Switching DNA-binding specificity by unnatural amino acid substitution

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ABSTRACT

The specificity of protein–nucleic acid recognition is believed to originate largely from hydrogen bonding between protein polar atoms, primarily side-chain and polar atoms of nucleic acid bases. One way to design new nucleic acid binding proteins of novel specificity is by structure-guided alterations of the hydrogen bonding patterns of a nucleic acid–protein complex. We have used Cl repressor of bacteriophage λ as a model system. In the λ-repressor–DNA complex, the ε-NH₂ group (hydrogen bond donor) of lysine-4 of λ-repressor forms hydrogen bonds with the amide carbonyl atom of asparagine-55 (acceptor) and the O₆ (acceptor) of CG6 of operator site O₁. Substitution of lysine-4 (two donors) by iso-steric S-(2-hydroxyethyl)-cysteine (one donor and one acceptor), by site-directed mutagenesis and chemical modification, leads to switch of binding specificity of λ-repressor from C:G to T:A at position 6 of O₁. This suggests that unnatural amino acid substitutions could be a simple way of generating nucleic acid binding proteins of altered specificity.

INTRODUCTION

A structural understanding of protein–nucleic acid interaction has advanced sufficiently for creation of engineered and artificially evolved DNA-binding proteins for some DNA sequences (1). Designed zinc finger proteins are now being applied for cell engineering and therapeutic purposes in some cases (2). However, a simple strategy for creating new nucleic acid binding proteins, in general, is still beyond reach. For example, an understanding of RNA recognition has not yet reached the sophistication needed for engineering RNA-binding proteins of novel specificity. A simple stereochemical rule for creating new recognition points on an existing protein or peptide scaffold would be of great benefit for designing RNA-binding proteins of novel specificity and allow creation of more diversified DNA-binding protein modules as well.

Several studies have looked into alterations and relaxations of specificity by mutating critical residues (substitution with natural amino acids). Some of these effects are weak (3–6). However, in other cases a specificity difference of several orders of magnitude is seen (7–13). Ebright et al. (7), Wharton and Ptashne (8) and Youderian et al. (9), reported that a single amino acid change in the recognition helix changes the binding specificity for a mutant operator by several orders of magnitude. Wharton and Ptashne (10) demonstrated that by changing the recognition helix of 434 repressor to that of the P22 repressor, by introducing several mutations, it is possible to completely switch the binding specificity from 434 operator to that of P22 operator. In many cases, recognition specificity is based on hydrogen bonding between amino acid side chains and nucleic acid bases, particularly with substituents in position 6 in purine and position 4 in pyrimidine. Base substitution within the same class (that is purine to purine and pyrimidine to pyrimidine) approximately preserves the size and shape of the base with concomitant switch of hydrogen bond donors to acceptors or vice versa in these positions. An example of such a switch is the position 6 of the purine ring which changes from a hydrogen bond donor (–NH₂) in adenine to a nearly iso-steric (sterically equivalent) hydrogen bond acceptor (6-Oxo) in guanine. Thus, an adenine is recognized at the 6-amino group by an amino acid side-chain, which is a hydrogen bond acceptor, substitution of an iso-steric amino acid with hydrogen bond donor properties may recognize guanine in place of adenine or vice versa. Such a simple substitution rule may allow relatively simple engineering of novel recognition specificity.

As a test case, we chose λ-repressor–O₁ complex to test the above-mentioned idea. Lysine-4 of λ-repressor is a crucial residue from point of view of operator recognition. In conjunction with Asn-55 it recognizes base pair CG6. Lysine-4 forms a crucial hydrogen bond with CG6, in which it recognizes the 6-Oxo group of the guanine by hydrogen bond donation from the ε-NH₂ group (Figures 1 and 2) while Asn-55 donates a hydrogen bond to purine N7 of CG6 base pair (14).

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This interaction is further stabilized by hydrogen bond between lysine-4 amino group and the side-chain carbonyl of Asn-55. If a TA base pair is substituted for CG6, an amino group replaces the oxo group at the 6 position of the purine ring, while retaining other recognition points. The two hydrogen atoms of this amino group make this group a hydrogen bond donor and may be recognized by a hydrogen bond acceptor, otherwise iso-steric with lysine-4. An ideal iso-steric group with above-mentioned properties will be where the \( -\text{NH}_2 \) group of lysine is replaced by a hydroxyl group (Figure 1). This will also preserve the hydrogen bond between lysine-4 and Asn-55, thus preserving the recognizing pair.

