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Nucleic acid melting by *Escherichia coli* CspE

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ABSTRACT

***Escherichia coli* contains nine members of the CspA family. CspA and some of its homologues play critical role in cold acclimation of cells by acting as RNA chaperones, destabilizing nucleic-acid secondary structures. Disruption of nucleic acid melting activity of CspE led to loss of its transcription antitermination activity and consequently its cold acclimation activity. To date, the melting activity of Csp proteins was studied using partially double-stranded model nucleic acids substrates forming stem–loop structures. Here, we studied the mechanism of nucleic acid melting by CspE. We show that CspE melts the stem region in two directions, that CspE-induced melting does not require the continuity of the substrate's loop region, and CspE can efficiently melt model substrates with single-stranded overhangs as short as 4 nt. We further show that preferential binding of CspE at the stem–loop junction site initiates melting; binding of additional CspE molecules that fully cover the single-stranded region of a melting substrate leads to complete melting of the stem.**

INTRODUCTION

In *Escherichia coli*, the CspA family includes nine homologous proteins, CspA to CspI. Four of these proteins are transiently and significantly induced upon temperature downshift from 37°C to 15°C (hence the name Csp, for Cold Shock Protein). None of the CspA homologues appears to be singularly responsible for cold-shock adaptation, since individual *csp* genes are dispensable at both normal and low growth temperatures (1). However, while all single, double or triple deletion mutants of *csp* genes grow at low temperature, a quadruple deletion strain (Δ*cspA*Δ*cspB*Δ*cspG*Δ*cspE*) is cold-sensitive for growth, indicating that a presence of at least one of these CspA family proteins is required to support growth at low temperature. Overproduction of any one of the nine *cspA* family genes, with the exception of *cspD*, complements the cold-sensitivity of the quadruple deletion strain (1), indicating that the functions of these proteins are redundant.

Many cellular functions have been attributed to CspE, a well-studied member of the *E.coli* CspA family. For example, the presence of camphor leads to chromosome decondensation. Overproduction of CspE leads to camphor resistance and chromosome condensation (2). CspC and CspE were shown to be involved in the regulation of expression of *rpoS*, a gene encoding a global stress response regulator, and *uspA*, a gene encoding a protein that is induced by numerous stresses (3). Hanna and Liu (4) demonstrated the interaction between CspE and the nascent RNA in transcription elongation complexes, suggesting that this protein is involved in transcription regulation. They also found that purified CspE interfered with Q-mediated transcription antitermination. CspA, CspE and CspC decrease transcription termination at several intrinsic terminators and also affect transcription pausing (5). CspE also impedes poly(A)-mediated 3′ to 5′ exonucleolytic decay by PNPase and inhibits both the internal cleavage and poly(A) tail removal by RNase E (6).

Among all these functions, the cold-shock function of Csp proteins is the focus of our study. The mechanism(s) through which the increase in the amount of CspA family proteins contributes to growth at low temperature is not fully known. *In vitro*, all CspA family proteins studied bind RNA or single-stranded DNA with low affinity and low specificity (7–9) and some act as nucleic acid chaperones, i.e. they can melt and/or destabilize nucleic acid secondary structure elements in an energy-independent fashion (5,10). This melting activity may be important at cold-shock conditions, when the stability of RNA secondary structure elements is increased, which could interfere with translation and transcription. Due to their RNA chaperoning activity, CspE, CspA and several other members of the family act as transcription antitermination factors (5,10). The physiological relevance of this function of CspE is suggested by an observation that CspE overexpression at normal temperature leads to increased expression of several promoter-distal genes of the *metY-rpsO* operon due to

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transcription antitermination (5). The products of these genes–NusA, IF2, RbfA and PNPase–are known to be involved in cold acclimation of cells.

The three-dimensional structure of CspA from *E.coli* (11–13) reveals five antiparallel β strands, β1 to β5, forming a β-barrel structure with two β-sheets. Two evolutionarily conserved RNA-binding motifs, RNP1 and RNP2, are located on the β2 and β3 strands. In CspE, the RNP1 Tyr10, Phe17 and Phe19 and RNP2 Phe30, His32 and Phe33 form a compact surface-exposed aromatic patch. By systematic mutational analysis of CspE aromatic patch residues we showed that Phe17, Phe30 and His32 are critical for the nucleic acid melting function, since substitution of these residues leaves the RNA-binding activity of CspE unaffected but abolishes the nucleic acid melting activity and, consequently, transcription antitermination and cold acclimation functions of the protein (10,14). Analysis of melting intermediates trapped by individual substitutions showed that Phe17 and Phe30 act at the earliest stages of melting, while His32 acts later and is necessary for the propagation of melting (15).

