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СТРУКТУРНО-ФУНКЦИОНАЛЬНЫЙ АНАЛИЗ БИОПОЛИМЕРОВ И ИХ КОМПЛЕКСОВ

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CHARACTERIZATION OF THE E-BOX BINDING AFFINITY TO SNAG-ZINC FINGER PROTEINS

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Members of the Snail/Gfi-1 domain family of zinc finger proteins are known to recognize the E-box sequence CANNTG, such as that found in the promoter of E-cadherin, however, no studies have shown that the internal "NN" dinucleotides can play a role in different binding affinities. We show via gel shift assays that only the sequences CACCTG and CAGGTG can be recognized more strongly by the SNAG-ZFP members such as Slug, Smuc, Snail, and Scratch while the other combinations of the internal nucleotides were bound weakly. All 16 possible dinucleotide combinations were tested by competition EMSAs to determine their relative binding affinities. The K_d value for the best-binding sequences was approximately 1.25×10^{-6} M, while the other interactions were less effective. Our study has shown for the first time how different internal dinucleotide combinations of the E-box can be recognized differently by different transcription factors and also sheds light into how this transcription factor binding site may participate in DNA-protein interactions.

Keywords: **Snail, E-Box, CANNTG, transcription factors, DNA-protein interaction**

The Snail/Gfi-1 (SNAG)-domain family of transcription factors is comprised of Cys_2His_2 zinc finger proteins (ZFPs) that act as transcriptional repressors via a conserved 21-amino acid domain at their N-terminal [1]. The C-terminal zinc fingers create a DNA-binding motif and bind to the upstream regulatory region of target genes in a sequence-specific manner [2], leading to selective inhibition of the transcription of the target gene. Several binding site consensus sequences have been derived for members of this family, including the well-studied E-box sequence CANNTG. E-box elements are also known to be bound by basic helix-loop-helix (bHLH) transcription factors that serve a wide range of functions, including muscle-related genes *Myf3–Myf6*, *MyoD*, and myogenin [3–5] as well as others like those found in photoreceptors in the eye and pancreas [6, 7].

Multiple transcription factors including several members of the SNAG family have shown some relation with E-box, either regulating or being regulated by it. SNAG members such as Snail, Scratch, and Slug bind to E-box elements of E-cadherin's proximal promoter site [8–10] in a way similar to SIP1 [11]. E-cadherin gene has two E-boxes in its proximal promoter region, both having a core CACCTG sequence [9]. This E-box can regulate bHLH transcription factors as does Smuc [12], and can itself be regulated by other transcription factors, as is the case with IA-1 [13]. The E-box element found within the IA-1 promoter is regulated by NeuroD1 and E47 heterodimers, critical transcription factors needed to start IA-1 transcription [13].

Apart from those previously mentioned, a binding site selected consensus sequence with the highly conserved core CAGGTG was identified for the *Drosophila* transcription factor SNAIL [14]. This is well reported since additional studies have shown that GCAGGTG, CACCTG, or sequences similar to them are the preferred sequences of Snail family members both in human and *Drosophila* models [15, 16]. Binding sites have been identified for other SNAG-ZFP family members such as Gfi-1 and IA-1, and ZBRK1 [13, 17, 19], however without any E-box relation.

The main goal of this research is to use the knowledge that most SNAG-ZFPs target E-box and determine whether the internal dinucleotides in the CANNTG sequence provide a stronger or weaker affinity to protein binding. Our preliminary data has shown a distinct variation in the strength of binding while additional analysis quantified K_d values for these SNAG-ZFP-E-box combinations. By varying the amount of unlabeled oligonucleotides as well as protein concentrations in different experiments, we are able to show that different members of the SNAG family can recognize different combinations of E-box at varying strengths.

EXPERIMENTAL

Bacterial expression and protein purification of GST-SNAG fusion proteins. Natural SNAG-domain family full-length transcription factors are shown in a

The text was submitted by the author(s) in English.