Modification of a cysteine that has been introduced by site-directed mutagenesis has been used as a strategy to introduce unnatural amino acids. In several cases mutation to a chemically similar amino acid has been used to derive the stringent requirement of a particular amino acid (17–22). We developed a strategy to create an amino acid, which is iso-steric with lysine but chemically dissimilar, through side-chain modification of a reactive cysteine. In this paper, we demonstrate that such a semi-synthetically repressor indeed recognizes the mutated DNA sequence.

**MATERIALS AND METHOD**

**Materials**

Acrylamide, bisacrylamide, TEMED, phenylmethylsulfonyl fluoride (PMSF), ethidium bromide, 2-iodoethanol, bromophenol blue, Commassie brilliant blue, hydroxyapatite, QAE–Sephadex A-50, Sephadex G-25, streptavidine agarose, EDTA, DTNB, DTT, BSA and ampicillin were purchased from Sigma Chemical Co. Tryptone and yeast extract were purchased from Himedia Laboratories Pvt. Ltd (India). Magnesium chloride, sodium chloride, calcium chloride, 2-mercaptoethanol, glycerol and Tris were purchased from Merck (India). X-gal, isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG), sodium MOPS, agarose, triethanolamine and SDS were purchased from SRL (India). N-iodoacetyl-N-biotinylhexylenediamine was purchased from Pierce (USA). Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim and Gibco BRL. Taq DNA polymerase primers and dNTPs were purchased from Generon (Bangalore, India). 5'-Amino linked oligonucleotides were from Microsynth (Switzerland).

**Site-directed mutagenesis**

The mutation from lysine-4 to cysteine-4 was performed by site-directed mutagenesis of the cl gene using PCR. A mutant upstream primer containing EcoRI site (5'-GTA TAG AAT TCACGT ATG AGC ACA AAA TGC AAA CCA TT-3') and a downstream primer complementary to non-coding region containing a BamHI site (5'-ACC ATT CTT CAT AAT TGG ATC CAT TTA CG-3') were used. The PCR was carried out in 0.5 ml Perkin-Elmer microcentrifuge tube. The reaction mixture was prepared by mixing template DNA, primers, dNTPs, Taq DNA polymerase and the reaction buffer (supplied by the manufacturer).

The reaction was carried out in Perkin-Elmer GeneAmp PCR System 2400 (USA) DNA thermal cycler. The PCR product was run in 1% agarose gel and the desired DNA band was cut. The PCR product was extracted using QIAGEN gel extraction kit (Qiagen, Germany). PCR product and plasmid vector were digested with restriction enzymes EcoRI and BamHI following Gibco BRL protocol. Ligation was carried out using T4 DNA ligase. Digestion of DNA with restriction endonucleases was carried out at 37°C for 3 h, in the buffer supplied by the manufacturer. The ligation of the EcoRI and BamHI digested PCR product with the EcoRI and BamHI digested pUC-19 vector was carried out at 23°C for 3 h using T4 DNA ligase. The ligated plasmid was transformed into competent DH5-α cells and colonies selected by blue-white selection. Five colonies were picked. One of those five

![Figure 1](image.png)

Upper panel shows the hydrogen bonding pattern of CG6 with lysine-4 and hydrogen bonding of lysine-4 with Asn-55. Lower panel is the hypothetical hydrogen bonding pattern of the mutant TA6 and S-(2-hydroxyethyl)-cysteine-4, along with Asn-55.
plasmids was fully sequenced in an automatic DNA sequencer and the desired mutation at K4C was confirmed. This plasmid having K4C λ-repressor gene was named pAM109.

pAM109 and pKK223-3 plasmids were digested with EcoRI and PstI restriction enzymes. The restriction fragment containing K4C gene was extracted from digested pAM109 using QIAGEN gel extraction kit following their protocol. The K4C gene was ligated into digested pKK223-3 using Boehringer Mannheim’s rapid ligation kit. The ligated product was transformed in Escherichia coli XL-1 blue I q strain following the previous method. Cells were grown on ampicillin Luria Agar plates at 37°C. Clone bearing colonies were confirmed through (i) plasmid isolation followed by agarose gel electrophoresis, (ii) restriction digestion of the plasmid by EcoRI and PstI restriction enzymes and (iii) after IPTG induction the over expression of 26 kDa protein in 15% SDS–polyacrylamide gel. Cells from one of the cloned colonies were stored in 20% glycerol at −80°C and the corresponding plasmid was named pAM209.

