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NUCLEIC ACID BINDING ACTIVITIES OF HUMAN COCKAYNE SYNDROME B PROTEIN AND IDENTIFICATION OF Ca^{2+} AS A NOVEL METAL COFACTOR

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Summary

Cockayne syndrome group B protein (CSB) is a member of the SWI/SNF2 subgroup of Superfamily 2 ATPases/nucleic acid translocases/helicases and is defective in the autosomal recessive segmental progeroid disorder Cockayne syndrome. We examine herein ATP dependent and ATP independent biochemical functions of human CSB. We report that Ca^{2+} is a novel metal cofactor of CSB for ATP hydrolysis, mainly through the enhancement of k_{cat}, and that a variety of biologically relevant model nucleic acid substrates can function to activate CSB ATPase activity with either Mg^{2+} or Ca^{2+} present. However, CSB lacked detectable ATP dependent helicase and single- or double-stranded nucleic acid translocase activities in the presence of either divalent metal. CSB was found to support ATP independent complementary strand annealing of not only DNA/DNA duplexes, but DNA/RNA and RNA/RNA duplexes, with Ca^{2+} again promoting optimal activity. CSB formed a stable protein:DNA complex with a 34mer double-stranded DNA in electrophoretic mobility shift assays, independent of divalent metal or nucleotide (e.g. ATP). Moreover, CSB was able to form a stable complex with a range of nucleic acid substrates, including bubble and “pseudo-triplex” double-stranded DNAs that resemble replication and transcription intermediates, as well as forked duplexes of DNA/DNA, DNA/RNA, and RNA/RNA composition, the latter two of which do not promote CSB ATPase activity. Association of CSB with DNA, independent of ATP binding or hydrolysis, was seemingly sufficient to displace or rearrange a stable pre-bound protein:DNA complex, a property potentially important for its roles in transcription and DNA repair.

Keywords

CSB/ERCC6; SWI/SNF2; Calcium; DNA-dependent ATPase; Nucleic acid binding

Introduction

Cockayne syndrome (CS) is a rare autosomal recessive segmental premature aging disorder characterized by cachectic dwarfism, cutaneous photosensitivity, sensorinural hearing loss, cataracts, loss of subcutaneous fat and neuropathologies; including white matter hypomyelination, central nervous system calcification, and microcephaly[1, 2]. In contrast to the

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photosensitive disease xeroderma pigmentosum (XP), patients with CS do not display any increased risk of cancer. CS has two strict complementation groups, CSA (*ERCC8, CKN1*) and CSB (*ERCC6*), while patients with combined XP-CS harbor mutations in XPB, XPD or XPG[3]. Mutations in the CSB gene account for ~80% of CS cases[4] and have also been found in DeSantis-Cacchione syndrome (DSC)[5], Cerebro-oculo-facio-skeletal syndrome (COFS)[6], and UV sensitive syndrome (UV^SS)[7].

The *ERCC6* gene is located on chromosome 10q21 and encodes a 4479 nt mRNA consisting of twenty-one exons[8]. The CSB protein is 1493 amino acids (~168 kDa) and has two putative nuclear localization signals, an acidic region, a glycine rich region, and seven sequence motifs characteristic of Superfamily 2 (SF2) ATPases/helicases/nucleic acid translocases[9]. CSB has been found to be essential for transcription coupled nucleotide excision repair (TC-NER) – a sub-pathway of NER that operates to remove RNA polymerase blocking lesions[10] – and is considered the human transcription coupled repair factor, similar in nature to the *Escherichia coli* protein Mfd[11]. CSB also appears to play a role in general transcription[12] and as an auxiliary factor in the base excision repair (BER) pathway[2]. CSB interacts with proteins involved in transcription, *i.e.* RNA polymerases (RNAP) I, and II[13, 14], the p44 and p62 subunits of TFIIH[15], and p53[16]; in NER, *i.e.* XPB, XPD, XPG[15], XAB2[17] and the p34 subunit of TFIIE[11]; and in BER, including PARP1[18], OGG1[19], and APE1[20].

A hallmark of CSB deficiency is sensitivity to ultraviolet (UV) light and a failure to recover RNA synthesis after UV exposure[21]. This latter defect is presumably reflective of inoperable TC-NER and/or faulty chromatin reorganization, typically mediated by CSB to facilitate transcription re-initiation[22]. CSB deficient cells are also sensitive to agents, such as 4-nitroquinoline (4-NQO) and N-acetoxy-2 aminofluorene (NA-AAF), which form bulky helix-distorting lesions repaired primarily by NER[23–25]. Moreover, CSB deficient cells are sensitive to ionizing radiation (IR)[26], paraquat[27], methyl methanesulfonate (MMS), and 5-hydroxymethyl-2'-deoxyuridine[20], suggesting that CSB plays a role in protection against or removal of BER substrates (e.g. base lesions, abasic sites, or single-strand breaks).

Previous biochemical work on CSB has shown that the protein possesses the ability to hydrolyze ATP to ADP plus inorganic phosphate (P_i) in a manner that is dependent on structured or double-stranded (ds) DNA, with DNA/RNA and RNA/RNA only weakly stimulating this function[11, 28, 29]. In addition, CSB has been found to change the nicked plasmid linking number by introducing negative supercoils in a seemingly ATP binding dependent manner[30, 31] and to pair complementary DNA strands in the absence of ATP[32]. CSB has also been reported to modify mononucleosome positioning, alter nucleosome arrays, and interact with histone tails[30], properties that are similar to other SWI/SNF2 chromatin remodeling enzymes[33]. Unlike SWI/SNF2 remodeling proteins, however, CSB was not able to change the topology of nucleosomal plasmid DNA[30]. Finally, CSB, in the presence of ATP or the non-hydrolyzable ATP analog (AMP-PNP), has been found by atomic force microscopy to wrap dsDNA around itself[31], perhaps explaining the ability of CSB to perturb nucleosomes.

Emerging evidence suggests that CSB is able to act in two modes, one that requires its ATPase activity, presumably in some remodeling aspect, and one that operates in an ATP independent fashion. Consistent with the ATP hydrolysis activity of CSB serving some function, *in vivo* complementation experiments indicate that an intact ATPase/SF2 core domain is required for rescue of 4-NQO sensitivity, UV-sensitivity, RNA synthesis recovery after UV, and prevention of UV induced apoptosis of CSB deficient cells[23, 34]. Additionally, CSB SF2 motif V/VI mutant cells have lower repair activity for 8-hydroxyguanine in DNA than comparable wild type complemented cells[35]. On the other hand, the CSB E646Q ATPase mutant only partially complements the MMS sensitivity of CSB deficient cells, suggesting that CSB functions, at least in BER, in two capacities: one involving its core ATPase-dependent activity and one

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invoking an ATP independent function that may engage the other regions of the protein, which perhaps mediate specific physical associations[20]. Furthermore, CSB is able to stimulate Ape1 AP site incision[20] and elongation of RNA polymerase I[36] in an ATP independent manner, indicating ATP independent functions of CSB in DNA repair and transcription. We have explored further the ATP dependent and ATP independent biochemical properties of CSB, focusing on its interactions with and manipulation of nucleic acid.

Results

Influence of divalent metals on DNA dependent ATP hydrolysis by CSB

Before proceeding with further biochemical characterization of CSB, we determined the optimal reaction parameters for the human enzyme. Specifically, we assayed the double stranded DNA (dsDNA) stimulated ATPase activity of CSB under two buffer conditions (Tris-Cl and HEPES-OH) at varying pH values. We found that CSB dsDNA ATPase activity was active over a wide pH range (6.5 to 8.5), under both buffer conditions (unpublished observations). With HEPES-OH pH 8.0, CSB consistently hydrolyzed the highest level of ATP to ADP and P_i, and thus, this platform was used for all subsequent experiments.