Previous studies of CspE-induced melting used a single-stranded DNA substrate model that, when folded, contained a 9 bp stem and a 61 nt loop. The extent of CspE-induced melting of this substrate was dependent on the concentration of CspE in the reaction: while melting initiation was detectable at the site of the stem–loop junction at the lowest CspE concentrations used, further propagation of melting required higher concentrations of CspE and complete melting was only achieved in the presence of a large excess of CspE over the DNA substrate. We explained these results by suggesting that melting is initiated by the binding of a single Csp molecule at the Y-shaped stem–loop junction site, and that the propagation of melting is due to a strain generated at the Y-junction when enough CspE protomers crowded in the topologically constrained single-stranded loop and 'push' outside, into the stem. According to this view, high concentrations of Csp needed for complete melting are the consequence of a large number of Csp monomers needed to cover the long loop of the melting substrate [note that a single Csp molecule is thought to occupy ~6 bases (16)]. In this work, we studied this hypothesis using melting substrates of different topologies and lengths. We show that although the 'strain' hypothesis is unlikely to be correct, melting substrates with shorter single-stranded regions are melted at dramatically lower concentrations of CspE and the melting is initiated at the stem–loop junction, as originally envisioned.

MATERIALS AND METHODS

Purification of the proteins

The *E.coli* cells harboring plasmids pET11a-*cspE* and pET11a-*cspE*-F30R were grown at 37°C to OD_{600} of 0.5 and the CspE expression was induced with 1 mM isopropyl β-D thiogalactopyranoside (IPTG) for 60 min at 37°C. The proteins were purified by Q-Sepharose and hydroxyapatite column chromatography as described previously (5).

$KMnO_4$ probing

The sequence of the original stem–loop DNA *invertbeaconseq* (15) was: 5′-TCCCAAGAACTCGCTCTCCAATCGCTGACAGCAGCCGCCACGTATCCCTTCGTGTCTATTTTTGTGGTGTTTCTTGGGA-3′. Based on sequence of this DNA, six 5′ overhang-stem DNA substrates with single-stranded regions of different lengths were created. To create the 61 nt, (substrate 1), 31 nt, 16 nt, 8 nt, 4 nt and 2 nt 5′ overhang-stem templates, an oligonucleotide: 5′-TCCCAAGAA-3′ was annealed with different labeled oligonucleotides (for 61 nt overhang: 5′-CTCGCTCTCCAATCGCTGACAGCAGCCGCCACGTATCCCTTCGTGTCTATTTTTGTGGTGTTTCTTGGGA-3′, for 31 nt: 5′-ACGTATCCCTTCGTGTCTATTTTTGTGGTGTTTCTTGGGA-3′, for 16 nt: 5′-TCTATTTTTGTGGTGTTTCTTGGGA-3′, for 8 nt: 5′-TGTGGTGTTTCTTGGGA-3′, for 4 nt: 5′-GTGTTTCTTGGGA-3′ and for 2 nt: 5′-GT TTCTTGGGA-3′). To create substrate (substrate 2) and to test melting from the 3′ end, a 5′ end labeled oligonucleotide 5′-TTCTTGGGA-3′ was annealed with oligonucleotide 5′-TCCCAAGAACTCGCTCTCCAATCGCTGACAGCAGCCGCCACGTATCCCTTCGTGTCTATTTTTGTG GTGT-3′. To create substrate with 14 bp stem and 16 nt overhang (substrate 3), a 5′ end labeled oligonucleotide 5′-TCTATTTTTGTGGTGTTTCTTGGTTTGGGA-3′ was annealed with oligonucleotide 5′-TCCCAAACCAAGAA-3′. The DNA templates were end labeled with [γ^{32}P]ATP using polynucleotide T4 kinase (New England Biolabs). Annealing was carried out by mixing respective two oligonucleotides in the presence of 25 mM Tris–HCl (pH 8.0) and 100 mM NaCl followed by boiling for 5 min and cooling gradually to 5°C. The DNA substrates were purified to ensure absence of unlabeled and/or single-stranded DNA that may act as competitor. The $KMnO_4$ probing reactions were carried out as described (15). The reaction mixtures (18 μl) containing labeled DNA template (0.2 μM) and CspE in 10 mM potassium phosphate buffer (pH 7.0) were incubated at 5°C for 5 min and were then treated with $KMnO_4$ (1 mM) for 20 s at 5°C. Reactions were terminated by the addition of β-mercaptoethanol to 330 mM, followed by phenol extraction, ethanol precipitation and 30 min treatment with piperidine (10% v/v) at 90°C. The reaction products were analyzed using 6–20% polyacrylamide/urea gels.

Hydroxyl radical footprinting

The DNA substrate with a 5′ single-stranded overhang and a 9 bp stem used in Figure 1A (substrate 1) was used as a substrate in hydroxyl radical footprinting performed as described by Heyduk *et al.* (17). The CspE-DNA-binding and hydroxyl radical cleavage reactions were carried out at 25°C for 5 min each. The reaction products were analyzed using 6% polyacrylamide/urea gels.