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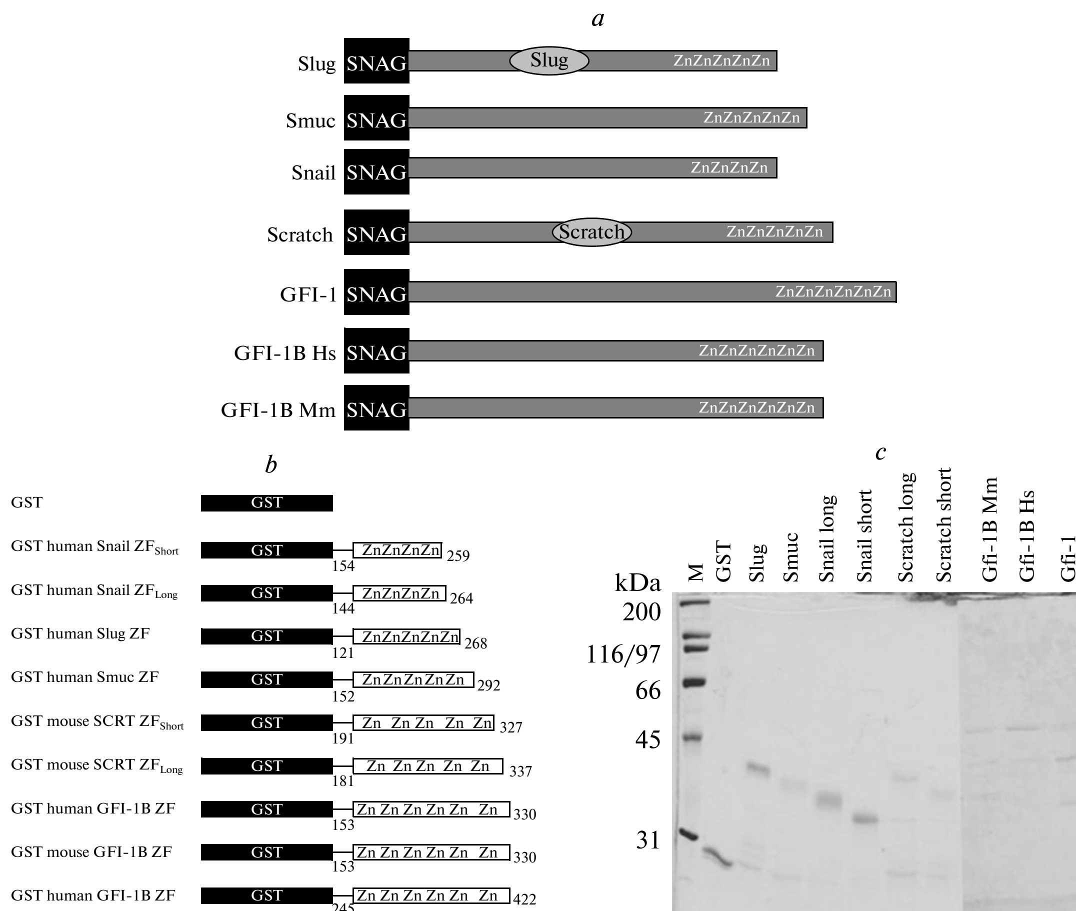

Fig. 1. *a* – Diagrammatic representation of naturally occurring SNAG-ZFPs used in the study are characterized by an N-terminal SNAG repression domain and C-terminal zinc finger DNA-binding domain. *b* – Diagrammatic representation of GST-fusion proteins denotes amino acid position of zinc finger regions amplified. *c* – Homogeneous, purified GST-SNAG-ZFP used for E-box characterization studies were run on an SDS-PAGE along with marker and a GST control.

diagrammatic representation in fig. 1*a*. Full-length genes were amplified from cDNA clones ("Open Bio-Systems") by PCR using primers ("Integrated DNA Technologies") flanking just the zinc finger region (table 1). The zinc finger regions in amino acids for each construct are as follows: Slug (121–268), Smuc (152–292), Snail long (144–264) and short (154–259), Scratch long (181–337) and short (191–327), Gfi-1 (245–422), Gfi-1B Hs (human) and Mm (mouse) (153–330). PCR products were run on an agarose gel, purified, and then digested with BamHI and SalI ("New England Biolabs").

GST fusion proteins were constructed using the PCR-amplified zinc finger products (fig. 1*b*). Recombinant constructs were ligated in frame with the GST affinity tag in the pGEX-4T2 plasmid vector. Ligated samples were transformed into competent *Escherichia coli* DH5α cells and transformants selected on LB/ampicillin plates were checked for positive inserts by EcoRI and BamHI ("New England Biolabs") digestion. Positive recombinant plasmids were transformed into the *E. coli* BL21 ("Invitrogen") host for protein expression. The *E. coli* was grown in LB containing ampicillin/kanamycin until OD_{595} = 0.6 and induced with 100 μM IPTG for 3 h at 30°C. Conditions for Gfi-1, Gfi-1B Hs and Mm were adjusted for a lower incubation temperature and longer induction time as these ZFPs were found to be difficult to collect in the soluble fraction. They were grown to OD_{595} = 0.3 and induced for 4 h for protein production.