**Isolation and characterization of K4C λ-repressor**

K4C λ-repressor protein was isolated from E.coli XL-1 blue strain carrying the plasmid pAM209, which contains Lys to Cys mutation at the 4th amino acid under the control of tac promoter. The cells were grown at 37°C, upto A590 = 0.6, then IPTG at a final concentration of 1 mM was added and grown further for 2 h. The cells were then harvested by centrifugation at 12 000 r.p.m. for 15 min and were re-suspended using 1.7 ml lysis buffer per gram of wet packed cells (SLA-1500). The cell
pellet was kept at -20°C. K4C λ-repressor was purified according to Johnson et al. (23), with some modifications. All volumes and amounts in the following purification have been normalized to 5 g of the starting cells. All steps were carried out at 0–4°C unless stated otherwise.

Frozen cells were thawed, suspended in 25 ml of lysis buffer containing 20 μg/ml of PMSF and disrupted by sonication. After sonication, (200 W; 30 s each time; 5 times with 5 min gap) the crude lysate was diluted to 100 ml by SB (23) containing 200 mM KCl and cellular debris were removed by centrifugation at 12 000 r.p.m. for 50 min. The repressor was precipitated with gradual addition of 5 ml of 10% polyethyleneimine, stirred for 1 h and precipitate was collected by centrifugation at 10 000 r.p.m. for 10 min. The polyethyleneimine pellet was re-suspended in 75 ml of SB containing 200 mM KCl and centrifuged at 10 000 r.p.m. for 10 min. Then the pellet was again re-suspended in 50 ml of SB containing 600 mM KCl, stirred for 10 min and centrifuged at 10 000 r.p.m. for 10 min. The supernatant containing the repressor was saved.

Repressor in the decanted supernatant was precipitated by gradual addition of 40 g of solid ammonium sulfate per 100 ml of the supernatant (i.e. 80% saturated) and pH was maintained at 8.0 by adding 3N KOH. The ammonium sulfate suspension was stirred for 2 h and the precipitate was collected by centrifugation at 13 000 r.p.m. for 45 min. The ammonium sulfate pellet was re-suspended in 5 ml of SB containing 50 mM KCl and was dialyzed overnight against 500 ml of SB containing 50 mM KCl. The dialyzed material was centrifuged at 12 000 r.p.m. for 10 min to remove any precipitated protein.

The supernatant containing the repressor was mixed with 50 ml of SB containing 100 mM KCl and 50 ml of pre-swollen QAE–Sephadex A-50 in SB containing 100 mM KCl and the mixture was stirred for 2 h at 4°C. It was then poured into a glass column and allowed to settle. The protein was eluted with a linear gradient of 150 ml of SB containing 100 mM KCl and 150 ml of SB containing 600 mM KCl. Flow rate was 0.5 ml/min and 6 ml fractions were collected. Fractions containing mutant repressor were identified by SDS–PAGE. The pooled fractions containing the mutant repressor were loaded on to a hydroxylapatite column and eluted by a gradient of 0.1–1.0 M potassium phosphate buffer (pH 6.8) containing 1.5 mM 2-mercaptoethanol (100 + 100) ml, flow rate was 0.5 ml/min and 3 ml fractions were collected. The mutant repressor pulled from the hydroxylapatite column was ~98% pure by criterion of electrophoresis on 15% SDS–polyacrylamide gel and gave dark single band (24) corresponding to a molecular weight of ~26 000 Da. The native repressor concentration was determined by using E1% (280) = 11.3 (25). Molar concentration was always calculated in terms of monomer subunit unless stated otherwise.