RESULTS

CspE can melt partially double-stranded structures

We chose to use DNA substrates due to following reasons, (i) CspE binds to RNA and single-stranded DNA with similar efficiency and specificity (7); (ii) CspE exhibits similar nucleic acid melting activity towards both the RNA and DNA substrates (10); (iii) the results of *in vitro* melting measured with the DNA substrates such as molecular beacons correlate well with transcription antitermination and cold acclimation function of CspE (5,10,15); (iv) it is convenient and cost-effective to work with DNA substrates; and (v) techniques such as

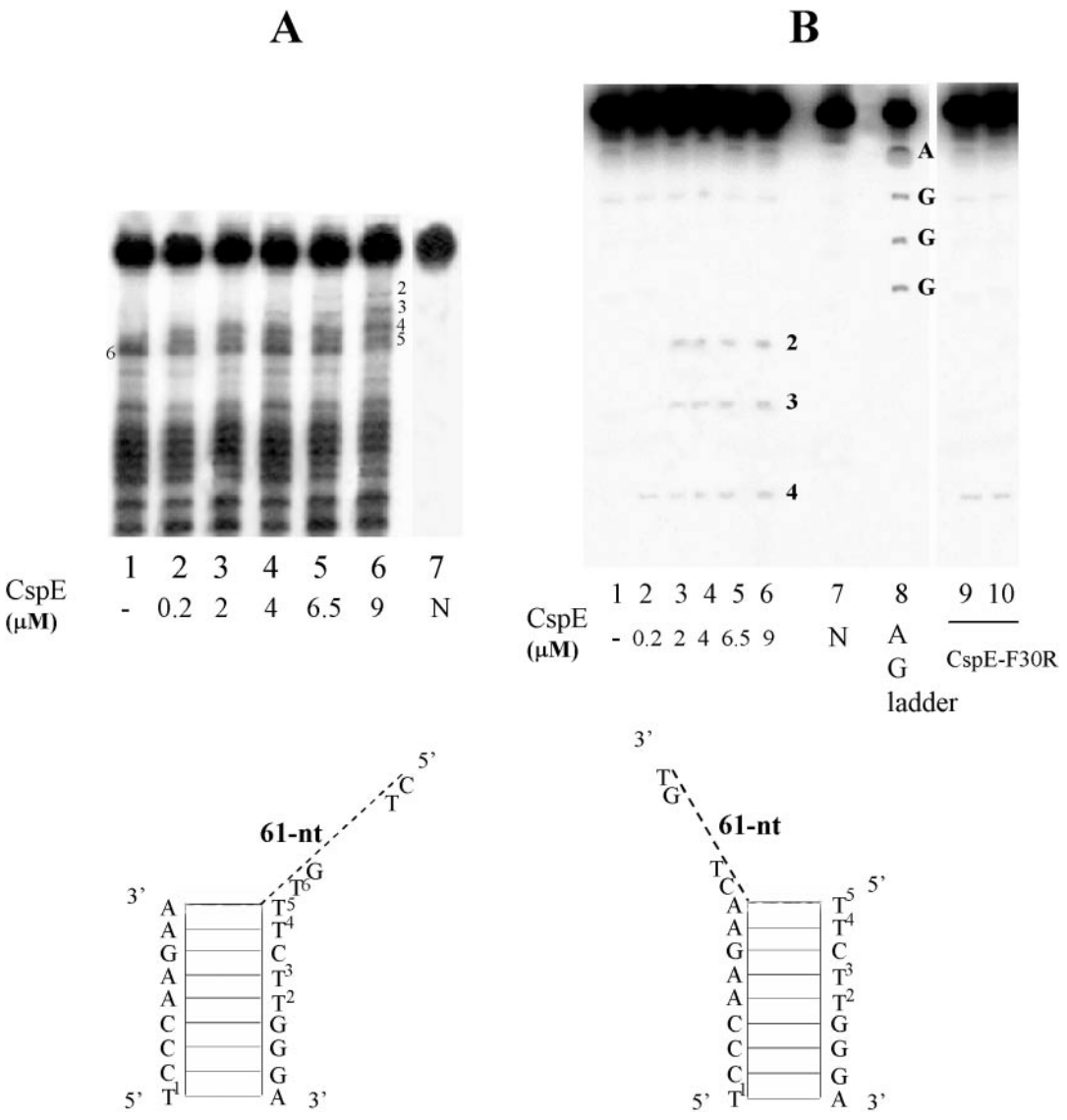

Figure 1. CspE-concentration-dependent melting of 5′ overhang-stem, and 3′ overhang-stem DNA substrates. (**A**) Melting of 5′ overhang-stem and (**B**) 3′ overhang-stem DNA substrates by CspE. The melting of stem structure of the respective DNA substrates with addition of CspE was followed with $KMnO_4$ probing as described in Materials and Methods. Lane 1, control reaction without CspE; lane 2–6, wild-type CspE: 0.2, 2, 4, 6.5 and 9 μM, respectively. Lane 7 designated by N represents naked DNA without $KMnO_4$ treatment. Thymines in the stem are numbered 1–5. A thymine residue that lies immediately outside the stem region for A is numbered 6. Schematic drawing of the stem structure of the respective DNA substrate is shown in the lower panel. The thymines are numbered as the corresponding ones in the upper panels. For (B) an AG ladder is shown in lane 8. Lanes 9 and 10 represent reactions carried out with CspE-F30R mutant protein (6.5 and 9 μM, respectively) instead of the wild-type CspE. Each gel is representative of experiment carried out at least three times.