Induced SNAG-ZFPs were purified using glutathione (GSH) sepharose affinity chromatography. A soluble fraction was obtained from the induced protein pellets by resuspending them in PBS/lysozyme (1 mg/mL), rotating at 4°C for 30 min then sonicating for 6–8–1 min pulses. These samples were centrifuged for 30 min at 4°C at 10000 rpm, and the resultant supernatant was centrifuged for an additional 20 min. The clear soluble fraction was passed through a chromatography column packed with PBS-washed Sepharose beads ("GE Healthcare"). The bound GST-fusion proteins were eluted 3 times in an elution buffer (10 mM GSH, 0.1% Triton X-100, 50 mM Tris-HCl, pH 8.0). Protein elutions were concentrated via

Table l. Primers used to amplify zinc finger containing regions

Name	Nucleotide sequence
Slug 5' Bam HI	5'-GTGGGATCCCATGCCATTGAAGCTGAAAAG-3'
Slug 3' SalI	5'-GTGGTCGACTCAGTGTGCTACACAGCAG-3'
Smuc 5' BamHI	5'-CACGGATCCTTTGAGTGCTTCCACTGCCAC-3'
Smuc 3' SalI	5'-CACGTCG ACTC AGGGGCCCGGGCAGCCAGC-3'
Snail long 5' BamHI	5'-GTGGGATCCGAGGCCAAGGATCTCCAG-3'
Snail long 3' SalI	5'-GTGGTCGACGCGGGGACATCCTGAG-3'
Snail short 5' BamHI	5'-GTGGGATCCTTCA ACTGCA A ATACTGC A-3'
Snail short 3' SalI	5'-GTGGTCGACGCAGCCGGACTCTTGG-3'
Scratch long 5' BamHI	5'-GTGGGATCCGGGTCGGGAGCCACGG-3'
Scratch long 3' SalI	5'-GTGGTCGACGGTTGCGGGGCCGCTAG-3'
Scratch short 5' BamHI	5'-GTGGGATCCCACGCGTGCGGAGAGTG-3'
Scratch short 3' SalI	5'-GTGGTCG ACGCAGGCTG ACTCGTAGTG-3'
Gfi-1 Hs 5' BamHI	5'-GTGGGATCCTGCACCCGCCTGCTGCTGG-3'
Gfi-1 Hs 3' SalI	5'-GTGGTCGACTCATTTGAGCCCATGCTGCG-3'
Gfi-1B Hs 5' BamHI	5'-GTGGGATCCAGCCTCCGCTACTCCCCAGG-3'
Gfi-1B Hs 3' SalI	5'-GTGGTCGACTCACTTGAGATTGTGCTGG-3'
Gfi-1B Mm 5' BamHI	5'-GTGGGATCCCGCCTCCGCTACTCTCCAGG-3'
Gfi-1B Mm 3' SalI	5'-GTGGTCGACTCACTTGAGATTGTGTTGACTCT-3'
IA-1 long 5' BamHI	5'-GTGGGATCCCACAAGTGCTCGCGCATCG-3'
IA-1 long 3' SalI	5'-GTGGTCGACCTAGCAGGCCGGACGCACAG-3'
IA-1 short 5' BamHI	5'-GTGGGATCCTACCGCTGCCCAGAGTGCG-3'
IA-1 short 3' SalI	5'-GTGGTCGACTCTATTCTCAG ACGGGTGG-3'

dialysis in Spectra/por molecularporous membrane tubing. Two four hour periods of dialysis in 1× PBS/100 mM PMSF followed by over 12 h in 1× PBS/10% glycerol/100 mM PMSF concentrated the proteins. Protein concentrations were estimated using Coomassie (Bradford) reagent (“Pierce”) and analyzed on SDS-PAGE to check integrity and homogeneity of purified GST-ZFPs (fig. 1*c*). In all cases, no additional refolding or renaturation were necessary for soluble protein production.

E-cadherin promoter and E-box characterization assays. GST-tagged SNAG-ZFPs were purified to homogeneity using column chromatography. Constructed E-cadherin promoter and E-box oligonucleotides (“Integrated DNA Technologies”) with varying internal NN nucleotide residues (table 2) were annealed in 10× NEB2 buffer (“New England Biolabs”) to a final concentration of 50 pm/λ by heating at 95°C for 5 min and slowly cooled to room temperature (~25°C) over several hours. Oligonucleotides were end-labeled with T4 polynucleotide kinase, its corresponding 10× buffer (“Fermentas”), and ^{32}P for 1 h at 37°C then purified via spin column (400 μL Sephadex beads, 2000 rpm, 5 min).

