control over the temporal and spatial pattern of expression. These interactions are characterized by binding constant (K_d) in the range of 10^{-6} and 10^{-12} M. The interactive forces at play are vast; they are poorly understood. Traditionally, the mobility-shift gel electrophoresis (GE) and the DNA footprinting (digestion of DNA with DNase I or a chemical agent) methods are used to study DNA–protein interactions. The latter method is used to study the protected or "buried" regions. The GE method is based on slow migration of the DNA–protein complexes through a low-ionic strength polyacrylamide gel medium than the fast moving free DNAs.

Here, we demonstrate the feasibility of an on-column capillary electrophoresis (CE) mobility-shift approach for DNA annealing, DNA–protein binding, and probing their binding constants. The DNA probe used is a CAG rich (CArG) binding site for serum response factor (SRF) (referred here as CArG dsDNA probe). The binding site, known as the serum response element (SRE), is a regulatory sequence found in several genes, including the *c-fos* gene and the human cardiac actin gene (1).

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotides used (Pharmacia, Piscataway, NJ) included the antisense strand containing the CArG specific binding site for SRF (labeled with fluorescein isothiocyanate on the 5′-end), 5′ ^{F}pG-C-T-T-G-C-C-T-T-A-T-T-T-G-G-T-C-C-C-C 3′ (^{F}CArG or antisense strand^{F}) and the unlabeled sense strand, 5′ pG-G-G-G-A-C-C-A-A-A-T-A-A-G-G-C-A-A-G-C. To control the nonspecific interactions, synthetic polymers from the same source were used: poly(dG:dC) (MW: 5.6×10^6; average length, 8,560 bp), poly(dI:dC) (MW: 2.5×10^5; average length, 383 bp), and poly(dI-dC:dC-dI) (MW: 4.0×10^6; average length, 6,175 bp).

Capillary electrophoresis. The CE equipment was described earlier (2, 3). Samples were applied to the column (having an uncoated active silica surface or a neutral-coated surface) by gravity flow from a height of 15 cm relative to the capillary outlet. The viscosity of the run buffer was controlled to 1.82 cP at 20°C to give a flow rate of 1.25 nL/s from that height. The CE buffer (Tris-borate-EDTA buffer, pH 8.3) with a medium-range polymer, e.g., hydroxyethyl cellulose (HEC, 0.25%, 12 kDa; Fig. 1) or a linear polyacrylamide (0.2%, 700 to 1,000 kDa), was synthesized according to Grossman (4) (Figs. 2 and

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5). The detection of the DNA probe and the complex was based on a fluorescence tag (emission at 514 nm) attached to one of the DNA strand. The fluorescence detection was enhanced by using an argon laser (excitation at 488 nm). For on-column annealing, the DNA strands were applied to a CE column sequentially, i.e., antisense strand^F followed by the sense strand to avoid introducing detectable contamination of the fluorescent-antisense solution with dsDNA.

Electrophoresis mobility (GE) shift assay. Poly(dI:dC) and poly-(dG:dC) were examined to determine the need for a nonspecific competitor. Eight reaction mixtures were prepared; a 5-μL sample from each reaction (25 μL) was analyzed in different lanes (Fig. 3a). The nuclear proteins (11.4 μg) were added to lanes 2 to 7; poly(dI:dC) (2 μL) to lanes 1 to 4; poly(dG:dC) (2 μL) to lanes 5 to 8, unlabeled CArG dsDNA probes (1 μL) to lanes 3 and 6, and unlabeled NF-κB dsDNA, (1 μL) to lanes 4 and 7. Buffer C (20 mM HEPES, 1.5 mM $MgCl_2$, 50 mM KCl, 20% Glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonic fluoride, 2 μg/mL leupeptin hemisulfate, and 2 μg/mL aprotinin, 10 μl), BSA (0.5 μL), and ^{32}P-labeled CArG dsDNA probe (1 μL) were added to the each reaction mixture. To detect for possible interference by the presence of fluorescence tag parallel experiments were carried out with the GE procedure by using ^{F}CArG dsDNA probe.

Determination of the binding constant. During the course of this work, several reports have appeared that in general deal with the determination of binding constants with CE (5–9); for a review article, see Hancock (10). Very recently, DNA-binding of small duplexes using CE method was also reported (11, 12). In this study, the empirical binding constant, K_d, was determined from the slope of Scatchard's plots: [D-P]/[D] vs [D-P], where [D-P] and [D] are the equilibrium concentrations (in mol · L^{-1}) of the bound probe and free probe, respectively—determined by peak areas in the CE method and by band intensities in the GE method. The plots produced a straight line in each case of the form $[D\text{-}P]/[D] = -(1/K_d)[D\text{-}P] + [P_o]$ for isolated and noninteracting binding sites. The term $[P_o]$ is derived from the intercept on the ordinate and represents the initial concentration of the SRF in the nuclear extract (for other methods of K_d determination see 13–17).