$KMnO_4$ probing can be used with DNA substrates and allow to monitor the extent of melting as the thymine residues in the melted stem can be clearly visualized allowing analysis of different stages of the melting process. Previously, we reported a DNA melting assay for Csp proteins using a synthetic DNA substrate with a 61 nt single-stranded loop region and a 9 bp stem. CspE-mediated melting of this substrate (*invertbeacon-seq*) was monitored with $KMnO_4$, an agent specific for thymines in single-stranded form (15). $KMnO_4$ modified thymines can be cleaved by piperidine and the cleavage sites can be revealed, after electrophoretic separation, by autoradiography. In the absence of CspE, the thymines in the loop region of the model substrate were accessible to modification, while thymines in the stem were protected, as expected. The addition of increasing concentrations of CspE led to gradual melting that initiated at the stem–loop junction site.

One mechanism which would be compatible with CspE-concentration-dependent extent of melting of the model stem–loop substrate envisions, the early and preferential binding of CspE on the Y-junction site between the loop and the stem (which initiates melting), followed by increased occupancy of the loop such that additional Csp molecules begin to 'push' on the Y-junction-bound Csp molecule and induce the propagation of melting. Such an 'internal strain' mechanism would imply that changing the topology of the substrate, by cleaving the loop region and leaving a 5′ or a 3′ overhang would interfere with melting. This prediction was tested in the experiment presented in Figure 1A and B. We created two

melting substrates by annealing of a 9 nt and a 70 nt synthetic DNA oligonucleotides. The resultant melting substrates corresponded to the original *invertbeaconseq* stem–loop substrate with the loop 'split' at either side of the last base pair of the stem. The two substrates differed from each other since one of them had a 5′ overhang (substrate 1, Figure 1A), while the other had a 3′ overhang (substrate 2, Figure 1B). We next tracked the progress of the split substrates' melting as a function of CspE concentration (Figure 1A and B). With substrate 1, the profile of melting was identical to that previously seen with the *invertbeaconseq* substrate. For substrate 2, T^4 became sensitive to modification with $KMnO_4$ at the lowest concentration of CspE (0.2 μM, Figure 1B, lane 2; note that since in substrate 2 the 9 nt oligonucleotide was labeled at the 5′ end, the radioactive fragment arising from $KMnO_4$ modification at T^5 runs off the gel and thus, no conclusions can be made about the state of the corresponding base pair). At 2 μM CspE, T^4, T^3 and T^2 became sensitive to $KMnO_4$ modification (Figure 1B, lane 3). The observed pattern of $KMnO_4$ modification in substrate 2 must have arised due to substrate melting, since a control experiment with 6.5 and 9 μM (Figure 1B, lanes 9 and 10, respectively) of a melting-deficient CspE mutant (CspE-F30R) (15), revealed that only T^4 became sensitive to modification with $KMnO_4$. We do not know the reason for the difference in the observed pattern of CspE concentration dependence for substrates 1 and 2. The results clearly indicate that the topologically constrained loop is not required for Csp-induced melting, making the 'crowding' mechanism of melting unlikely. The results also indicate that Csp-induced melting is bidirectional, i.e. it can proceed in both 3′ to 5′ and 3′ to 5′ directions.

Correlation between effective melting concentration of CspE and the length of the single-stranded region of the melting substrate

We next investigated the dependence of CspE-induced melting on the length of the single-stranded 5′ overhang. The following was the rationale behind this experiment. As can be seen from Figure 1, high concentrations of CspE are required for complete melting of the substrates used. This can be due to intrinsically low affinity or low melting efficiency of CspE. Alternatively, long single-stranded overhangs may effectively titrate CspE from the site of the melting action, which is located at the junction site between the single-stranded and double-stranded DNA. If the latter were true, one would expect that shortening of the single-stranded region would allow complete melting to occur at lower concentrations of CspE. Figure 2 presents the results of an experiment where a series of melting substrates with varying lengths of the 5′ single-stranded overhang was used in CspE-induced melting assay. In addition to substrate 1 of Figure 1B, substrates with 31 nt, 16 nt, 8 nt, 4 nt and 2 nt 5′ overhangs were used (the stem sequence remained the same in all of these substrates). The substrates were combined with increasing amounts of CspE and probed with $KMnO_4$. For the 31 nt overhang substrate, thymines up to T^4 and T^3 became susceptible to modification at 0.2 and 2 μM CspE, respectively. At 4 μM CspE, all four thymines in the stem became susceptible to modification, and the efficiency of modification was further increased at 6.5 μM CspE. In the experiment with the 16 nt overhang substrate, thymines up to T^4 became sensitive to $KMnO_4$ in the presence of as low as 0.1 μM CspE. The minimum concentration of CspE required for complete melting was 2 μM; higher concentrations of CspE only led to increased efficiency of T^2 modification. An additional band of unknown origin appeared between T^2 and T^3 at high (6.5 μM) concentration of CspE and was not further investigated. When the overhang's length was reduced to 8 nt, T^2 was efficiently modified in the presence of as little as 0.1 μM CspE and became more prominent in the presence of 0.2 μM CspE. The control lane (no CspE added to the reaction), showed faint background bands for T residues in the stem region for this substrate, however, the effect of CspE addition was prominent as seen from the figure. For the 4 nt overhang substrate, 0.1 μM CspE was sufficient to completely melt the substrate. Thus, shortening of the single-stranded overhang dramatically lowers the amount of CspE required for complete melting of the substrate, as is schematically illustrated in Figure 2, where the minimal concentrations of CspE needed for complete melting of different substrates is plotted. With the 2 nt overhang substrate, background modification of stem thymines was observed even in the absence of CspE, and only a very slight increase in T^4 and T^5 modification was observed in the presence of high (2 and 4 μM) concentrations of CspE, indicating that no efficient CspE-induced melting occurred.