We also examined at two concentrations (1 and 4 mM) the effect of various divalent cations on the ATP hydrolytic activity of CSB. $Ca^{2+} > Mg^{2+} \geq Mn^{2+} > Zn^{2+}$, Fe^{2+} were found to activate dsDNA dependent ATPase activity – in some cases in a concentration specific manner – whereas Co^{2+} was not (Fig. 1a). To our surprise, Ca^{2+}, at both concentrations tested, was able to promote the greatest CSB dsDNA dependent ATP hydrolysis, ~ 2.5 – 3 fold over Mg^{2+}. An ATPase motif II mutant protein, E646Q, was unable to hydrolyze ATP in the presence of either Mg^{2+} or Ca^{2+} and dsDNA, indicating that intact SF2 motifs are required for DNA dependent ATP hydrolysis and that the activity with either metal is intrinsic to CSB (Fig. 1b).

Time course studies indicated that Ca^{2+} promoted ATP hydrolysis by CSB more effectively throughout the reaction period (Fig. 1c). To further characterize the mechanism by which Ca^{2+} stimulates CSB dsDNA dependent ATPase activity, we determined the kinetic parameters for ATP hydrolysis in the presence of either metal ion. Using pUC19 as the effector DNA, we found that the K_m values for ATP were similar: 16.5 μM with Mg^{2+} and 27.6 μM with Ca^{2+}. V_{max} values were 0.06 μM product min^{-1} with Mg^{2+} and 0.2 μM product min^{-1} with Ca^{2+}, indicating a ~ 3 fold increase in k_{cat} specifically.

It has been shown that CSB requires DNA with some double-stranded nature to stimulate its ATP hydrolysis activity in the presence of Mg^{2+}[11, 28, 29, 37]. We examined CSB ATPase function when presented with a variety of model substrates in the presence of Mg^{2+} or Ca^{2+} (see Experimental Procedures). Substrates used were a true ssDNA (19T), a supercoiled plasmid (pUC19), a short dsDNA duplex (34G/34C), a dsDNA containing an 18 nucleotide bubble (54FE/54C), and two “pseudo-triplex” substrates (54FE/54C/18D and 54FE/54C/18R) designed to mimic a DNA replication bubble or a transcription bubble, respectively (see Table 1 for oligonucleotide sequences). Consistent with prior results[11, 28], the single-stranded oligonucleotide (19T) poorly stimulated the ATPase activity of CSB under either metal condition (Fig. 2). Conversely, with each metal, supercoiled plasmid DNA stimulated CSB catalyzed ATP hydrolysis most significantly, with little difference seen in comparison to the other duplex-containing substrates (e.g. compare pUC19 to 34G/34C) in the case of Mg^{2+} (Fig. 2, top) or with a <3-fold difference in the case of Ca^{2+} (Fig. 2, bottom). No unique substrate preference was uncovered when Ca^{2+} was the divalent cation instead of Mg^{2+}, although a slightly different profile was observed.

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The effect of divalent metals on nucleic acid complementary strand annealing by CSB

Previously, it had been demonstrated that CSB was able to pair two partially complementary DNA strands that form a fork structure when annealed in the presence of Mg^{2+}, a function that may be relevant to its participation in various DNA transactions[32]. We sought to examine if divalent metal was required and whether substitution of Ca^{2+} for Mg^{2+} would affect, in any way, the ability of CSB to pair two partially complementary DNA oligonucleotides, DNA50 and DNA49 (Table 1). We found that (i) CSB does not require divalent metal to hybridize complimentary DNA/DNA strands, (ii) Ca^{2+} stimulates DNA/DNA strand pairing by CSB, and (iii) Mg^{2+} negatively impacts the annealing function relative to the no metal control (Fig. 3a and b). Similar to the ATP hydrolysis reactions, substitution of Ca^{2+} for Mg^{2+} increased the DNA/DNA pairing activity of CSB maximally ~ 3 fold.

We next asked whether this strand annealing function of CSB extended to DNA/RNA complementary hybrids (see Materials and Methods), which would be expected to arise during processes such as transcription. Similar to the DNA/DNA strand pairing results above, we found that CSB was able to promote the formation of double stranded DNA/RNA hybrids (DNA50 and RNA49; Table 1) more efficiently (~2-fold) in the presence of Ca^{2+} relative to Mg^{2+} (Fig. 3c). It is interesting to note that Mg^{2+} alone appeared to promote more efficient strand annealing than Ca^{2+} alone (*i.e.* without CSB), particularly in the case of the DNA/DNA and, to a lesser extent, the DNA/RNA reactions, making the Ca^{2+} stimulation of the CSB strand pairing activity even more dramatic (Fig. 3). Finally, we observed that CSB was able to promote strand annealing of partially complementary RNA oligonucleotides (RNA50 and RNA49; Table 1) in the presence of Mg^{2+} or Ca^{2+}; in this case, either divalent cation supported CSB facilitated strand pairing to similar levels (data not shown).

CSB binds a variety of nucleic acid structures independent of divalent metals or nucleotide

We next sought to elucidate whether CSB could form a stable complex with oligonucleotide duplexes and whether divalent metal or nucleotide status would affect complex formation. Previous DNA binding by CSB was shown using a 90mer and Mg^{2+} containing buffer[11]. We found that CSB DNA binding did not require any divalent metal and that binding to the short 34mer duplex substrate (34G/34C) was consistently, albeit only slightly, better in buffer lacking divalent metal (Ca^{2+} or Mg^{2+}) (see representative binding gel in Fig. 4a). Apparent dissociation constants (K_D) for CSB DNA binding were 10.7±0.6 nM without metal, 13.7±1.1 nM with Ca^{2+}, and 20.5±5.2 nM with Mg^{2+}. In addition, nucleotide status (*i.e.* addition of ATP, ADP, or AMP-PNP) did not dramatically affect DNA binding by CSB and, consistent with previous observations[11], nucleotide was not required for CSB:DNA binary complex formation (data not shown).

In addition to 34mer dsDNA binding by CSB (Fig. 4a), we examined whether CSB could bind stably to alternate nucleic acid structures that might arise *in vivo*. We utilized duplex substrates that stimulated CSB ATP hydrolysis activity (54FE/54C, 54FE/54C/18D, 54FE/54C/18R; Table 1) and that were hybridized in the complementary strand annealing assays (DNA50/DNA49, DNA50/RNA49, RNA50/RNA49; Table 1). Interestingly, CSB was able to bind all six substrates (Fig. 4b), although the DNA/RNA hybrid (DNA50/RNA49) and the RNA/RNA duplex (RNA50/RNA49) did not promote significant ATP hydrolysis by CSB (data not shown). Apparent K_D values for each substrate are as follows: 3.2±0.6 nM for 54FE/54C, 2.2 ±0.2 nM for 54FE/54C/18D, 3.8±0.2 nM for 54FE/54C/18R, 5.9±0.6 nM for DNA50/DNA49, 13.4±3.3 nM for DNA50/RNA49, and 3.6±1.5 nM for RNA50/RNA49 (Fig. 4c and d). The data indicate that CSB has slightly higher DNA binding affinity or stability for bubble containing DNA structures over small (34mer) dsDNAs (see for instance 54FE/54C/18D *vs.* 34G/34C without metal, ~ 5 fold difference in K_D) and that CSB shows little difference in

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preference for the forked duplexes of varying nucleic acid composition (compare DNA50/DNA49, DNA50/RNA49, and RNA50/RNA49).

CSB is neither a nucleic acid helicase nor a single- or double -strand nucleic acid translocase

CSB belongs to the SWI/SNF2 subgroup of SF2 helicases and nucleic acid translocases[38, 39]. As such, we examined whether CSB possessed any ATP dependent nucleic acid unwinding or nucleic acid translocation activities similar to other characterized SF2 enzymes[40].