RESULTS AND DISCUSSION

Formation of dsDNA in a CE column. The ability to resolve single-stranded DNA (ssDNA) from pre-annealed double-stranded DNA (dsDNA) by CE is studied by using a fluorescein-labeled antisense strand (antisense strand^F). The complementary strands are pre-annealed at 25°C for 5 min. The material is applied immediately to an *uncoated* column. The sample is resolved into two components (Fig. 1b). The early peak is characterized as the antisense strand^F (Fig. 1a) and the one emerging later as the dsDNA (Fig. 1b). The sense strand has no fluorescence label, thus it cannot be observed in these experiments. Formation of more dsDNA is observed as the reaction mixture is incubated for a longer time. For example, the antisense strand^F peak decreased as the mixture is incubated for 20 min (Fig. 1c). The remaining material (25%) of the strand^F changes into dsDNA after 40 min (Fig. 1d).

Instantaneous *on-column* annealing of the complementary strands can be achieved by using either a *coated* capillary column (with hydrophilic elements, Fig. 2) or an *uncoated* column (with bare silica surface). The antisense DNA strand^F migrates as a homogeneous component (Fig. 2a). A direct and sequential

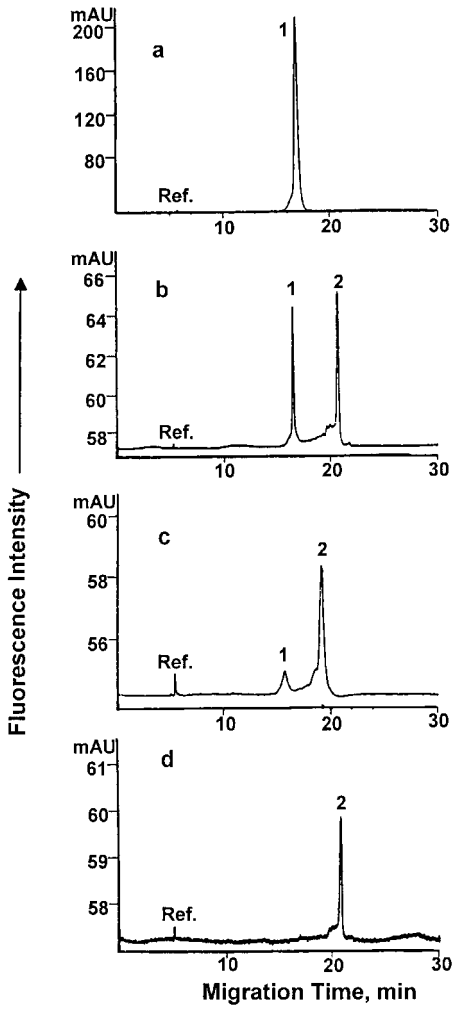


FIG. 1. Separation of ssDNA and preannealed dsDNA strands by CE. (a) A fluorescent-labeled antisense strand (80 fmol) (1). (b) Labeled antisense strand (1) and unlabeled sense strand in an equal proportion (80 fmol each) mixed and applied immediately to the column to form dsDNA (2). (c) The sample prepared as in (b). The mixture incubated prior to application to the column at 20°C for 20 min. (d) The mixture is same as in (b) but incubated for 48 min. The separations are carried out in an *uncoated* capillary (75 μm i.d., 50 cm long, 30 effective length) in a 24 mM NaH_2PO_4, 12 mM $Na_2B_4O_7$ buffer, pH 8.0 containing 0.25% HEC by using 200 V/cm and LIF detector. The ssDNA strand was prepared (2 mg/mL) in 10 mM TES, 100 mM NaCl and 1 mM EDTA, pH 7.5 (NET buffer).

application of the two strands in an equal proportion to a coated column yields dsDNA as shown in Fig. 2b. The results indicate no trace of any un-annealed antisense strand^F (compare with the results in Fig. 2a). The two strands are applied with a 5-s time interval. Similar results are observed irrespective of the sequence in which the strands are applied. Application of a larger amount of antisense strand^F (1.7:1.0 vs 1:1 ratio) results in two peaks (Fig. 2c). An earlier emerging peak of dsDNA is followed by the antisense strand^F. A comparison of the on-column annealing was carried out with those obtained *after* annealing the DNA probe.