Next we tested the effect of titration by single-stranded DNA added *in trans* on CspE-induced melting. The melting substrate with an 8 nt 5′ overhang was combined with an equimolar amount of a 31 nt oligonucleotide whose sequence corresponded exactly to the single-stranded 5′ overhang of the 31 nt overhang melting substrate used in Figure 2, followed by the addition of two concentrations of CspE. As can be seen from Figure 3, the addition of the 31 nt oligonucleotide had no effect on the extent of the 8 nt overhang substrate melting, which was complete at both the low and high concentrations of CspE used. In contrast, only partial melting of the 31 nt overhang melting substrate was observed at the concentrations of CspE used (Figure 2). Therefore, it appears that single-stranded DNA added *in trans* is unable to effectively compete for CspE binding with the partially double-stranded melting substrate. This implies that after the first CspE molecule is bound at the double-stranded and single-stranded DNA junction site, it nucleates the binding of additional CspE molecules to the single-stranded DNA located *in cis*, which ultimately leads to complete melting.

CspE initiates melting by binding at the junction site between the single-stranded and double-stranded DNA

The above experiments are consistent with an idea that CspE first binds at the stem–loop junction to initiate melting. In order to test this possibility, we carried out hydroxyl radical footprinting of the 61 nt 5′ arm 9 bp DNA. The results are shown in Figure 4. The control reaction without CspE (lane 2) shows uniform set of bands arising from hydroxyl radical cleavage of the radioactively labeled DNA with 61 nt 5′ overhang and 9 bp stem (substrate 1). CspE, at low concentration protects the stem–loop junction region from cleavage (lane 3). Vertical lines next to the lanes 3 and 4 show the areas protected from hydroxyl radical cleavage at the lowest and intermediate concentrations of CspE. The protected area in lane 3 is ~6 nt in