First, we tested a large number of nucleic acid substrates for ATP dependent CSB catalyzed strand separation under the same buffer conditions where CSB was able to hydrolyze ATP in the presence of dsDNA, examining reactions that contained either Mg^{2+} or Ca^{2+} (see Materials and Methods). We examined DNA/DNA and DNA/RNA substrates that contained a free 5' or a free 3' end, as well as substrates that formed DNA/DNA, DNA/RNA, RNA/DNA, and RNA/RNA forked duplexes. In other words, all combinations of single-strand nucleic acid loading platforms and double-stranded nucleic acid duplexes were explored (Supplemental Fig. 1, top two tiers). CSB was found to have no ATP dependent unwinding activity on these substrates (see examples in Supplemental Fig. 2). We also tested DNA:RNA hybrid oligonucleotides where there was a nucleic acid switch at a single-strand:double-strand junction (Supplemental Fig. 1, third tier). CSB, again, displayed no unwinding activity on any of these substrates (data not shown). Finally, we examined a set of bubble and "pseudo-triplex" substrates (Supplemental Fig. 1, bottom tier) for CSB unwinding activity and observed no strand separation by the protein (data not shown).

Second, we asked whether CSB could translocate along either single-stranded or double-stranded nucleic acid, again under conditions in which CSB was active as a DNA dependent ATPase. Using 50mer DNA or RNA oligonucleotide strands that harbor a centrally located biotinylated nucleotide pre-bound to streptavidin, we found no evidence that CSB was able to translocate along these ssDNA or ssRNA molecules to release the stretpavidin (Supplemental Fig. 3). We also employed a standard triplex DNA displacement assay to determine whether CSB was able to translocate along dsDNA (see Materials and Methods). Briefly, the dsDNA substrate consists of a polypurine tract to which a shorter third polypyrimidine DNA strand is bound via Hoogsteen base pairing. Many, but not all, SWI/SNF2 family proteins have been demonstrated to translocate along dsDNA and release a triplex strand in an ATP dependent manner[41]. When CSB and ATP were incubated with the triplex substrate, no CSB dependent release of the third radiolabeled strand was observed in the presence of either Mg^{2+} (Fig. 5) or Ca^{2+} (data not shown), although in the presence of Ca^{2+} alone, the triplex was somewhat destabilized. In parallel reactions with Mg^{2+}, we incubated a known dsDNA translocase *Sacchromyces cerevisiae* Rad54 (yRad54) with the triplex substrate plus ATP and were able to observe strand displacement, supporting the conclusion that human CSB is unable to translocate along dsDNA (Fig. 5). We note that the translocase assay with CSB was performed under conditions found to be optimal for CSB ATPase activity, and that CSB ATPase function was less robust under the conditions used for yRAD54.

dsDNA binding by CSB promotes removal or rearrangement of a protein pre-bound to DNA

We next asked, in a heterologous system, whether DNA binding by CSB could perturb a stable protein:DNA complex. Prior studies on stand-alone SWI/SNF2 proteins (*i.e.* not large chromatin remodeling complexes) have indicated that they can remove specific proteins (TBP by Mot1 and Rad51 by Rdh54) from DNA in an ATP dependent manner[42, 43]. Towards this end, we obtained a mutant *Bam*HI protein, *Bam*HI_{E111A}, which can bind its recognition sequence (5'-GGATCC-3'), yet lacks endonucleolytic activity[44]. In our experimental design, *Bam*HI_{E111A} was prebound in excess to a 42mer dsDNA duplex containing a single *Bam*HI restriction site. When *Bam*HI wild-type (*Bam*HI_{WT}) enzyme was added, endonuclease activity

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of *Bam*HI$_{WT}$ was reduced to <10% in the presence of the prebound mutant protein (Fig. 6a, lanes 1 and 8, and Fig6b). When wild-type CSB was added in increasing amounts simultaneously with *Bam*HI$_{WT}$ enzyme, increasing incision activity by the wild-type endonuclease was observed, indicating that CSB was able to remove or rearrange the binding interface of the prebound *Bam*HI$_{E111A}$ to allow for cleavage (Fig. 6a, lanes 3–7). Notably, addition of ATP to the reaction did not have any appreciable effect, either positively or negatively, on the ability of CSB to activate *Bam*HI$_{WT}$ endonuclease cleavage (Fig. 6a, lanes10–14). As ATP alone was found to inhibit *Bam*HI$_{WT}$ activity (Fig 6a, lane 8) restoration of the cleavage event was likely mediated both by CSB dsDNA dependent ATP hydrolysis (*i.e.* removal of ATP) and by *Bam*HI$_{E111A}$ protein displacement/rearrangement. The lack of a direct effect of ATP on CSB stimulation is seemingly consistent with the results obtained above, where DNA binding by CSB of the 34mer duplex was not affected by the presence of nucleotide (e.g. ATP, ADP, AMP-PNP).

Utilizing the SF2 motif II mutant CSB$_{E646Q}$ protein, which can bind but not hydrolyze ATP[29], we found that, in the absence of ATP, increasing concentrations of CSB$_{E646Q}$ were also able to restore *Bam*HI$_{WT}$ cleavage activity (to levels similar to those seen with wild-type CSB), supporting that this property of CSB functions independent of ATP (Fig. 6c). In the presence of ATP, however, CSB$_{E646Q}$ was able to only partially relieve the inhibition by mutant *Bam*HI$_{E111A}$, suggesting that ATP binding (*i.e.* sequestration) in the absence of hydrolysis incompletely alleviated the inhibition of ATP on *Bam*HI$_{WT}$ endonuclease activity. In all, the ability of CSB to remove or rearrange the binding interface of the prebound *Bam*HI$_{E111A}$ does not depend on ATP binding or hydrolysis, as both wild-type CSB and CSB$_{E646Q}$ were able to promote *Bam*HI$_{E111A}$ displacement/rearrangement in the absence and presence of ATP.

We also observed that, independent of ATP, CSB was able to stimulate *Bam*HI$_{WT}$ restriction endonuclease activity (compare lane 1 to 7 or 8 to 14), somewhat reminiscent of the ability of CSB to stimulate Ape1 AP site incision activity[20]. We sought to determine whether this stimulation, as well as whether the CSB protein removal/rearrangement activity, was due to a direct CSB:*Bam*HI physical interaction. Towards this end, we designed an *in vitro* pull down assay. Specifically, an α-His$_6$ antibody was coupled and crosslinked to Protein A/G agarose, which allowed for capture of His$_6$-tagged CSB protein. When Ape1 protein was incubated with the crosslinked α-His$_6$ antibody Protein A/G agarose and CSB, we were able to detect a direct physical interaction between these two proteins, as evidenced by CSB-dependent pull-down (Supplementary Fig. 4, lane 12), consistent with previous findings[20]; Ape1 alone did not interact with the crosslinked α-His$_6$ antibody Protein A/G agarose beads (Supplementary Fig. 4, lane 9). When *Bam*HI was incubated with crosslinked a-His6 antibody Protein A/G agarose and CSB, we did not observe a direct physical interaction between the two proteins (Supplementary Fig. 3, lane 6), and *Bam*HI did not interact with the crosslinked α-His$_6$ antibody Protein A/G agarose (Supplementary Fig. 4, lane 3). These results strongly support that the CSB protein removal/rearrangement activity and its ability to stimulate *Bam*HI restriction endonuclease activity is independent of a direct protein:protein interaction and likely the result of a CSB:DNA interaction.