### Determination of sulfhydryl content

K4C λ-repressor protein was mixed with 5 mM DTT and was kept for 1 h at 4°C and exhaustively dialyzed against degassed 0.1 M potassium phosphate buffer (pH 8.0) containing 1 mM EDTA. The protein solution was placed in a sample cuvette and the dialysate buffer was placed in a reference cuvette of a double-beam spectrophotometer. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) reacts with free sulfhydryl groups of proteins to form thionitrobenzoate anion for each –SH group. The concentration of this strongly colored anion is determined from its absorbance at 412 nm (ε = 13 600 M−1 cm−1 at pH 8). DTNB solution was added to each cuvette to a final concentration of 0.1 mM and the reaction was followed for 30 min. The determination of exposed cysteines of K4C λ-repressor protein were followed at 6 μM protein by reaction with 0.1 mM DTNB.

### Chemical modification of K4C by 2-iodoethanol

A total of 50 μl of 100 mM DTT (final concentration 3.3 mM) was added to 1.5 ml K4C λ-repressor protein (50 μM stock) and was kept for 2 h at 4°C. A 25 ml Sephadex G-25 column was equilibrated with the required amount of degassed reaction buffer [50 mM triethanolamine–HCl (pH 7.8) containing 50 mM NaCl, 1 mM EDTA and 10% glycerol]. Then the DTT and protein mixture was passed through a Sephadex G-25 column using the above-mentioned buffer to remove excess DTT and the reduced K4C λ-repressor was collected as 1.2 ml fractions. The fractions containing K4C λ-repressor were pooled in a screw cap glass vial (5 ml of 13 μM protein).

### Circular dichroism (CD)

To see if any secondary structural elements change in the modified K4C λ-repressor, the far UV-CD spectra of modified K4C λ-repressor and wild λ-repressor were measured in a JASCO J-600 spectropolarimeter in a 1 mm path length cuvette at 25°C, between 250 and 200 nm. The bandwidth was 2 nm and scan speed was 20 nm/min. Samples were prepared at 50 mM Tris–HCl buffer (pH 7.5), containing 0.1 mM EDTA and 200 mM KCl and the protein concentration were modified 2.4 μM for K4C λ-repressor and 2 μM for wild-type λ-repressor. The results are reported as mean residue ellipticity, with the unit of deg cm² dmol⁻¹.
Fluorescence spectroscopy

The wavelength maximum of tryptophan fluorescence of modified K4C λ-repressor (concentration 2.4 μM) and wild λ-repressor (concentration 2 μM) were measured in a Hitachi F3010 spectrofluorometer at 25°C and the experiments were carried out in 1 cm pathlength quartz cuvette. The buffer was 50 mM Tris–HCl (pH 7.5), containing 0.1 mM EDTA and 200 mM KCl. The excitation wavelength was at 295 nm, the excitation and emission bandpasses were 10 nm each.

Fluorescein isothiocyanate (FITC) labeling of oligonucleotide

Oligonucleotides with S' amino link (X = hexyl amino group) were purchased from Microsynth (Switzerland). Sequence of oligonucleotides was as follows: Operator O1: 5'-XTCGACATACCACTGGCCGTGATATGGATCC-3' and 5'-XGGGATCCATATCAGCCGACGTGATATGGATCC-3'; Mutated O1 (CG6 to TA at the consensus half site): 5'-XTCGACATACCACTGGCCGTGATATGGATCC-3' and 5'-XGGGATCCATATCAGCCGACGTGATATGGATCC-3'.

For preparation of FITC labeled, oligonucleotides were labeled in 500 μl solution containing 1 M sodium carbonate/bicarbonate buffer (pH 9): DMF:water [high-performance liquid chromatography (HPLC) grade] = 5:2:3. Reaction was carried out for 20 h at 25°C. After incubation, the reaction was loaded onto a Sephadex G-25 column (pre-equilibrated in 0.1 M phosphate buffer (pH 8.0)) and eluted with same buffer. The oligonucleotides and their complementary strands were annealed by heating at 80°C followed by cooling slowly at room temperature.