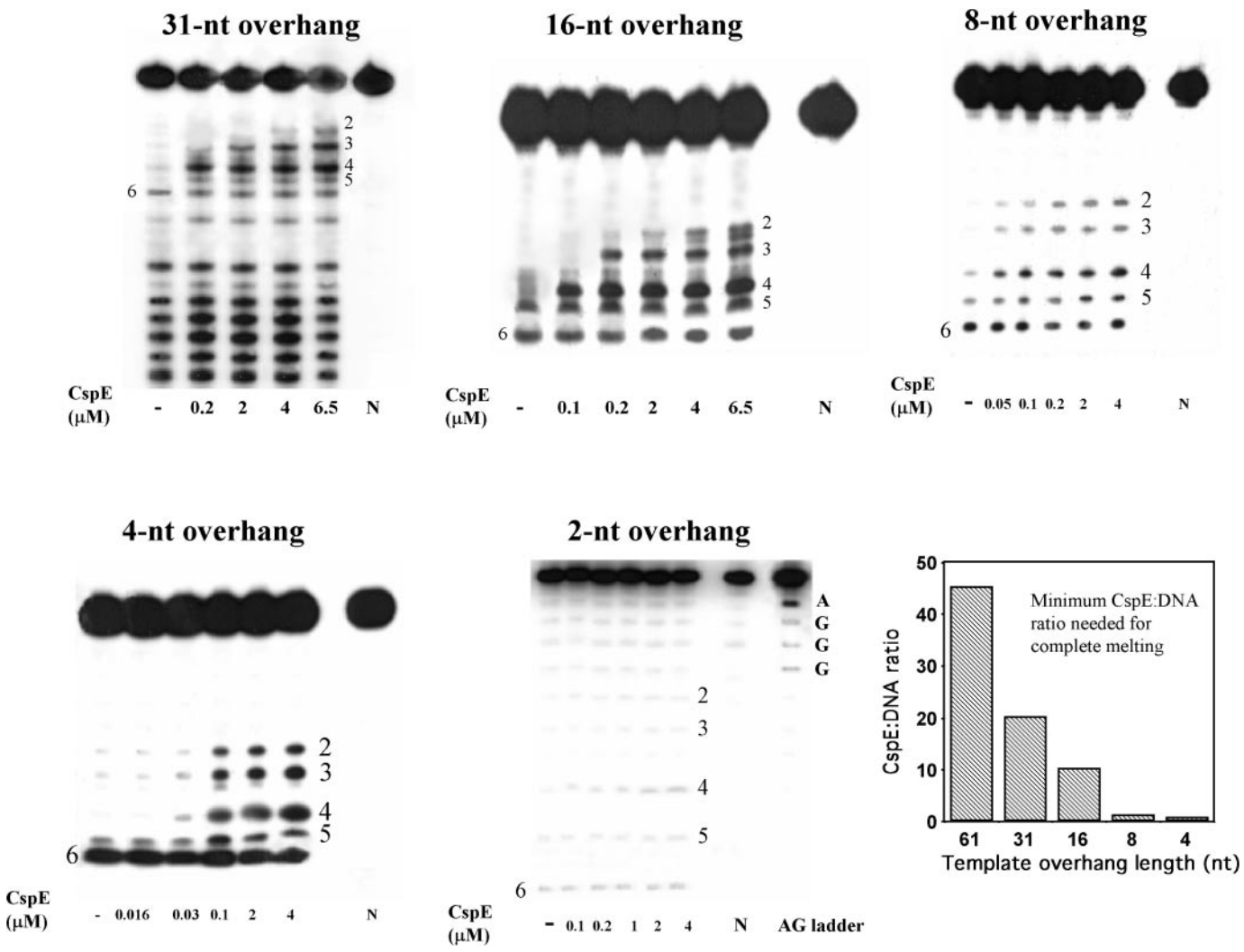

Figure 2. CspE-concentration-dependent melting of 5′ overhang-stem DNA substrates of varying overhang lengths. Various versions of the 5′ overhang-stem DNA substrates were created as described in Materials and Methods. These substrates have identical stem region and 5′ overhang of varying lengths such as 31 nt, 16 nt, 8 nt, 4 nt and 2 nt. The melting of stem structure of the respective DNA substrates with addition of CspE was followed with $KMnO_4$ probing as described in Materials and Methods. Thymines in the stem that become sensitive to $KMnO_4$ due to melting are numbered 2–5. A thymine residue that lies immediately outside the stem region is numbered 6. Concentrations of CspE used are shown. Lane designated by N represents naked DNA without KMnO4 treatment. For 2 nt 5′ overhang containing substrate, an AG ladder is shown. Each gel is representative of experiment carried out at least three times. The graph representing minimum CspE:DNA ratio required to melt each DNA substrate versus the length of the 5′ overhang of the respective substrate is created by taking into account the results from Figure 1A and all the gels shown in Figure 2.

length and thus is likely due to the binding of a single CspE protomer (16). Higher concentration of CspE leads to further protection of the single-stranded arm region (lane 4) and at the highest concentration of CspE used, almost the entire DNA is protected (lane 5). At low or intermediate concentrations (0.2 and 4 μM, lanes 3 and 4, respectively), CspE is not expected to completely melt the stem (see lanes 2 and 4 in Figure 1A). Indeed, a band arising due to hydroxyl radical cleavage in the stem and indicated by an arrow in Figure 4 is not protected at these concentrations of CspE. However, at higher concentration of CspE when the stem is expected to be completely melted (see lane 6 in Figure 1A) and therefore bound by CspE, the band becomes protected from hydroxyl radical cleavage (lane 5, Figure 4). Therefore we conclude that CspE preferentially binds at the junction site between the single-stranded and double-stranded DNA; this initial interaction leads to binding of additional CspE protomers that cover the entire single-stranded region followed by complete melting of the stem region.

CspE cannot completely melt DNA substrates with longer stems

$KMnO_4$ probing allows to monitor melting of the stem with respect to thymines only. Thus, melting of substrates used in our study, all of them containing an identical 9 bp stem, could be followed only up to the fourth thymine counting from the end of the stem (5′-. . .GGGA-3′ being the terminal residues). However, our previous studies using a molecular beacon based on the *invertbeaconseq* substrate showed that CspE completely opens the 9 bp stem (10,15). To test if CspE can melt DNA substrates with longer stems, we created a version of