To address whether CSB induces structural changes (*i.e.* bending or kinking) in DNA upon binding, and whether these structural changes, if observed, are dependent on the "motor" ATPase function of CSB, we carried out footprinting studies with the 34G/34C duplex substrate. Unfortunately, attempts to perform DNA footprinting in solution failed, as addition of chemical footprinting (e.g. iron-EDTA, H_2O_2, ascorbic acid) or crosslinking (gluteraldehyde, even as low as 0.01%) reagents disrupted the CSB:DNA complex (data not shown). This fact suggests that, at least *in vitro*, CSB DNA binding is sensitive to fluxes in metal ions, oxidation and/or charge, and limited our ability to utilize different solution-based footprinting strategies. We therefore employed an *in situ* footprinting technique. In these

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experiments, CSB-DNA binding reactions were resolved on a native polyacrylamide gel, and free and CSB bound nucleic acid was subjected to hydroxyl radical attack using 1,10-phenanthroline-copper [$(OP)_2Cu$] footprinting reagents. These studies, however, did not uncover a difference in the digestion pattern of bound and unbound DNA, regardless of whether ATP or AMP-PNP was added to the binding reactions (Supplemental Fig. 5). We presume that the lack of a CSB binding footprint on DNA and the absence of $(OP)_2Cu$ hypersensitive/resistant sites is due to sequence independent binding by CSB and the fact that the protein does not induce a detectable structural change in dsDNA. This conclusion is consistent with previous experiments using DNaseI to interrogate the interaction of CSB with DNA, where no footprint or hypersensitive/resistant sites were observed[30].

Discussion

CSB is a member of the SWI/SNF2 subgroup of SF2 ATPases/nucleic acid translocases/helicases based on sequence homology of its core ATP binding and hydrolysis motifs[38, 39]. It is a dsDNA dependent ATPase that operates over a wide physiological pH range and is operational in the presence of several divalent metal ions (Fig. 1). We examined herein ATP dependent and ATP independent biochemical activities of CSB, focusing on its interactions with and manipulation of nucleic acid.

Despite the fact that Mg^{2+} is generally considered to be the relevant metal cofactor for many ATPases *in vivo*, CSB ATPase function is significantly more active in the presence of Ca^{2+}, regardless of the DNA cofactor (Fig. 2). To our knowledge, this is the first instance of Ca^{2+} promoting ATP hydrolysis in a Superfamily 1 or 2 (RecA-like domain containing) enzyme. In fact, the yeast homolog of CSB, Rad26, was found to require Mg^{2+} for ATP hydrolysis, but was unable to utilize Mn^{2+}, Zn^{2+}, Co^{2+}, or Ca^{2+} as cofactors[45]. Characterization of the kinetic parameters of CSB dsDNA dependent ATPase activity indicates that Ca^{2+} exerts an effect on the enzyme turnover, increasing k_{cat} ~ 3 fold. The significant effect of Ca^{2+} on human CSB ATPase function is noteworthy given that this divalent metal plays a prominent role in neuronal function and that CS patients characteristically exhibit neuroabnormalities[1, 46]. It is tempting to speculate that loss of CSB function would be most evident in cells where Ca^{2+} concentration is highest.

Previous analysis of nucleic acid cofactors for CSB catalyzed ATP hydrolysis indicated that dsDNA was a prerequisite and that CSB displayed a slight (2-fold) preference for cofactors containing a small (10 nt) bubble[37]. We report herein that alternate model substrates, e.g. DNA replication and transcription mimics, serve as cofactors for ATP hydrolysis by CSB, in the presence of either Ca^{2+} or Mg^{2+} (Fig. 2). Furthermore, while CSB had been shown to exhibit Mg^{2+} dependent, ATP independent DNA/DNA strand annealing activity[32], we report that (i) this activity can occur independent of metal, yet is most efficient with Ca^{2+} present and slightly impaired by the addition of Mg^{2+} relative to the no metal control, and (ii) CSB can hybridize DNA/RNA (as well as RNA/RNA) duplexes, most efficiently in the presence of Ca^{2+}. Although complementary DNA/DNA strand annealing activities have been ascribed to many human RecQ family *bona fide* helicases (RecQ1[47], WRN[48], BLM[49], and RecQ5[50]), no *in vivo* functions for this activity – antagonistic to their DNA unwinding function – have been defined, and alternate nucleic acid preferences for these enzymes have not been reported. For CSB, it is conceivable that the DNA/DNA or DNA/RNA strand annealing activity plays a role: (a) in promoting the formation of duplex substrates during repair; (b) in facilitating the movement of the active bubble structure during transcription, akin to the transcription bubble rewinding by *E. coli* Mfd[51]; or (c) during homologous recombination, which requires complementary strand pairing at several phases.

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Since nucleic acid binding requirements and the substrate range for SWI/SNF2 proteins have not been analyzed extensively, we evaluated the effect of metal cofactors, nucleotides, and substrate composition on CSB:DNA complex formation. EMSAs revealed that CSB bound stably to a diverse collection of nucleic acid substrates, and that inclusion of either Mg^{2+} or Ca^{2+} only slightly affected the affinity of CSB for dsDNA (Fig. 4). Moreover, addition of ATP, ADP, or AMP-PNP did not overtly affect the DNA binding properties of CSB, indicating that neither ATP binding nor hydrolysis are required for the stable interaction of CSB with DNA. Our data also indicate that CSB has a slight nucleic acid binding preference for dsDNA substrates that contain both single-stranded and double-stranded regions, such as the model replication and transcription bubbles (54FE/54C/18D and 54FE/54C/18R), implying that CSB is active, both for complex formation and ATP hydrolysis, at sites where these conformations are formed *in vivo*. Furthermore, it had been shown previously that DNA/RNA hybrid duplexes and RNA molecules are poor cofactors for ATP hydrolysis by CSB[28]. We demonstrate here that CSB is able to stably interact with DNA/DNA, DNA/RNA, and RNA/RNA forked duplexes in EMSAs, suggesting that CSB may impart function, independent of ATP hydrolysis, via binding to nucleic acid substrates. The indiscriminate manner in which CSB is able to interact with a diverse array of nucleic acids implies a wide range of activities *in vivo* and is consistent with CSB being implicated in a variety of cellular processes (e.g. TC-NER, BER, and transcription).

It is clear that under the conditions examined in the studies herein, CSB is unable to separate nucleic acid strands. Whereas previous studies tested simple DNA/DNA substrates for CSB helicase activity[11, 28], we examined many nucleic acid combinations/structures for potential ATP dependent helicase and nucleic acid translocase functions (see examples in Supplemental Fig. 1–3, and Fig. 5). Given that the CSB ATPase core (*i.e.* Motifs I through VI) primary amino acid sequence, secondary structure, and predicted tertiary structure falls under the SF2 SWI/SNF2 subgroup and that, to date, no SWI/SNF2 family member has been shown to possess ATP dependent unwinding or single-strand translocase activities, the absence of these properties for CSB was not surprising. What was unexpected, however, was that CSB apparently lacks dsDNA translocase activity under conditions in which it is active as an ATPase. Indeed, many SWI/SNF2 members possess the ability to translocate along dsDNA in an ATP dependent manner; including FancM (mutated in Fanconi Anemia)[52], Rad54 (involved in recombination)[53], Rdh54 (a yeast Rad54 paralog)[54], and SSO1653 (an archaeal SWI/SNF2 protein)[55]; and the chromatin remodeling motors SWI/SNF[56], RSC[57], and ISWI[58]. That said, other SWI/SNF members appear to lack classic translocase activity. For example, *S. cerevisiae* Mot1p (a homolog of human BTAF1) uses energy from ATP hydrolysis to "grip" DNA proximal to promoters, causing dissociation of TBP, consequently inhibiting transcription initiation[42]. Thus, it is not unprecedented that SWI/SNF2 members can use the energy from ATP hydrolysis for activities other then movement along dsDNA[59]. Moreover, modeling of the CSB core using the *Sulfolobus solfataricus* SWI/SNF2 co-crystal structure with DNA (PDB ID: 1Z63) indicates that the amino acid side chain contacts seen between the SSO1653 ATPase domain and DNA are likely not the same for a CSB-DNA complex (Supplemental Fig. 6). This observation implies that, at least for the ATPase core, these two proteins interact with nucleic acid differently and may in part explain why the biochemical properties of CSB differ from this and other SWI/SNF2 family members.