Operator titration of wild λ-repressor and its iso-steric analogue

All fluorescence studies were done in a Hitachi F3010 spectrofluorometer at 25°C and the experiments were carried out in 1 cm pathlength quartz cuvette. Anisotropy experiments were performed using Hitachi (650-10/40) polarization accessory. The excitation wavelength was at 480 nm and emission was at 530 nm, the excitation and emission bandpasses were 10 nm each. Fluorescein labeled wild O1 and mutated O1 (the CG6 of consensus site has replaced by TA6) were titrated with increasing concentrations of both wild-type λ-repressor and S-(2-hydroxyethyl)-cysteine containing λ-repressor separately up to 350 nM protein concentrations. Anisotropy values were calculated at each point according to the equation and plotted against different protein concentrations. The titrations were performed in 50 mM Tris–HCl buffer (pH 7.5), containing 1 mg/ml BSA, 0.1 mM EDTA and 250 mM KCl. The binding isotherms obtained were fitted to a single site binding equation of the dimer coupled with monomer–dimer equilibrium using a MATLAB program written by the authors. The dimer–monomer dissociation constant was taken as 20 nM.

RESULTS AND DISCUSSION

The strategy to create an S-(2-hydroxyethyl)-cysteine containing λ-repressor at position 4 relies on the fact that λ-repressor has no reactive cysteines (26). Thus, site-directed mutation of K4 to C, followed by reaction with 2-iodoethanol may create an amino acid, which is almost iso-steric with lysine but having an OH instead of the ε-NH2. K4C mutant cI gene was created by PCR using one mutagenic end primer and another end primer. The mutation was confirmed by sequencing of the whole gene. The mutant gene expressed well and was purified as described previously (25).

The mutant protein showed one reactive sulphydryl group per subunit upon reaction with DTNB (Table 1). The reaction kinetics of C4 was rapid, indicating exposed nature of the sulphydryl group. The K4C λ-repressor was labeled with 2-iodoethanol, followed by reaction with an iodoacetamido derivative of biotin to block the unreacted sulphydryl groups. The reaction mixture was then passed through a column of streptavidin-agarose to remove all biotin-modified protein. This leads to isolation of λ-repressor that has S-(2-hydroxyethyl) cysteine in both subunits. The purified S-(2-hydroxyethyl)-cysteine-4 containing λ-repressor was characterized by ten rounds of N-terminal Edman sequencing, CD and fluorescence spectroscopy. N-terminal sequencing revealed an unknown amino acid at position 4, while the other nine are consistent with the wild-type sequence. λ-repressor has three tryptophans at 129, 142 and 230. They are sensitive probes of the tertiary structure of the protein. Figure 3 shows the tryptophan emission spectra of the wild-type and S-(2-hydroxyethyl)-cysteine-4 λ-repressor. The emission maximum and fluorescence intensities (after corrections for concentration differences) are very similar (emission maximum of 339 nm in both cases), indicating no significant changes in the tertiary structure due to modification. The tryptophans in λ-repressor are all situated in or around the

![Figure 3. Fluorescence emission spectra of wild-type (solid line; 2 μM) and S-(2-hydroxyethyl)-cysteine-4 λ-repressor (dashed line; 2.4 μM). Experimental details are in Materials and Methods.](http://nar.oxfordjournals.org/)

Table 1. Reaction of K4C and wild-type λ-repressor with DTNB

<table>
<thead>
<tr>
<th>Type of protein</th>
<th>Protein concentration (μM)</th>
<th>Observed OD₄₁₂</th>
<th>No. of exposed –SH group/subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>K4C</td>
<td>6</td>
<td>0.083</td>
<td>1.04</td>
</tr>
<tr>
<td>Wild type</td>
<td>6</td>
<td>0.019</td>
<td>0.23</td>
</tr>
</tbody>
</table>

C-terminal domain and thus report the integrity of the structure there. The DNA-binding N-terminal domain is devoid of tryptophan but consists of several helices. The far UV-CD spectrum of the whole protein is dominated by the N-terminal helices (26) and thus the CD spectra is a sensitive monitor for the conformation of the N-terminal domain. Figure 4 shows the CD spectra of the wild-type and the S-(2-hydroxyethyl)-cysteine-4 λ-repressor. The spectra are similar indicating that there is no significant conformational perturbation in the N-terminal domain due to mutation and chemical modification. Taken together, we may conclude that the protein remains in native-like conformation.