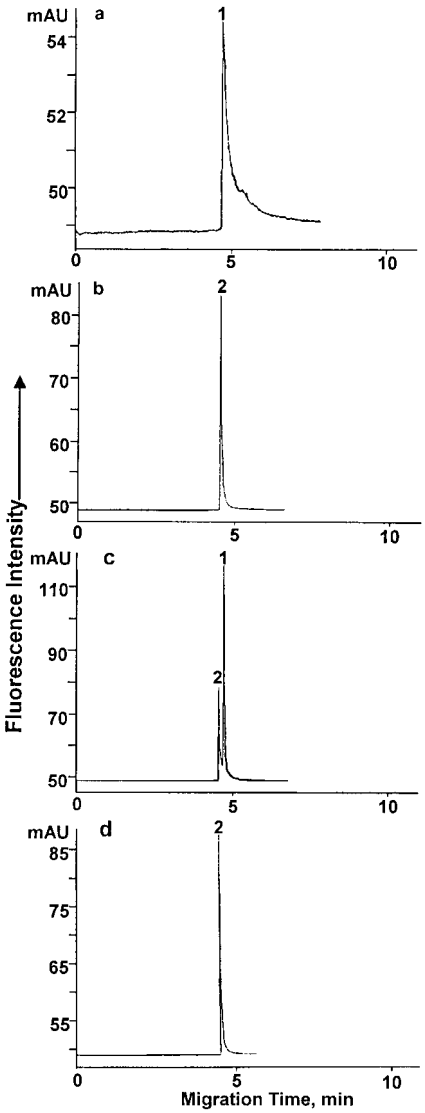


FIG. 2. Annealing of complementary DNA strands on-column using a neutral capillary. (a) A fluorescent-labeled antisense strand (30 fmol) (1). (b) The labeled-antisense strand (60 fmol) (1) is applied followed by the unlabeled-sense strand (60 fmol). The samples, two strands of equal amounts, are applied sequentially to the column without premixing. Only one peak (2) representing dsDNA is observed. (c) A sequential application of the sample as in (b) but has a 67% additional labeled strand. The labeled-antisense strand (30 fmol) (1) application is applied followed by the unlabeled-sense strand (18 fmol). (d) For comparison with *on-column* annealing, a *preformed* dsDNA is applied to the column as in Fig. 1(d). The separations are carried out in a coated capillary column (75 μm i.d., 50 cm long, 30 effective length) in a 0.3 M Tris-base–2 mM EDTA, pH 8.3 containing 0.2% linear polyacrylamide, using 400 V/cm and LIF detector.

The two annealing methods produced identical results (compare Figs. 2b and 2d). Under the UV detection conditions (260 nm), both antisense and sense DNA strands, in addition to dsDNA were observed at different ratios of the complementary strands (Table I).

The CE method offers direct and rapid annealing of the DNA strands without need of prior incubation (Figs. 1 and 2). The dsDNA can be made and analyzed on-column by running the complementary strands at 20°C by the CE method (Fig. 2). The traditional annealing method yields unwanted peaks (results of CE using UV detection not shown). The kinetics of annealing is favorable and the time required to initiate the experiment (sample injection etc., ~5 s) appears to be appropriate for the dsDNA formation.

Study of nonspecific binding in mobility shift by flat gel and CE methods. The DNA–protein interaction, though often highly specific, also results in the binding of nonspecific proteins with the DNA probe. To remove the nonspecific proteins from the complex, a competing duplex polymer such as poly(dI:dC) or poly(dG:dC) is commonly added to the reaction mixture. The polymer interacts and removes the proteins without diminishing the specific interaction between the DNA probe and the cognate protein. This method is routinely used in the GE procedure. To evaluate the need of competing DNA in the CE method, poly(dI:dC) (lanes 1 to 4, Fig. 3a) and poly(dG:dC) (lanes 5 to 8, Fig. 3a) were first tested by the GE method. Poly(dG:dC), as noted in Fig. 3a (lane 5) enhanced the specific binding between the DNA probe and the cognate protein by a factor of eight over poly(dl:dC) (lane 2, Fig. 3a, and Table II). However, a large proportion of the DNA probe binds nonspecifically (39%) with the proteins if poly(dG:dC) is the only competitor in the reaction mixture (second band in lane 5, Fig. 3a). The nonspecific interactions can be avoided by adding poly(dI:dC) to the reaction (lane 2, Fig. 3a). The nonspecific binding is also not observed in the presence of poly(dG:dC) with a heterologous oligonucleotide (NF-κB; second band absent in

TABLE I

Mobility of Antisense and Sense DNA Strands and Their Annealing Analyzed by CE and Monitored by UV[a]

Sample applied to the column	Ratio of antisense to sense strand	Mobility, average of 3 analyses (min)		
		Antisense strands	Sense strand	dsDNA
Sense strand			13.7	
Antisense strand		13.0		
Antisense + sense strands (1:1)	1 to 1	None	None	15.3
Antisense + sense strands (1:2)	1 to 2	trace	13.4	15.4

Note. An application of the two strands in an equal proportion to an uncoated column yields only the annealed dsDNA. However, if the sense strand concentration is doubled, an additional peak of this strand is observed as expected. The migration time of the components changes from experiment to experiment, but constant in relation to an internal marker, such as uridine (ca. 6 min).