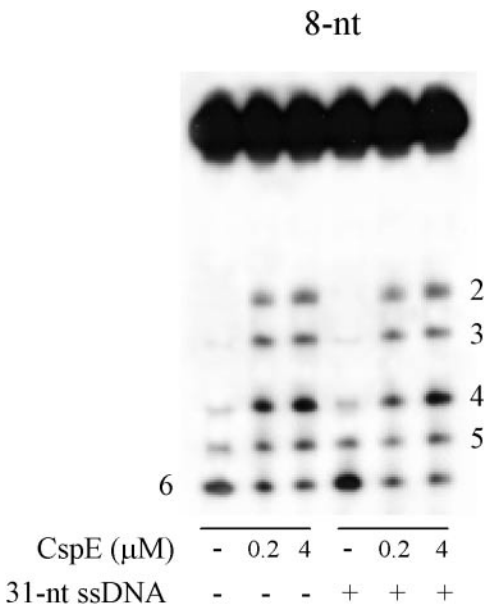

Figure 3. Effect of single-stranded DNA added *in trans* on CspE-induced melting. Melting of stem structure of a DNA substrate with 9 bp stem and 8 nt 5′overhang (same as used in Figure 2) by CspE was followed by $KMnO_4$ probing in presence and absence of a 31 nt single-stranded DNA. The sequence of 31 nt single-stranded DNA is identical to that of the 31 nt 5′ overhang of the DNA substrate used in Figure 2. Thymines in the stem that become sensitive to $KMnO_4$ due to melting are numbered 2–5. A thymine residue that lies immediately outside the stem region is numbered 6. Concentrations of CspE used are shown. The gel is representative of experiment carried out at least three times.

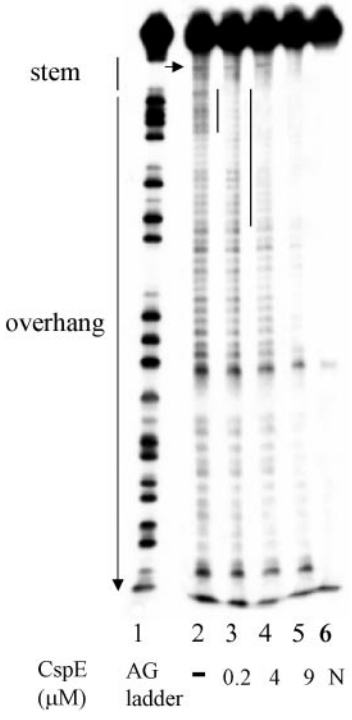

Figure 4. CspE initiates melting by binding at a junction site between the single-stranded and double-stranded DNA. Hydroxyl radical footprinting of the substrate with 61 nt 5′ overhang and 9 bp stem (substrate 1) was carried out without (lane 2) and with different concentrations of CspE (lanes 3–5: 0.2, 4 and 9 μM, respectively) as described in Materials and Methods. Lane 6 represents naked DNA without the hydroxyl radical cleavage.

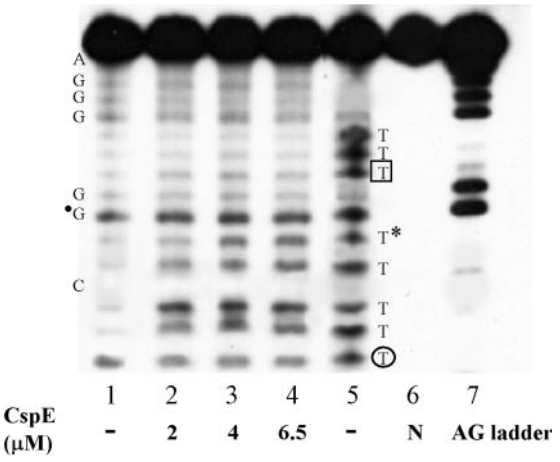

Figure 5. CspE-concentration-dependent melting of a substrate with 14 bp stem. Melting of stem structure of a DNA substrate with a 14 bp stem (substrate 3) with the addition of CspE was followed with $KMnO_4$ probing as described in Materials and Methods. This DNA has a 16 nt 5′ overhang. Lane 1, control reaction without CspE; lane 2–4, wild-type CspE: 2, 4 and 6.5 μM, respectively. Lane 5 represents $KMnO_4$ probing reaction using only the single-stranded (ss) DNA (labeled oligonucleotide) and lane 6 designated by N represents naked DNA without $KMnO_4$ treatment. AG ladder is shown in lane 7. The thymines up to the residue marked with an asterisk are sensitive to $KMnO_4$ due to CspE-induced melting. A thymine residue that lies immediately outside the stem region is encircled. The gel is representative of experiment carried out three times.

substrate 1 containing a 16 nt 5′ overhang and 14 bp stem. The sequence of this substrate, substrate 3, is derived from the *invertbeaconseq*, except that the 'GGTTT' sequence is inserted before the 3′ end-proximal GGGA residues of the longer oligonucleotide, and the complementary sequence is inserted in the shorter oligonucleotide. As can be seen from Figure 5, the control reaction without CspE (lane 1) shows a low background of $KMnO_4$ sensitive thymines in the stem; other bases also appear to be sensitive to the probing reagent for reasons unknown. To make sure that the melting substrate is properly annealed, we also probed radioactively labeled, single-stranded longer oligonucleotide that was used to make substrate 3 (lane 5). As expected, thymines became modified by $KMnO_4$ with high efficiency and all stem thymines could be accounted for (an AG chemical sequencing ladder was used as a marker, lane 7). For unknown reasons, the G residue marked with a filled circle was also found to be sensitive to $KMnO_4$. A thymine residue that lies immediately outside of the stem region is encircled in Figure 5 and was sensitive to $KMnO_4$ in all reactions including the control reaction without CspE (lane 1), as expected. Importantly, even at the highest CspE concentrations used here, 4 and 6.5 μM (Figure 5, lanes 3 and 4, respectively), melting of the substrate 3 stem only up to the thymine residue marked with an asterisk was observed. At lower CspE concentration (2 μM), the efficiency of modification of this thymine was lower (lane 2). Increasing CspE concentration up to 9 μM did not lead to further melting (data not shown). The results thus show, that the length of the stem region imposes limitations on the melting activity of CspE. Though the use of $KMnO_4$ limits our ability to pinpoint the exact boundary of melting, there is no melting beyond a thymine enclosed by a square in Figure 5.