E-cadherin promoter oligonucleotide binding reactions consisted of 2× NEBB (nuclear extract binding buffer) without NaCl and 100 ng of purified protein. Binding reactions for E-box assay consisted of 100 ng of purified protein, 200 000 cpm radiolabeled E-box oligonucleotides, and 5× NEBB (100 mM HEPES pH 7.5, 2.5 mM DTT, 50% glycerol, 25 mM $MgCl_2$, 0.25 mM $ZnSO_4$) to a salt concentration of 75 mM for 20 min at room temperature. Electrophoretic Mobility Shift Assays (EMSAs) were run at 4°C on a 5% DNA polyacrylamide gel for 1.5 h at 400 V in 0.5× TBE buffer, transferred onto Whatman paper, dried in vacuum at 80°C for 45 min, and processed for autoradiography at –80°C.

Competition assays and K_d determination. Proteins and unlabeled oligonucleotides were titrated at various concentrations. For each data set, an equivalent amount of labeled DNA was added to each reaction and then titrated with increasing amounts of unlabeled oligonucleotide. Unlabeled oligonucleotides from 80 nM to 51.2 μM in two-fold and ten-fold increments were added to the reaction with 100 ng standard of protein at room temperature for 20 min to allow the reaction to reach equilibrium, followed by addition of labeled oligonucleotide at an equal amount to the lowest

Table 2. Oligonucleotides used for E-cadherin promoter and E-box dinucleotide assays

Primer	Oligonucleotide sequence
E-cad E-box forward	5'-GGAACTGCAAAGCACCT-GTGAGCTTGCGG-3'
E-cad E-box reverse	5'-CCGCAAGCTCACAGGT-GCTTTGCAGTTCC-3'
EBX-AA forward	5'-CAAAGCAAATGTGAG-3'
EBX-AA reverse	5'-CTCACATTTGCTTTG-3'
EBX-AT forward	5'-CAAAGCAATTGTGAG-3'
EBX-AT reverse	5'-CTCACAATTGCTTTG-3'
EBX-AG forward	5'-CAAAGCAAGTGTGAG-3'
EBX-AG reverse	5'-CTCACACTTGCTTTG-3'
EBX-AC forward	5'-CAAAGCAACTGTGAG-3'
EBX-AC reverse	5'-CTCACAGTTGCTTTG-3'
EBX-TA forward	5'-CAAAGCATATGTGAG-3'
EBX-TA reverse	5'-CTCACATATGCTTTG-3'
EBX-TT forward	5'-CAAAGCATTTGTGAG-3'
EBX-TT reverse	5'-CTCACAAATGCTTTG-3'
EBX-TG forward	5'-CAAAGCATGTGTGAG-3'
EBX-TG reverse	5'-CTCACACATGCTTTG-3'
EBX-TC forward	5'-CAAAGCATCTGTGAG-3'
EBX-TC reverse	5'-CTCACAGATGCTTTG-3'
EBX-GA forward	5'-CAAAGCAGATGTGAG-3'
EBX-GA reverse	5'-CTCACATCTGCTTTG-3'
EBX-GT forward	5'-CAAAGCAGTTGTGAG-3'
EBX-GT reverse	5'-CTCACAACTGCTTTG-3'
EBX-GG forward	5'-CAAAGCAGGTGTGAG-3'
EBX-GG reverse	5'-CTCACACCTGCTTTG-3'
EBX-GC forward	5'-CAAAGCAGCTGTGAG-3'
EBX-GC reverse	5'-CTCACAGCTGCTTTG-3'
EBX-CA forward	5'-CAAAGCACATGTGAG-3'
EBX-CA reverse	5'-CTCACATGTGCTTTG-3'
EBX-CT forward	5'-CAAAGCACTTGTGAG-3'
EBX-CT reverse	5'-CTCACAAGTGCTTTG-3'
EBX-CG forward	5'-CAAAGCACGTGTGAG-3'
EBX-CG reverse	5'-CTCACACGTGCTTTG-3'
EBX-CC forward	5'-CAAAGCACCTGTGAG-3'
EBX-CC reverse	5'-CTCACAGGTGCTTTG-3'

concentration for another 20 min at room temperature to reach equilibrium again. Conversely, proteins were added from 6.25 to 400 ng in two-fold increments. Reactions were made in 5× NEBB (–NaCl + DTT) to a concentration of 50 mM NaCl, bound as above, and loaded on a 5% DNA polyacrylamide gel for EMSA.