[a] DNA samples analyzed in an uncoated capillary column. A mixture of the two DNA strands applied immediately to the column. For experimental details see legends to Fig. 1.

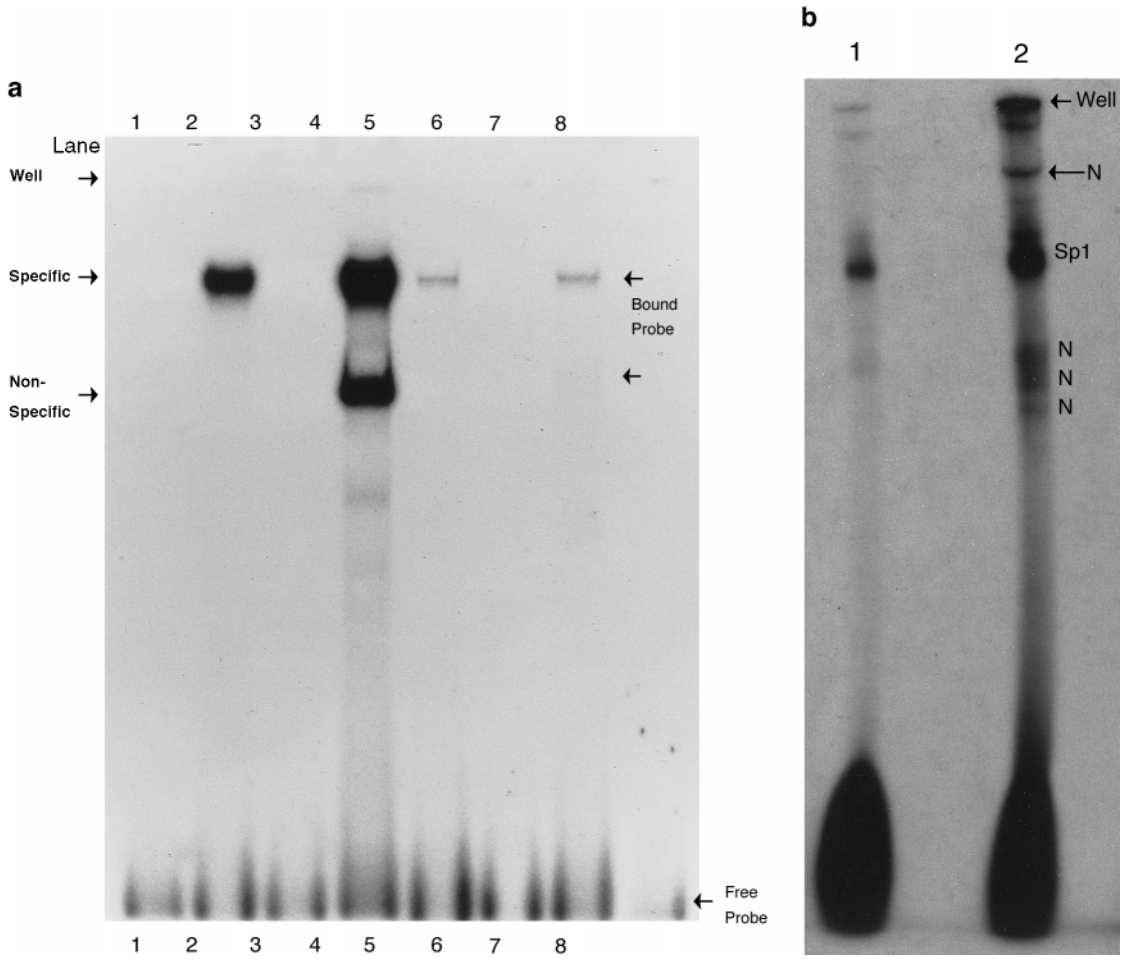


FIG. 3. (a) Evaluation of competing dsDNAs [poly(dI:dC) versus poly(dG:dC)] for removing nonspecific binding of proteins by gel-mobility-shift assay (MSA) analysis. Both homologous and heterologous promoter binding elements (DNA probes), and C2 myotube nuclear extract (3 mg) are used for the analysis. Lanes 1 to 4 contain poly(dI:dC) and lanes 5 to 8 contain poly(dG:dC). In addition, lanes 2 and 5 contain no competing homologous or heterologous promoter elements. Lanes 3 and 6 contain unlabeled (cold) SRE as competitor for specific binding. Lanes 4 and 7 contain a dsDNA having the NF-κB binding element as control for specific binding of serum response factor. Lanes 1 and 8 served as negative control (contain no nuclear extract). For other details see Table II. (b) Effect of poly(dI:dC) in the binding of Sp1 to its element (Xian, 1994). A mixture of Sp1 dsDNA binding element and a nuclear extract (20–25 mg) from mouse fiber cells was incubated with poly(dI:dC) (lane 1) and without poly(dI:dC) (lane 2). Polyacrylamide gel electrophoresis was performed in a low salt, 9% native polyacrylamide gel and 45 mM Tris—borate–EDTA buffer (3 h). N, nonspecific binding; Sp1, specific binding; well, loading well. Poly(dI:dC) (12.8 A_{260} units/mg) and poly(dG:dC) (8.0 A_{260} units/mg) were dissolved separately in NET buffer (legend to Fig. 1) to of 2.1 μg/μL and warmed up to 45°C for 5 min to ensure the duplex formation. The solutions were stored at −16°C. The DNA–protein complex was formed, prior to analysis according to the method described by Gustafson *et al.* (1989). The binding reaction mixture was incubated at 20°C for 15 min. A 2.8 μL of 10× gel loading buffer (30% Ficoll, 5 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol prepared in 445 mM Tris base, 445 mM boric acid and 10 mM Na_2EDTA buffer, pH 8.3) was added and mixed immediately before loading the samples into the gel wells. Electrophoresis was performed in 44.5 mM Tris base, 44.5 mM boric acid and 1 mM Na_2EDTA buffer, pH 8.3.