DISCUSSION

Elucidation of the function(s) of CspA and its homologues has been a subject of much recent effort. In our work, we use *E.coli* CspE as a prototypical member of this group. The distinct functions of CspE depend on its nucleic acid binding and melting activities (10). For example, mutants of CspE that have lost nucleic acid melting activity cannot antiterminate transcription and thus cannot support cold acclimation of cells. However, the RNA-binding activity of these mutants is intact and these are still capable of regulation of proteins such as RpoS at physiological temperatures, an activity that depends solely on its RNA-binding activity. In this work, we set up to learn more about the molecular mechanism of CspE-induced melting and how it relates to the physiological melting substrates, intrinsic transcription terminators.

Our previous studies suggested that Csp molecules bind to the single-stranded loop region of a model stem–loop DNA melting substrate and initiate the melting of the stem. Both the DNase I protection studies and $KMnO_4$ probing showed the dependence of melting on CspE concentration (15). As one Csp molecule covers ~6 nt, several CspE molecules were needed to cover the 61 nt loop region in the original synthetic DNA substrate used in our studies. This gave rise to a hypothesis that binding of CspE molecules in the loop region creates a 'strain', which forces the stem to open. In the present study we used a 'split' version of our *invertbeaconseq* that we used earlier to determine if CspE-induced 'strain' is required for melting as no such strain is expected to accumulate in 'split' substrates. The results show that CspE is fully proficient in melting partially duplex substrates with both the 5′ and the 3′ overhangs. Thus, binding of CspE molecules does not lead to creation of the strain that was presumed to be a prelude to melting. The concentration of CspE required for complete melting is inversely proportional to the length of the overhang, presumably because fewer CspE molecules are required to cover shorter overhangs. Interestingly, for the overhang whose lengths are between 61 to 8 nt, the melting process is gradual, with only partial opening of the stem at low concentrations of CspE. This suggests that though the first CspE may preferentially bind at the single- to the double-stranded DNA junction, resulting in partial initial opening, the entire single-stranded region needs to be covered by CspE for full opening of the stem. In contrast, melting of the 4 nt overhang substrate is an all-or-none process, indicating that the binding of a single CspE protomer, perhaps in a fixed position relative to the single-stranded and double-stranded DNA junction site is sufficient to induce complete melting. This must be a consequence of the fact that the 4 nt overhang is close to the 6 nt binding site for a single CspE protomer. By the same reasoning, the inability of CspE to melt the 2 nt overhang substrate must be due to the fact that the length of the overhang is too short to allow even a single CspE to bind.

The results obtained with single-stranded DNA added *in trans* suggest that the first CspE molecule bound serves as an efficient nucleus for the binding of additional CspE protomers to the same melting substrate. The complete melting could be a consequence of a strain generated by 'pushing' between CspE molecules bound to the single-stranded region. However, the strain does not act by increasing the topological stress within the substrate, as studies with split substrates demonstrate. The hydroxyl radical footprinting showed that this hypothesis is correct as the CspE first binds at the junction between the single-stranded and double-stranded DNA and then progressively covers the single-stranded loop region leading to complete melting.

For experimental simplicity, our work was performed with DNA substrates. Conclusions drawn using DNA substrates correlated well with physiological function of CspE (10,15). The physiological substrate of CspE appears to be RNA, specifically, the nascent intrinsic transcription terminator RNA as it emerges from the RNA polymerase. Since most *E.coli* intrinsic transcription terminators are characterized by a ~5–7 stem and a ~4 nt loop (18), these seem to present preferred melting substrates for CspE and should be melted in an all-or-none manner. The physiological concentration of CspE is around 30 μM (19), which increases upon cold-shock. In fact, upon cold-shock, the cellular concentration of cold-shock-inducible CspA homologues is in the millimolar range. As seen from this study, very low concentration of Csp would be required to melt the intrinsic transcription terminators, and also single-stranded DNA does not compete efficiently *in trans* with the melting substrate, so competition by single-stranded nucleic acids should not interfere with their melting function *in vivo*. Future work on the specificity of CspE (and other Csp proteins) interactions with terminator structures of various sequences should allow to determine if different Csp proteins can target specific terminators *in vivo*.

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