We explored herein if CSB possessed the ability to remove or rearrange a protein bound to DNA. CSB was indeed able to dissociate or reorganize *Bam*HI_{E111A} that was pre-bound to its cognate site, most likely through an interaction with DNA, as no direct physical association could be detected between CSB and the restriction enzyme (Fig. 6 and Supplementary Fig. 4). Intriguingly, we found that this property of CSB was ATP independent, and not affected by ATP binding or hydrolysis. Other SWI/SNF2 proteins that remodel chromatin (SWI/SNF, RSC, ISWI, and Rad54 among others) and those that remove specific proteins from DNA

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(Mot1p (BTAF)) require ATP for their protein displacement activity. Nonetheless, the ATP independent protein removal/rearrangement activity of CSB is likely important in (i) DNA repair to uncover occluded lesions and allow for the repair machinery to recognize and excise the target damage, (ii) transcription to clear a path for RNAP elongation, and (iii) transcription initiation to promote binding of *trans* acting initiation factors to their regulatory elements. Studies are underway to delineate the precise ATP dependent and independent tasks of CSB, with a focus on the potential impact of post-translational modifications, other protein binding partners, and the unique N-(amino acids 1 to 501) and C-terminal (amino acids 1013 to 1493) regions of CSB in modulating or directing protein function *in vivo* and *in vitro*. Such studies are imperative given the recent evidence that a normally suppressed triplex displacement activity of full-length *E. coli* Mfd is activated upon binding to RNAP or following deliberate removal of an auto-inhibitory domain[60].

Materials and Methods

Proteins

Recombinant wild-type and E646Q CSB proteins were expressed in Sf9 insect cells via a baculoviral system as previously described[28]. Purification was performed by Ni^{2+} affinity, heparin, and typically anion exchange (source Q) chromatography, which allowed for isolation of protein to near homogeneity. The *Bam*HI mutant protein E111A ($BamHI_{E111A}$)[44] was graciously provided by Drs. Michael Dalton and Lydia Dorner of New England Biolabs (Ipswitch, MA). *Saccharomyces cerevisiae* Rad54 (yRad54) was kindly provided by Dr. Patrick Sung (Yale University, New Haven, CT). Wild-type *Bam*HI and T4 polynucleotide kinase were purchased from New England Biolabs.

Oligonucleotides

Oligonucleotides for complementary strand annealing, helicase, and single-strand nucleic acid translocase assays 61 were a gift from Dr. Zvi Kelman (University of Maryland Biotechnology Institute, Rockville, MD) and/or purchased from IDT (Coralville, IA). $(TC)_{20}$ oligonucleotide was a kind gift from Dr. Weidong Wang (National Institute on Aging, Baltimore, MD), as was plasmid pBSCR[52].

ATPase Assay

Buffers for standard ATPase reactions contained 20 mM HEPES-OH pH 8.0, 0.05 mM ATP, 40 μg/mL BSA, and 1 mM DTT. Reactions were supplemented with 1 or 4 mM divalent metal as indicated. ATPase reactions employed CSB and 12.5 μCi γ-^{32}P ATP. Reactions were incubated for 1 hr at 30°C with 150 ng of nucleic acid substrate. Reactions were stopped by the addition of 0.5 M EDTA. ATP hydrolysis was analyzed by Polyethyleneimine-thin layer chromatography (PEI-TLC) using 1 M formic acid/0.8 M LiCl. PEI-TLC plates were analyzed and quantitated on a Typhoon phosphoimager using OptiQuant TL software. Reactions for time course kinetics were performed as above, except that 10 μL aliquots were taken at the time points specified. For kinetic parameter determinations, CSB (150 ng, 900 fmol, 90 nM) was incubated with varying amounts of unlabeled ATP (500, 100, 50, 10, 5, 1 μM) for 1 hour at 30°C and processed as above. K_m and V_{max} parameters were calculated from Lineweaver-Burk plots.

Complementary strand annealing assay

Oligonucleotides (DNA50 or RNA50) were labeled at the 5' end with ^{32}P. Unincorporated γ-^{32}P ATP was removed by centrifugation through a Bio-Rad micro bio spin P30 column. Labeled oligos were stored in 20 mM HEPES-OH pH 8.0. Buffers for strand annealing contained 20 mM HEPES-OH pH 8.0, 40 μg/mL BSA, 1 mM DTT and either no metal, 1 mM

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Ca^{2+} or 4 mM Mg^{2+}. DNA/DNA, DNA/RNA, and RNA/RNA strand pairing reactions contained 100 fmol each DNA50 plus DNA49, DNA50 plus RNA49, or RNA50 plus RNA49 oligonucleotides, respectively, and 75 ng (450 fmol, 20 nM) CSB. Reactions were kept on ice and initiated by the addition of CSB and incubation at 30°C for the times specified. Reactions were stopped by the addition of Stop buffer containing 35 mM EDTA, 0.6% SDS, 25% glycerol, 0.05% bromphenol blue and 0.05% xylene cyanol. Reactions were run on 12% native polyacrylamide-TBE gels at 160 V for 2 hr. Substrates and products of strand pairing reactions were visualized and quantitated by phosphoimager analysis.

EMSA

Buffers for EMSA reactions contained 20 mM HEPES-OH pH 8.0, 50 mM KCl, 200 μg/mL BSA, 5 mM DTT, and 5% (v/v) glycerol. Where mentioned, 1 mM Ca^{2+} or 4 mM Mg^{2+}, and 2.5 mM ATP, ADP, or AMP-PNP final concentration, were added. Binding reactions contained 50 fmol of substrate and 15, 50, 150, 250, 350, or 500 fmol CSB. Binding reactions were incubated at 30°C for 30 min, then immediately loaded onto a native 4% polyacrylamide-TBE gel and run at 15 mA for 90 min at 4°C. Substrates and products were visualized and quantitated by phosphoimager analysis as above. Nucleic acid saturation binding curves were plotted (% substrate bound *vs.* nM CSB concentration) and K_D values were calculated by non-linear curve fitting using the Hill equation in Origin 7.0 (Northampton, MA).

dsDNA translocation assay

$(TC)_{20}$ was labeled at the 5'end with ^{32}P. Plasmid pBSCR was linearized by digesting with *Sca*I. Triplex substrate was created by incubating 10 pmol labeled $(TC)_{20}$ oligonucleotide with 15 pmol linearized pBSCR plasmid in Triplex buffer (33 mM Tris-Acetate pH 5.5, 66 mM Potassium acetate, 100 mM NaCl, 10 mM $MgCl_2$, and 0.4 mM Spermine), and incubated for 1 hr at 56°C and then overnight at room temperature. Unincorporated γ-^{32}P ATP and unannealed $(TC)_{20}$ were removed via centrifugation as above. Labeled substrate was stored in 33 mM Tris-Acetate pH 5.5, 5 mM $MgCl_2$. Buffers for CSB triplex displacement assays contained 20 mM HEPES-OH pH 8.0, 40 μg/mL BSA, 1 mM DTT, 2.5 mM ATP and either 4 mM Mg^{2+} or 1 mM Ca^{2+}. Buffer for yRad54 triplex displacement contained 40 mM Tris-HCl pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 2 mM dithiothreitol, 2% glycerol, and 100 ng/μL bovine serum albumin. Increasing concentrations of CSB [10 ng (60 fmol, 3 nM), 30 ng (180 fmol, 9 nM), 100 ng (600 fmol, 30 nM), and 300 ng (1.8 pmol, 90 nM)] or yRad54 [18 ng (180 fmol, 9 nM), 60 ng (600 fmol, 30 nM) and 180 ng (1.8 pmol, 90 nM) were incubated with 100 fmol triplex substrate at 30°C for 1 hr. Reactions were stopped by the addition of Stop buffer containing 0.9% SDS, 40% glycerol, 0.1% bromphenol blue, and 0.1% xylene cyanol. Reactions were run on 12% polyacrylamide-Tris-acetate-$MgCl_2$ gel containing 25% glycerol at 160 V for 4–5 hr. Substrate and product were visualized and quantitated by phosphoimager analysis as above.