lane 7, Fig. 3a and Table II). Poly(dI:dC) appears to be the more affective as a nonspecific competitor. An additional DNA probe and protein mixture is analyzed to confirm this conclusion. An examination of a dsDNA probe containing an Sp1 binding site with nuclear proteins (20–25 mg) from mouse fiber cells confirms the versatility of poly(dI:dC) for removing nonspecific binding (Fig. 3b). Lane 1 contains the competitor, while the lane 2 has none. The nonspecific binding is nearly eliminated in the presence of poly(dI:dC). The use of poly(dI:dC) and poly(dG:dC) for controlling nonspecific protein binding in CE was also studied. The results (not shown) indicated no additional or differences in the peak (DNA–protein complex) shape or peak shift with or without the addition of 1 μg of poly(dI:dC) or poly(dG:dC) in the reaction mixture. This confirms that no competing (nonspecific) polymer is required for the proposed CE method.

Effect of fluorescein tag on DNA–protein interaction. The radioactive labeling for the CE method was found inappropriate earlier in this laboratory (18), hence the

TABLE II

Effect of Competing Polymers in Removing Nonspecific Binding Proteins from DNA–Protein Complex

Lane	Reaction components	Observations	Intensity	
			Band 1[d]	Band 2[e]
1	Poly(dI:dC) only	Control; no binding.	0	0
2	Poly(dI:dC) + proteins[a]	Specific binding.	1,640	0
3	Poly(dI:dC) + proteins[a] + cold CArG([b])	Binding but competing with the cold probe.	0	0
4	Poly(dI:dC) + proteins[a] + NF-κB([c])	Specific binding as in lane 2.	1,160	0
5	Poly(dG:dC) + proteins[a]	Specific and nonspecific bindings.	13,790	8,740
6	Poly(dG:dC) + proteins[a] + cold CArG ([b])	Binding but competing with the cold probe.	0	0
7	Poly(dG:dC) + proteins[a] + NF-κB ([c])	Specific binding as in lane 2.	4,590	0
8	Poly(dG:dC) only	Control; no binding.	0	0

[a] Protein, mixture of proteins derived from nuclear extract of C2 myotube muscle cells.
[b] Cold CArG, indicates CAG-rich serum response element carrying no label.
[c] NF-κB, mammalian RNA polymerase II transcription factor which recognizes a sequence in the κ immunoglobulin light chain of B cells (Alberts, 1989).
[d] Specific DNA–protein binding.
[e] Nonspecific DNA–protein binding.

use of the fluorescein tag on the DNA–protein binding was examined by GE. Two sets of experiments were carried out (results not shown). In one set only [^{32}P]-antisense strand with an unlabeled sense strand was used. The other set contained this radioactive strand and a sense strand, but labeled with fluorescein. The two sets of experiments also contained other control DNA sequences for comparison. The reaction composition of the experiments was identical to that described earlier for Fig. 3a. The presence of the fluorescein tag on DNA produced identical results to those with the radioactive label. Hence, a fluorescein tag in the CE method work can be used without any adverse effects.