RESULTS

GST-SNAG-ZFPs showed functionality and preferentially bind to certain E-box sequences

Naturally occurring SNAG-domain family transcription factors are shown in a diagrammatic representation in fig. 1*a*. The GST-tagged recombinant SNAG-ZFP proteins such as Slug, Smuc, Snail long and short, Scratch long and short (short refers to zinc fingers alone while long refers to zinc fingers flanked by additional amino acids to enhance proper folding), Gfi-1, and Gfi-1B are shown diagrammatically in fig. 1*b*. The GST-tagged recombinant SNAG-ZFP proteins were expressed in *E. coli* and purified to homogeneity. Once the integrity of pure proteins was ascertained, they were taken for experimental procedures. All these recombinant proteins were tested for their ability to bind to the E-cadherin promoter. This assay revealed as expected that only the Slug, Smuc, Snail, and Scratch ZFP proteins were able to recognize and bind to the E-box element of the E-cadherin promoter, while Gfi-1B could not, thus ensuring functionality of our GST-tagged fusion proteins (fig. 2). Gfi-1 and Gfi-1B are the non-E-box binding members of the SNAG-ZFP family.

In our preliminary binding assays we tested three oligonucleotides containing different compositions of E-boxes such as CAGGTG, CACCTG and CATGTG. Of these only the first two efficiently bound all the SNAG-ZFP recombinant proteins while the CATGTG did not make any complex (data not shown). Therefore, in the next series of experiments we wanted to test the influence of internal NN residues of the E-box "CANNTG" sequence. Using all 16 NN combinations, EMSAs were performed to see the effect, if any, of differential interaction. Fig. 3 shows that a sequence of CAGGTG and CACCTG will strongly interact with the SNAG-ZFPs, whereas other sequences such as CAACTG and CATATG do not interact at all while CAAGTG and CACGTG sequences interact with weak affinity. Also of note is that sequences that follow CACNTG seem to be preferentially bound. It is possible that the first cytosine plays a stronger role in the protein-DNA recognition than other nucleotides.

These results suggest that particular nucleotides play a role in strong binding with proteins. Although it is not necessary for the internal nucleotides of E-box to be GG or CC for the binding to occur, these combinations may strengthen contact between protein and DNA. Possibly, the strongest interaction between a SNAG-ZFP and a target gene is one that has a GG or CC E-box dinucleotide. This agrees with the previously discussed studies, which noted that GCAGGTG, CACCTG, or sequences similar to these can be recognized preferentially by Snail family members [14–16]. Flanking nucleotides may also be necessary to facilitate proper binding of a factor.

During our studies, we also observed that the SNAG-ZFPs do not have the same binding properties amongst each other. CAGGTG and CACCTG sequences are strongly bound, while CAACTG and CATATG sequences are weakly bound, however Snail was less likely to interact with most of the E-box oligonucleotides when compared to other family members. Scratch and Smuc were able to bind in every case, indicating that they may interact with E-box sequences more readily than Slug or Snail.

Snail-CAGGTG interaction maintains the greatest binding affinity

In order to quantify the binding affinities of the SNAG ZFPs for the different E-box sequences, increasing amounts of unlabeled oligonucleotides were added to obtain a competing effect (fig. 4). EMSA gels were analyzed to derive a K_d value for the best representative of SNAG-ZFP and E-box (as described in Letovsky and Dynan, 1989) [20]. We observed the most strongly bound dinucleotide combination, CAGGTG, was found to have a K_d of approximately 1.25×10^{-6} M with Snail (fig. 4). This data along with the data presented in fig. 3, indicates that the rest of the proteins and the E-box sequences bind to a lesser extent than this derived value. This value is slightly higher compared to other zinc finger protein to DNA binding affinities reported earlier [20, 21], but the interaction still provides effective binding. Additional series of EMSAs have been done for other SNAG-ZFPs (Slug, Smuc, Snail, and Scratch) and E-box dinucleotide combinations (GG, CC, CT, AG, and CG) based on those with the greatest ability to bind as seen in the E-box characterization assay described in fig. 3 (data not shown).

Nature of E-box sequence can control the strength of protein binding

We investigated whether the same SNAG-ZFP can bind differently to different E-box dinucleotide combinations. We tested CACCTG and CAGGTG combinations with Snail-ZFP and observed that they bound very differently as seen in fig. 5. This confirms that DNA-protein interactions can vary depending on the nature of the SNAG-ZFP and the nature of the E-box sequences used in binding assays.