Restriction enzyme site accessibility assay

Buffers for restriction enzyme site accessibility assays contained 20 mM HEPES-OH, pH 8.0, 50 mM KCl, 200 μg/mL BSA, 5 mM DTT, and 5% (v/v) glycerol and 4 mM Mg^{2+}. 50 fmol of substrate (42BamHI/42Comp) was incubated with 400 ng (20 pmol, 850 nM) *Bam*HI_{E111A} at 30°C for 15 min with or without 2.5 mM ATP; we note that ATP addition had no effect on the final pH of the reaction mixture. Subsequently, CSB or the CSB SF2 motif II mutant CSBE646Q (50, 150, 250, 350, or 500 fmol) were added to the reactions, followed immediately by addition of 1 U *Bam*HI wild-type (*Bam*HI_{WT}) enzyme and incubation at 30° C for 30 min. Stop buffer (95% formamide, 20 mM EDTA, 0.5% bromphenol blue, and 0.5% xylene cyanol) was then added and samples were heated at 95°C for 3 min and then loaded onto a 15% polyacrylamide-urea denaturing gel and electrophoresed at 225 V for 90 min.

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Substrates and products were visualized and quantitated by phosphoimager analysis using OptiQuant TL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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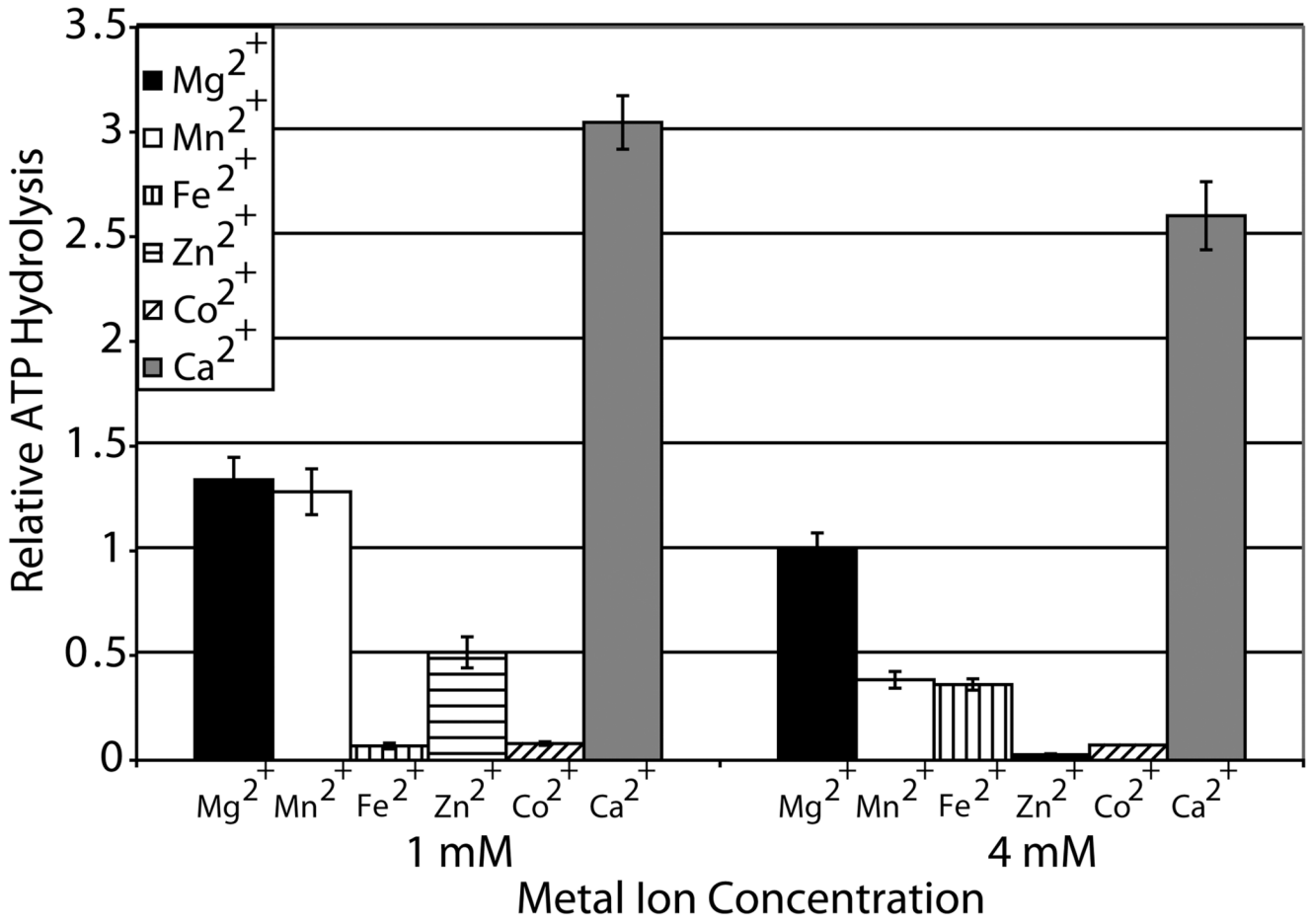