Effect of a competing protein and poly(dI-dC:dC-dI) on DNA–protein binding. A reaction mixture containing a DNA probe and proteins (same as in Fig. 3a) was mixed with (or without) a competing protein (BSA) and a nonspecific competitor, poly(dI-dC:dC-dI) and the mixture separated by the CE method. The two sets of experiments produced identical results as judged by the peak area of the DNA–protein complex and the absence of any additional peak (results not shown for brevity). The results indicate that only specific interaction occurs between the DNA probe and its cognate protein. No competitor is required to remove any unwanted nonspecific proteins. However, if there are other complexes present, for example those between the proteins and the nonspecific competitor, they are not observed, as the two elements are unlabeled. These elements, if present, cannot interfere with the quantification of the DNA–protein complex of interest.

Application of micellar electrokinetic CE. To study the separation of DNA strands and the protein complex, different forms of CE were examined. Migration of the analytes in micellar electrokinetic CE (MEKC) was studied. This mode of CE involves the use of an anionic detergent that can form micelles inside the capillary column (19). The separation is performed in an uncoated capillary column with a SDS buffer. The amount of SDS was optimized to maintain the active form of the SRF. A LIF detector monitors the peaks (Fig. 4). The results indicated that the antisense strand[F] was clearly resolved into two components, representing the two distinct conformations of the oligomer (Fig. 4a, peaks 1 and 1′). The two species were

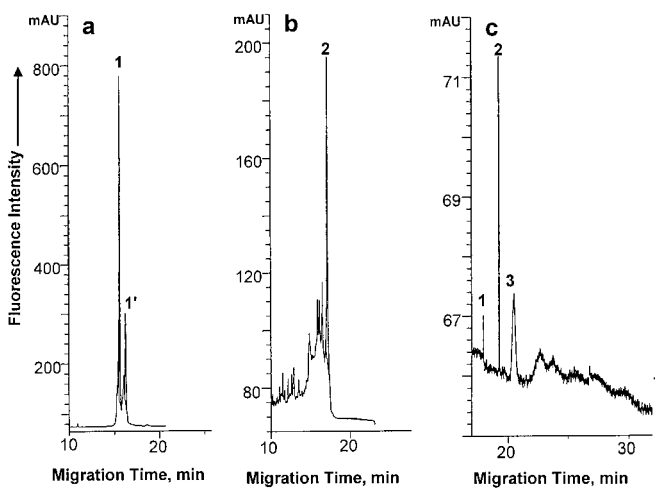


FIG. 4. Separation of DNA and DNA–protein complex by micellar electrokinetic chromatography (MEKC) in an uncoated capillary (total amount of DNA used is 1.66 pmol). (a) Migration of labeled sense strand[F]. Peaks 1 and 1′, the two forms of labeled sense strand. (b) Migration of annealed dsDNA (2). (c) Separation of SRE dsDNA probe complexed with its specific protein (3), present in a nuclear extract from mouse myoglobin cells. The separations are performed by CE using an uncoated capillary (as in Fig. 2) and 25 mM $Na_2BaO_7 - H_3BO_3$ buffer, pH 9.0 containing 20 mM SDS.

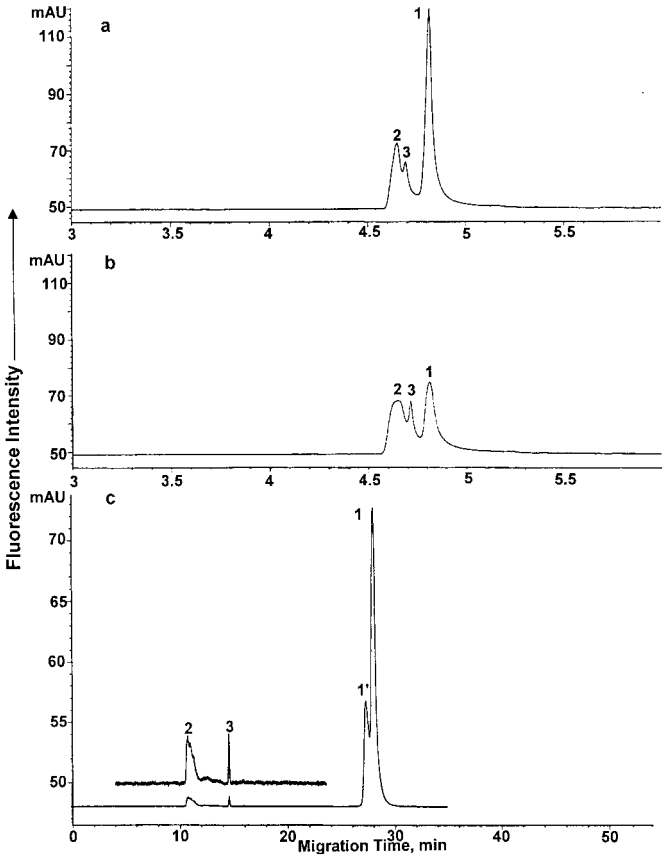
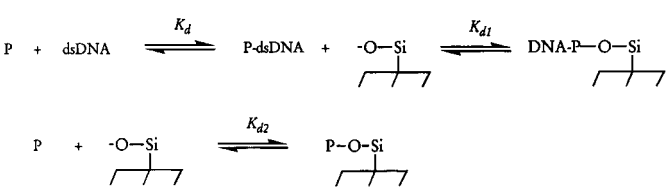