As shown in our preliminary E-box characterization assay (fig. 3), some dinucleotide combinations bind better than others, confirming expected results. When comparing the binding fraction for the same protein Snail versus the amount of unlabeled E-box sequences, we find that a better competing effect was observed for CAGGTG than CACCTG as described for other transcription factors in various other studies [22–24]. The preferential specificity of the GG combination indicates that Snail will more likely bind this sequence better than an E-box containing CC.

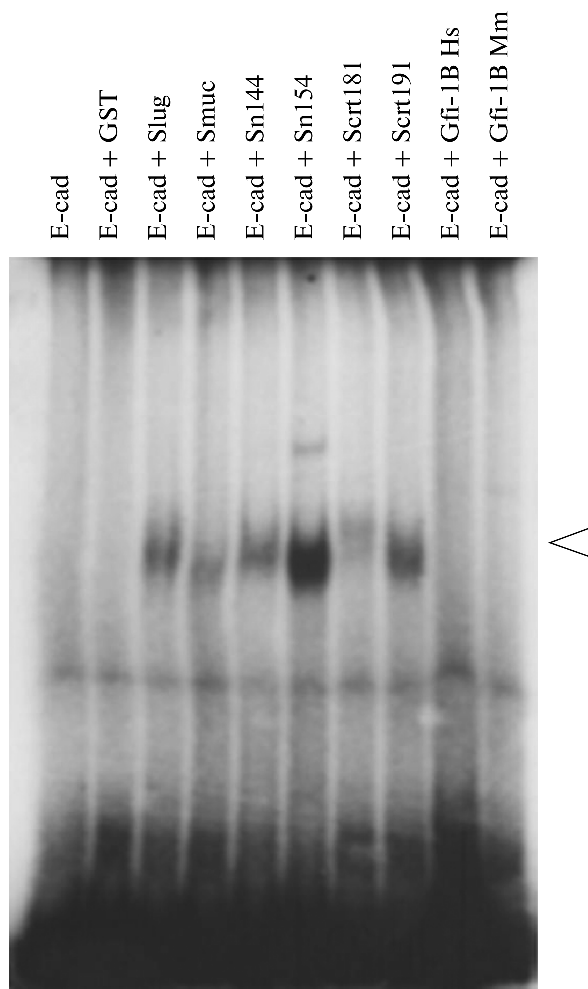

Fig. 2. Gel mobility shift assay. An EMSA with E-cadherin promoter and GST-SNAG-ZFPs shows functionality of the purified proteins. The open arrowhead indicates DNA-protein complexes for E-box binders or no interaction with those that do not bind to the promoter. E-cadherin free probe and a GST-tag control were also included.

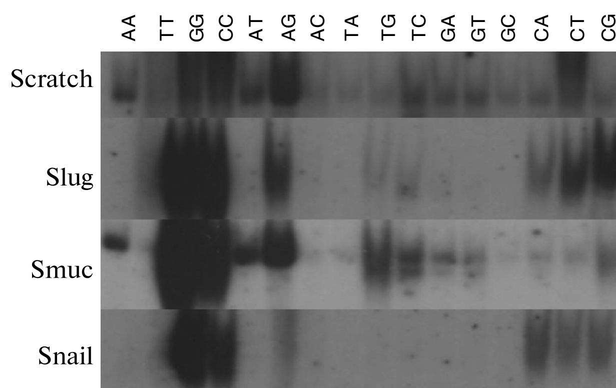

Fig. 3. E-box binding assay with 16 possible internal dinucleotide residues reveal that internal NN nucleotides dictate stronger or weaker binding.