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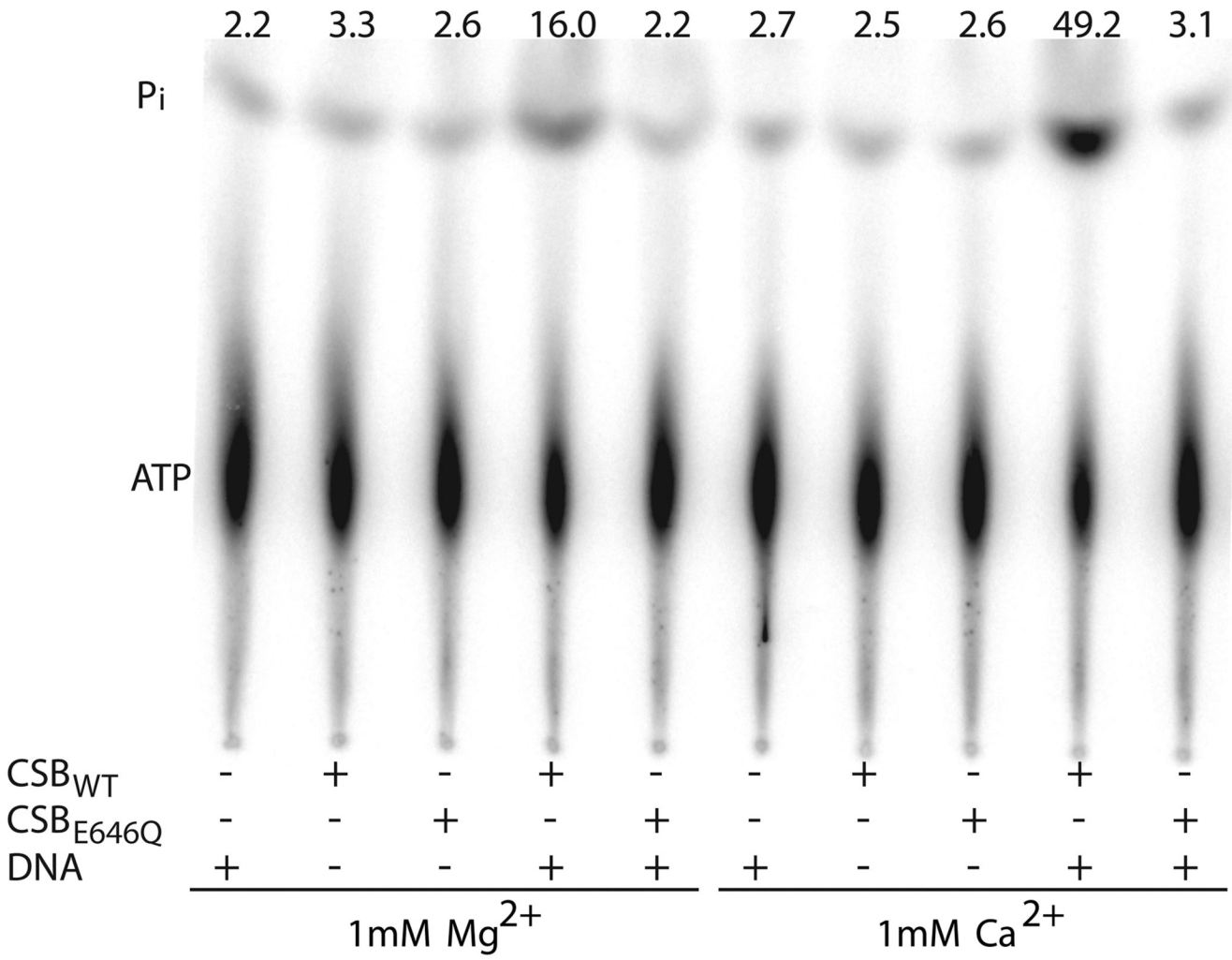

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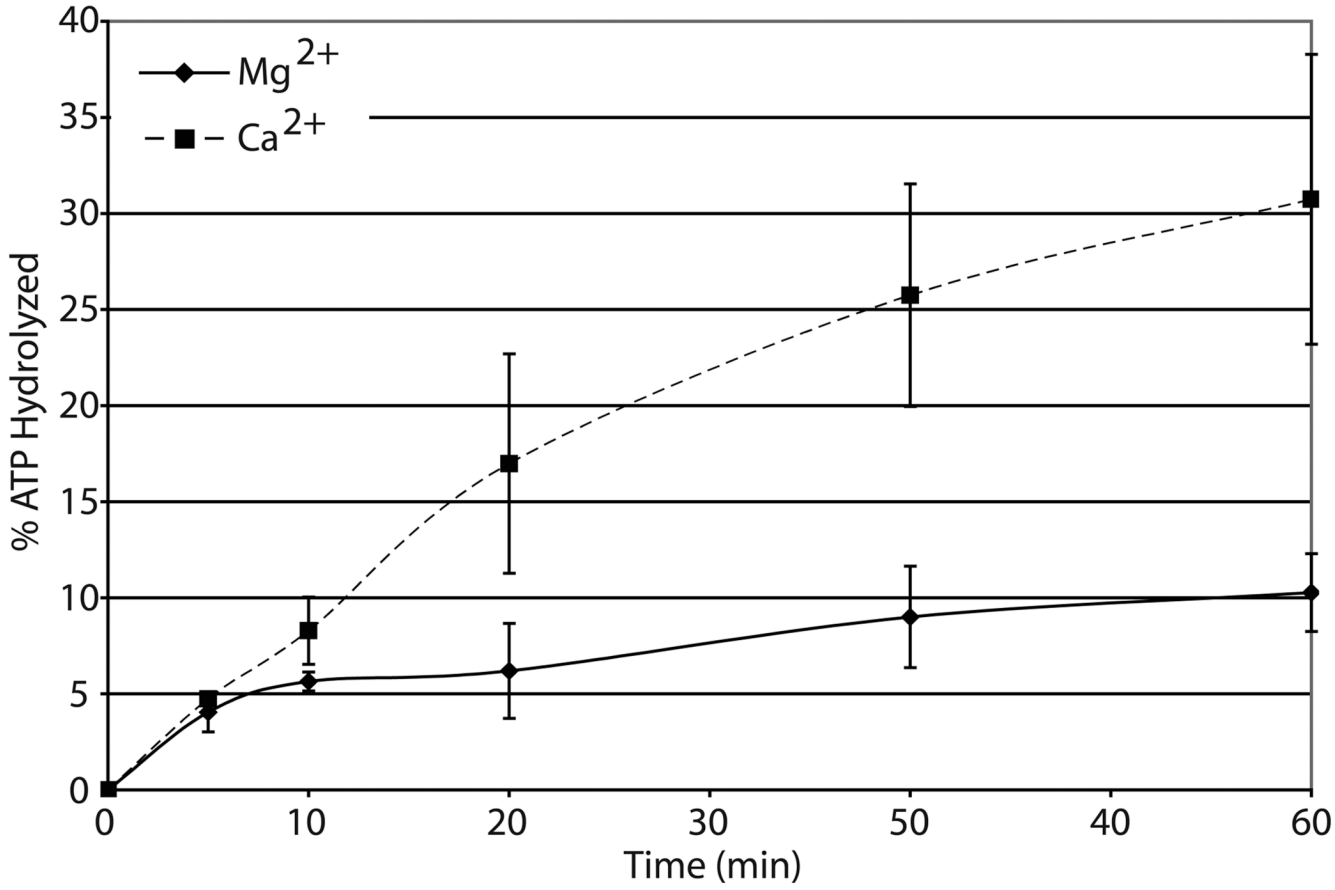

Fig. 1. Ca^{2+} is a novel metal cofactor for DNA dependent ATP hydrolysis by CSB

(a) Graph of relative ATP hydrolysis by CSB in the presence of various divalent metal ions at two concentrations (1 mM or 4 mM). Average values are plotted with standard deviations of at least 3 independent experiments. All values are relative to the 4 mM Mg^{2+} reaction, the metal condition typically used to assess CSB ATPase function [28]. (b) CSB ATP hydrolysis is dependent on active SF2 motif and the presence of dsDNA for both 4 mM Mg^{2+} and 1 mM Ca^{2+}. PEI-TLC plate of ATP hydrolysis by wild-type CSB or Motif II mutant (E646Q) in the presence or absence of dsDNA (pUC19) and divalent metal (Mg^{2+} or Ca^{2+}). Shown above is the average percent ATP hydrolyzed, calculated from at least 3 independent experiments. (c) Graph of CSB ATPase time course kinetics in the presence of Mg^{2+} or Ca^{2+}. Average values are plotted with standard deviations of at least 3 independent experiments.