FIG. 5. Formation of DNA–protein complex on-column: influence of binding protein contents and the column length. (a) Samples are applied successively in the order and for the time indicated: nuclear extract in Fig. 3a (5 s), run buffer (2 s), fluorescein-labeled antisense strand (5 s), and finally sense strand (3 s). (b) Samples injected successively as in (a), but with twice the amount of the nuclear extract (10 s). Separations in (a) and (b) are performed by using a 50-cm-long (effective length, 30 cm) neutral capillary column and other conditions as described in Fig. 2. (c) Experiment in (b) is repeated with a longer neutral capillary column (100 cm, effective length 80 cm). Peaks 1 and 1′, the two forms of labeled antisense strand; 2, dsDNA; 3, DNA–protein complex.

present in a ratio of 2.4 to 1. Preannealed DNA strands were resolved along with uncharacterized fluorescent-labeled species in Fig. 4b. A reaction mixture consisting of preannealed DNA with excess sense strand and other components (BSA and competing DNA) is resolved in Fig. 4c. This mode of CE can thus resolve all three components, namely, antisense strand^F, dsDNA, and the DNA–protein complex (peaks 1, 2, and 3, respectively).

Sequential formation of dsDNA and DNA–protein complex in CE capillary without additives. The DNA–protein complex formation following ssDNA annealing is studied while varying the amount of the binding protein. In Fig. 5a, the sample contains only 50% of the nuclear proteins compared to the sample used in Fig. 5b. Further addition of the nuclear extract (as measured by relative peak heights) appears to yield more DNA–protein complex (peak 3) and less antisense strand^F (peak 1). A longer capillary column (50 cm vs. 100 cm) produced excellent resolution of all three species (Figs. 5a and 5b vs Fig. 5c). The dsDNA (peak 2), DNA–protein complex (peak 3) and the two different forms of the antisense strand^F (see peaks 1 and 1′ in a peak area ratio of 3 to 1) are well resolved (Fig. 5c). Two such forms of the antisense strand were also observed in MEKC for the sense strand^F (Fig. 4a). Despite no competitors in the reaction mixture, no nonspecific binding is observed in the CE method. In general, the protein moves slower than that the DNA probe. Application of the protein, followed by the DNA probe results in enhanced binding. As DNA migrates, it encounters the protein plug; the binding takes place if the binding constant of the DNA and protein is high enough to offset the electrophoretic (or electroosmotic) destabilizing force. Indeed, there appears to be no need for including polymer duplexes in the reaction mixture to avoid the nonspecific association. The nonspecific interactions in GE can also, in part, result from restrictive movement of DNA and protein inside the limited volume of the gel pore, thus resulting in associations between unwanted DNA and non-cognate proteins. This phenomenon is often called the "caging effect". Restricting the DNA and the protein into a small volume provides opportunity for nonspecific binding energy to overcome the entropic factor.

Differences in binding determined by different methods. The DNA–protein contents and the binding affinities were found higher in an *uncoated* capillary than the *coated* one (or GE method). Differences in the K_d values can be rationalized. In an uncoated capillary, three different equilibriums exist (Scheme I). Therefore, the SRF protein (P) can possibly interact with three different entities. It can interact with the DNA probe, yielding P-dsDNA. The P-dsDNA complex can in turn interact with the capillary surface silanols. Finally, P can interact directly with the capillary silanols. The interaction of the complex with the capillary silanols (K_{d1}) can destabilize the DNA–protein complex. Moreover, the silanols can also react with the basic surface R groups of the protein. The interaction of the protein with the silanols (K_{d2}) can lower the effec-



SCHEME I. Three different equilibriums exist in an uncoated capillary column. They involve the protein, SRF: one with the DNA and two with the capillary column surface (P, protein; dsDNA, double-stranded DNA).