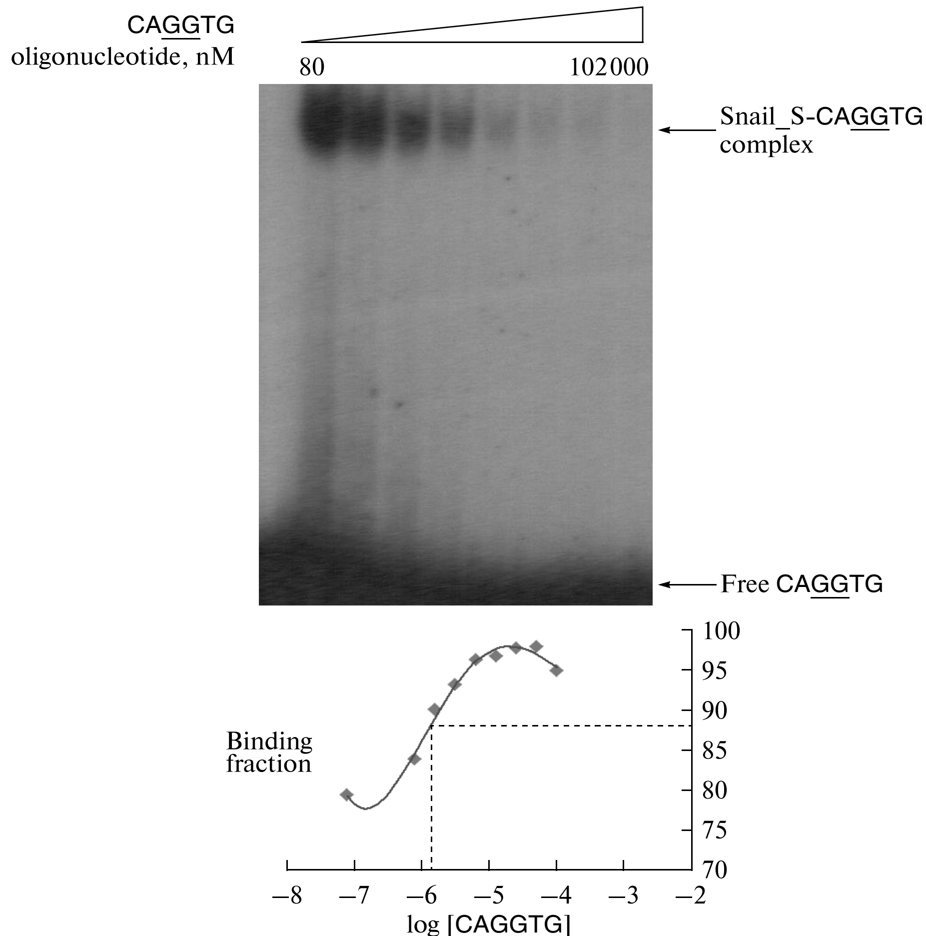

Fig. 4. Competition between labeled and unlabeled CAGGTG oligonucleotide with purified Snail ZFP allows for K_d determination. Increasing unlabeled CAGGTG decreases DNA-protein complex (top), and the half maximal inhibitory concentration for the Slug-CAGGTG complex derived from EMSA studies indicates the K_d value (bottom).

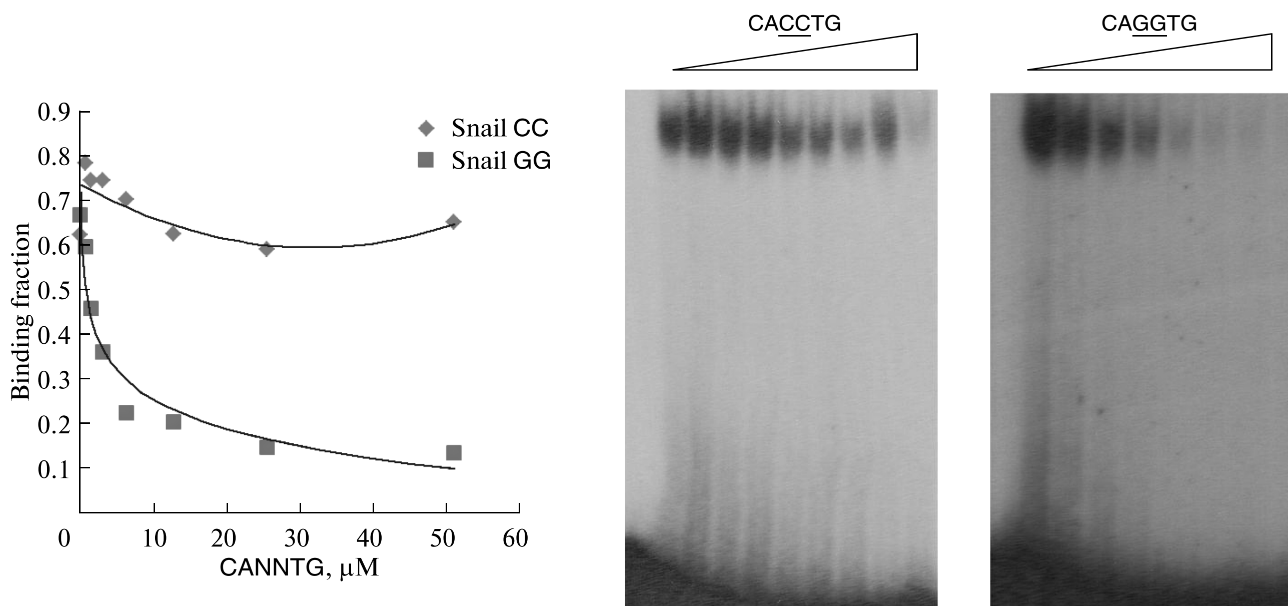

Fig. 5. Comparison of Snail-ZFP binding to different E-box combinations show variation in binding intensities. Addition of different dinucleotide combinations have differential effects when bound to the same SNAG-ZFP.