Fluorescence anisotropy is now widely used to study protein–DNA interaction (27). Figure 5 shows binding of wild-type λ-repressor with wild-type O₁₁ operator site containing oligonucleotide duplex. The binding was measured by fluorescence anisotropy of an attached fluorescein probe to the 5' ends of the duplex oligonucleotide. The binding was conducted at somewhat elevated ionic strengths to offset any possible non-specific binding. The ionic strength used is not incompatible with physiological conditions. The binding isotherm was fitted to a binding equation as described in Koblan and Ackers (28,29). The initial anisotropy of the duplex oligonucleotide and amplitude of increase is consistent with those obtained previously (30). The dissociation constant thus obtained was 56.2 nM for wild-type repressor–O₁₁ complex. The dissociation constant of a similar synthetic O₁₁ containing oligonucleotide–wild-type λ-repressor complex was determined by isothermal titration microcalorimetry and found to be 4.2 nM at 210 mM monovalent salt and pH 7.0 (31). Koblan and Ackers (28,29) have determined dissociation constant of O₁₁–wild-type λ-repressor complex as a function of monovalent salt concentration and pH. After extrapolation of dissociation constant obtained by microcalorimetry to 300 mM monovalent cation (including tris) concentration and pH 7.5, a value of ~35 nM was predicted for the dissociation constant of O₁₁–wild-type λ-repressor complex at pH 7.5 and 300 mM monovalent cation (linear extrapolation of data presented in Koblan and Ackers to 300 mM monovalent salt concentration yielded a factor of three weakening when compared to that at 210 mM). Similar extrapolation from pH 7.5 yields a factor of about four weakening when compared to pH 7.

Figure 4. CD spectra of wild-type (solid line; 2 μM) and S-(2-hydroxyethyl)-cysteine-4 λ-repressor (dashed line; 2.4 μM). Experimental details are in Materials and Methods.

Figure 5. Upper panel reflects the anisotropy increase of 5' fluorescein labeled synthetic duplex oligonucleotide containing either wild-type O₁₁ operator site (open circles) or TA6 mutant (solid circles), with wild-type λ-repressor. Lower panel reflects the anisotropy increase with S-(2-hydroxyethyl)-cysteine-4 λ-repressor. The symbols have the same meaning as above. The oligonucleotides were end-labeled with fluorescein as described. The titration procedure is also given in the Supplementary Data. Each point is an average of eight independent titrations.

Under identical conditions, wild-type λ-repressor does not bind the CG6−−TA6 mutant operator (Figure 5). This is consistent with base pair substitution experiments (32). Figure 5 also shows the binding of S-(2-hydroxyethyl)-cysteine-4 containing λ-repressor with wild-type O₁₁. There is little binding in the concentration range tested indicating importance of having a lysine ε-NH₂ in position 4. The deletion of first six amino acids of λ-repressor abolishes operator
binding indicating importance of lysine-4 (33). The S-(2-hydroxyethyl)-cysteine-4 modified λ-repressor however binds tightly to CG6—TA6 mutant O1 as indicated by increased anisotropy. When the binding isotherm was fitted to the binding equation, a dissociation constant of 9.82 nM was obtained for S-(2-hydroxyethyl)-cysteine-4 modified λ-repressor complex. This indicates that S-(2-hydroxyethyl)-cysteine-4 modified λ-repressor regains all the binding energy that is lost upon TA6 substitution.

Since both the dimers contain S-(2-hydroxyethyl)-cysteine-4 modifications, while the mutant operator carries only a mutation in the consensus half site, it is likely that putative K4 interaction with the symmetrically related CG pair in the non-consensus half site does not contribute significantly to the binding energy. This is consistent with the fact that the N-terminal arm of the subunit that contacts the non-consensus half site remains disordered even at low temperatures in the crystal and substitution of a TA base pair in the non-consensus half have little effect on the binding energy (see Figure 2) (10,32).

Although the sterically equivalent substitution made here is incorporated by chemical modification, the enormous progress that is being made in recent years in incorporating artificial amino acids in vivo (34) should allow us to soon make non-natural iso-steric amino acid substitutions, in vivo, with ease. This may open up new possibilities of engineering new DNA-binding proteins and engineering of the genetic programs, not hitherto accessible.

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Conflict of interest statement. None declared.

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