TABLE III

Binding Constants of DNA–Protein Complex Determined by Different Methods

DNA probe and binding protein	Binding constant, M (SD)	Binding protein present, M	References
SRE and SRF[b]	$0.8\ (\pm 0.1) \times 10^{-6}$	$0.17\ (\pm 0.07) \times 10^{-6}$	CE uncoated cap.[c]
SRE and SRF[b]	$2.5\ (\pm 0.1) \times 10^{-6}$	$0.38\ (\pm 0.53) \times 10^{-6}$	CE coated cap.[d]
SRE and SRF[b]	$2.8\ (\pm 0.4) \times 10^{-6}$	$0.29\ (\pm 0.30) \times 10^{-6}$	GE method[a]
SM50 probe and P3A2	1.7×10^{-9}	3.0×10^{-8}	Calzone *et al.* (20)
Cylla Z probe and P3A1	3.5×10^{-8}	4.1×10^{-9}	Höög *et al.* (21)
SpP3A2 probe and rSpP3A2	1.9×10^{-7} (weak site)	Not reported	Xian *et al.* (22)
SpP3A2 probe and rSpP3A2	3.9×10^{-8} (strong site)	3.0×10^{-8}	Xian *et al.* (22)

[a] SD, standard deviation derived from several data points: uncoated, 3; coated capillary, 3 and polyacrylamide gel electrophoresis (PAGE) method, 10 experiments.
[b] Values determined from this work. SRE, serum response element (DNA); SRF, serum response factor (protein).
[c] An uncoated capillary column used for analyses (see legend to Fig. 4 for details).
[d] A hydrophilic-coated capillary column used for analyses (see legend to Fig. 5 for details).

tive concentration of the free SRF. The SRF protein concentration determined by different electrophoretic methods (Table III) appears to suggest that the protein–silanol reaction (K_{d2}) does exist in bare capillary columns. A lower protein concentration is noted in an uncoated CE column. The silanols in an uncoated capillary compete nonspecifically with free DNA. However, specific interaction of free DNA and protein is energetically favored resulting in the protein–DNA complex. Apparently, proteins must have preference to bind with cognate DNA over the silanols. Negatively charged DNA may offer enhanced binding sites for specific proteins than the free silanols. Possibly the binding between the complex and the silanols may be insignificant (K_{d1}). When a preformed complex (Fig. 2d) is applied to the column, only very few basic protein surface residues should be available to bind with the silanols. The crude nuclear protein extract also contains other proteins. They may preferentially bind to silanols over DNA (K_{d2}-type equilibrium). If there is such binding, it cannot be detected under present detection system. Generally, irreversible interactions with silanols are often suppressed while working with proteins in CE separations. An addition of hydrophilic groups (mono-layer) modifies the capillary surface. The hydrophilic surface tends to decrease the binding of both proteins (K_{d2}) and DNA–protein complexes (K_{d1}) to the capillary surface. Interestingly, very similar K_d values are obtained by using coated capillary and classical gel electrophoresis methods (Table III).

Significance of relative K_d values for transcription factors. Strong DNA–protein interactions are usually characterized by very low dissociation constants, i.e. in the order of 10^{-9} M or less (see Table III for example). In this study, the interaction of SRF and CArG dsDNA appears to be weak (10^{-6} M, see Table III) although the concentration of the SRF is in the order of 10^{-7} M. The results suggest that high affinity is apparently not important in transcriptional activation by SRF. Based on the results in Table III, we can draw some general conclusions that support common physiological interpretations (23). For example, when the K_d value is high, DNA-binding transcription factor binds to its target weakly. Hence, a large number of the copies of the transcription factor must be required for optimal expression of the gene to meet the cellular requirement. On the other hand, if K_d value is low, then less number of copies of the transcription factor may be produced. However, when concentration of the binding protein is high and the observed binding is also high, this could result in the accumulation of harmful or even lethal gene products. Conversely, a low concentration of the protein and weak binding could result in product deficiency.

Here, we have shown that a mixture of ssDNA, dsDNA, and protein–DNA complexes can be resolved by the CE methods. The proposed methods provide powerful tools for characterization and quantification of the DNA–protein complex without increasing the ionic strength of the sample matrix and loss of DNA to nuclease activity present in the crude nuclear extract. The results from the CE method are comparable to those from the gel-mobility-shift procedure. The method is very simple, rapid and permits immediate DNA duplex formation inside the capillary, and does not require the use of any nuclease inhibitors or a high-salt solution. It is highly sensitive, precise, and adaptable to various experimental systems, and needs only a very small amount of the material, i.e., binding proteins and DNA probe. It does not require use of any radioisotopes. The electrophoretic band density measured in the gel method is often not homogenous across the region and yields unreliable results. The CE method, on the other hand, offers instant quantification of the peak area and high degree of sensitivity. Appropriate separation conditions can be easily determined to eliminate nonspecific protein binding and use of chasers (polynucleotides, BSA). Thus, achieving only

the sequence-specific DNA–protein complex and the unbound excess DNA probe. The benchmark of this work is the ability to anneal complementary ssDNA's and quantification of specific DNA–protein interactions in a single CE experiment with powerful specificity and speed.

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