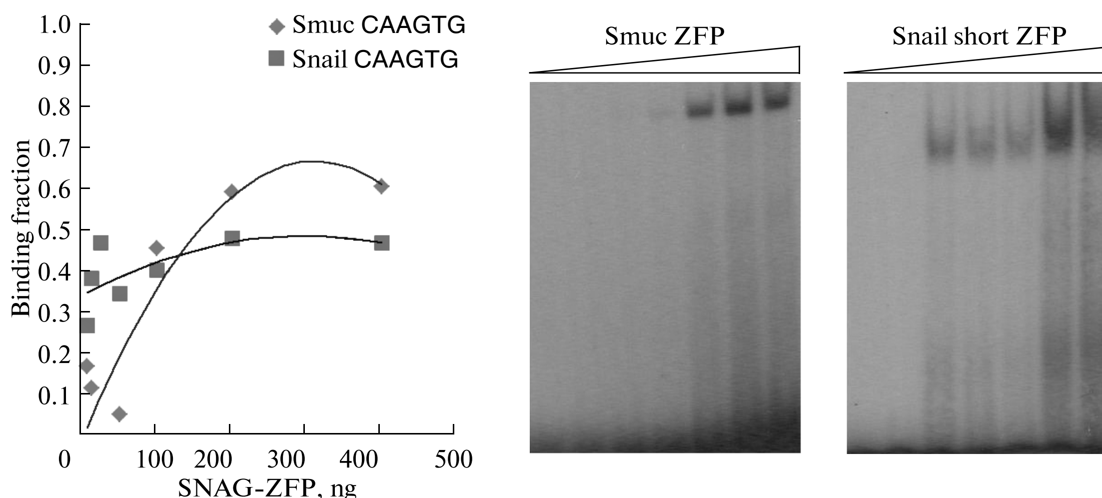

Fig. 6. Increasing amounts of SNAG-ZFP have varied effects when bound to the same E-box sequence. Representative EMSAs are shown on the right for Smuc and Snail short CAAGTG binding. Graph shows variation in binding when different SNAG proteins are bound to the same E-box dinucleotide combination.

Nature of the protein can dictate the strength of binding for the same E-box sequence

To decipher if different proteins can differ in their binding affinities to the same E-box, binding fractions were compared for different SNAG-ZFPs. We tested Smuc and Snail zinc finger proteins against the same E-box, CAAGTG. As expected, increasing quantity of protein to a constant amount of radiolabeled E-box oligonucleotides showed increased DNA-protein interaction (fig. 6). However, when it comes to the ability to bind, we find that the same E-box dinucleotide combination is bound more readily by some proteins than others. In this experiment, the CAAGTG sequence was more readily bound by Smuc than Snail (fig. 6), which also indicates that Smuc has higher affinity to CAAGTG sequence than Snail. While Smuc exhibited a dose-dependent increase in complex formation, Snail did not show this effect.

DISCUSSION

Based on gel mobility shift assay experiments, several variants of the E-box sequence were tested to determine which combination of the internal dinucleotides would show the strongest binding with members of the SNAG-family of transcription factors. The CACCTG and CAGGTG combinations showed the greatest affinities for all the SNAG-ZFP members tested. This could be why the E-cadherin promoter element E-boxes are the widely recognized binding sites for multiple transcription factors. Although the other 14 dinucleotide combinations were less preferred for SNAG-ZFPs, other transcription factors might be recognizing those sequences more robustly.

Quantitative data derived from the EMSAs reveal additional information about the ability of the SNAG-ZFPs to bind to the various E-box sequences. This information can be used to suggest possible binding sites for the SNAG-ZFPs and to contribute to the overall knowledge of this E-box sequence and its relation to this family of transcription factors. Because it is a highly recognized string of nucleotides, other families of transcription factors may also act in a similar manner and be bound by the properties of the internal dinucleotides.

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