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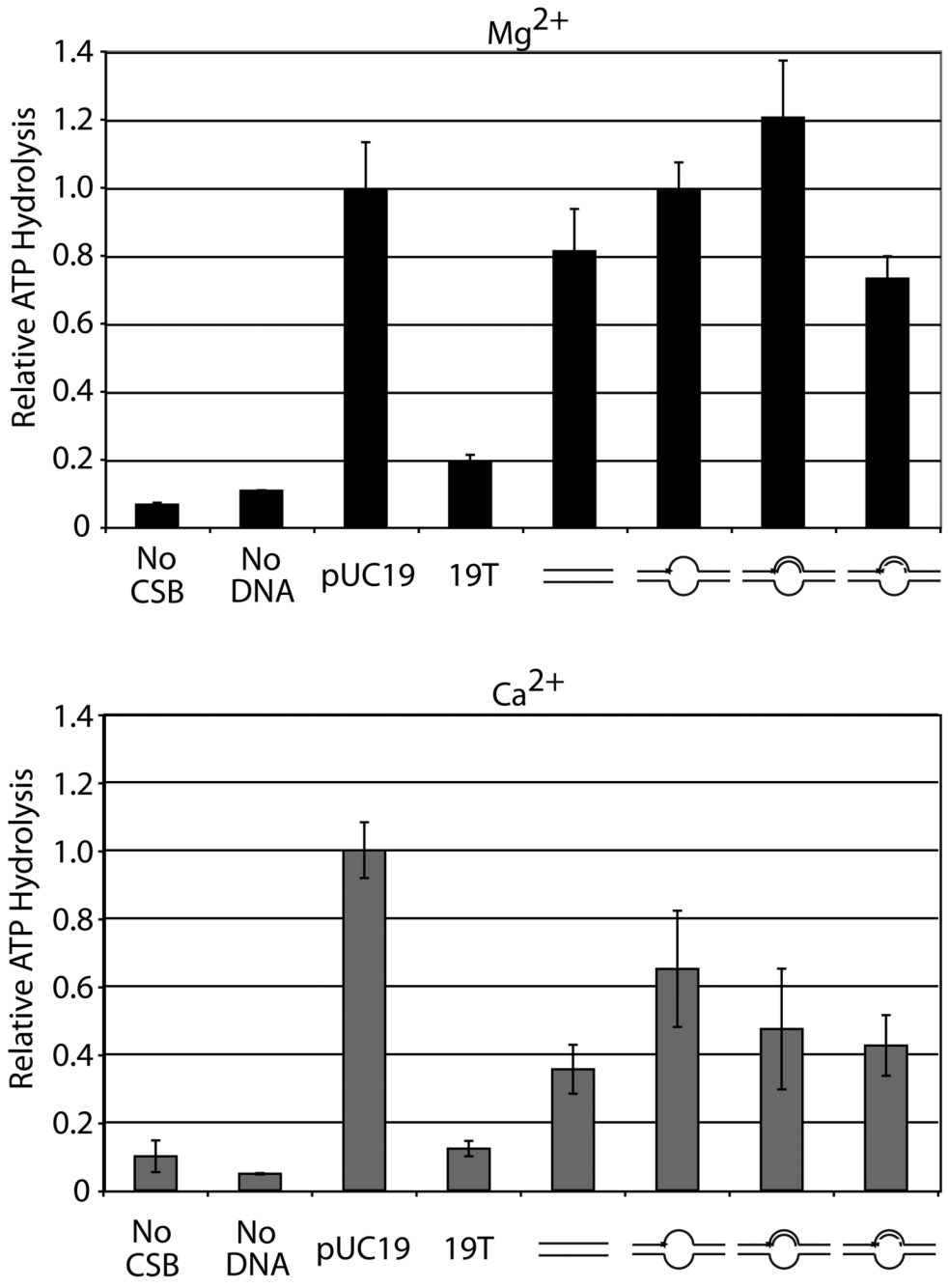

Fig. 2. Alternate model nucleic acid substrates serve as cofactors for CSB dependent ATP hydrolysis in the presence of Mg^{2+} (top) or Ca^{2+} (bottom)

CSB ATPase assays were performed as described in Experimental Procedures. Average relative values (to pUC19 reaction under each metal condition) are plotted with standard deviations of at least 3 independent experiments. Substrate depictions are displayed underneath each graph: from left to right pUC19, 19T, 34G/34C, 54FE/54C, 54FE/54C/18D, and 54FE/54C/18R. Solid lines represent DNA and dashed lines represent RNA. (see Table 1 for oligonucleotide details).

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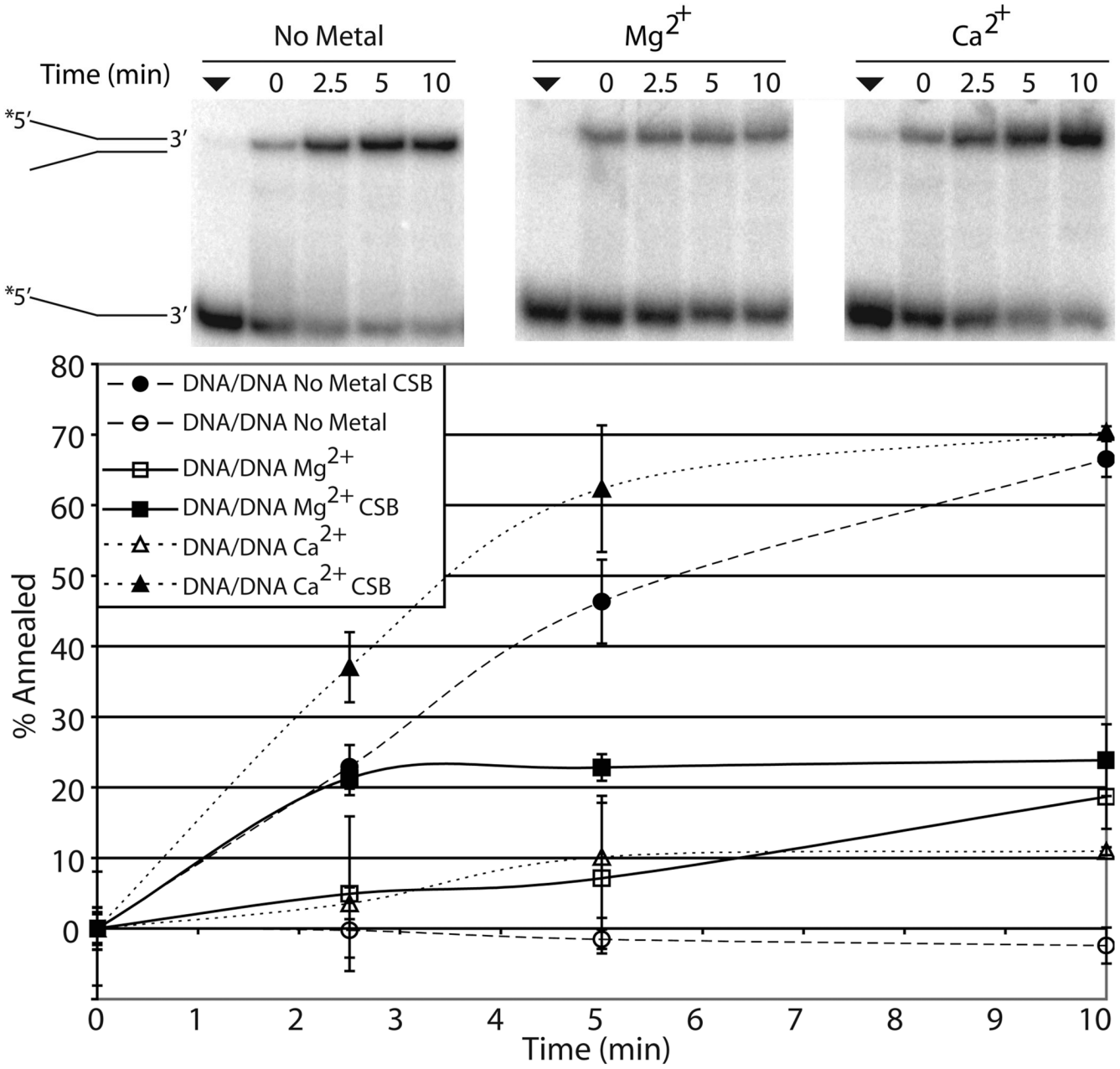

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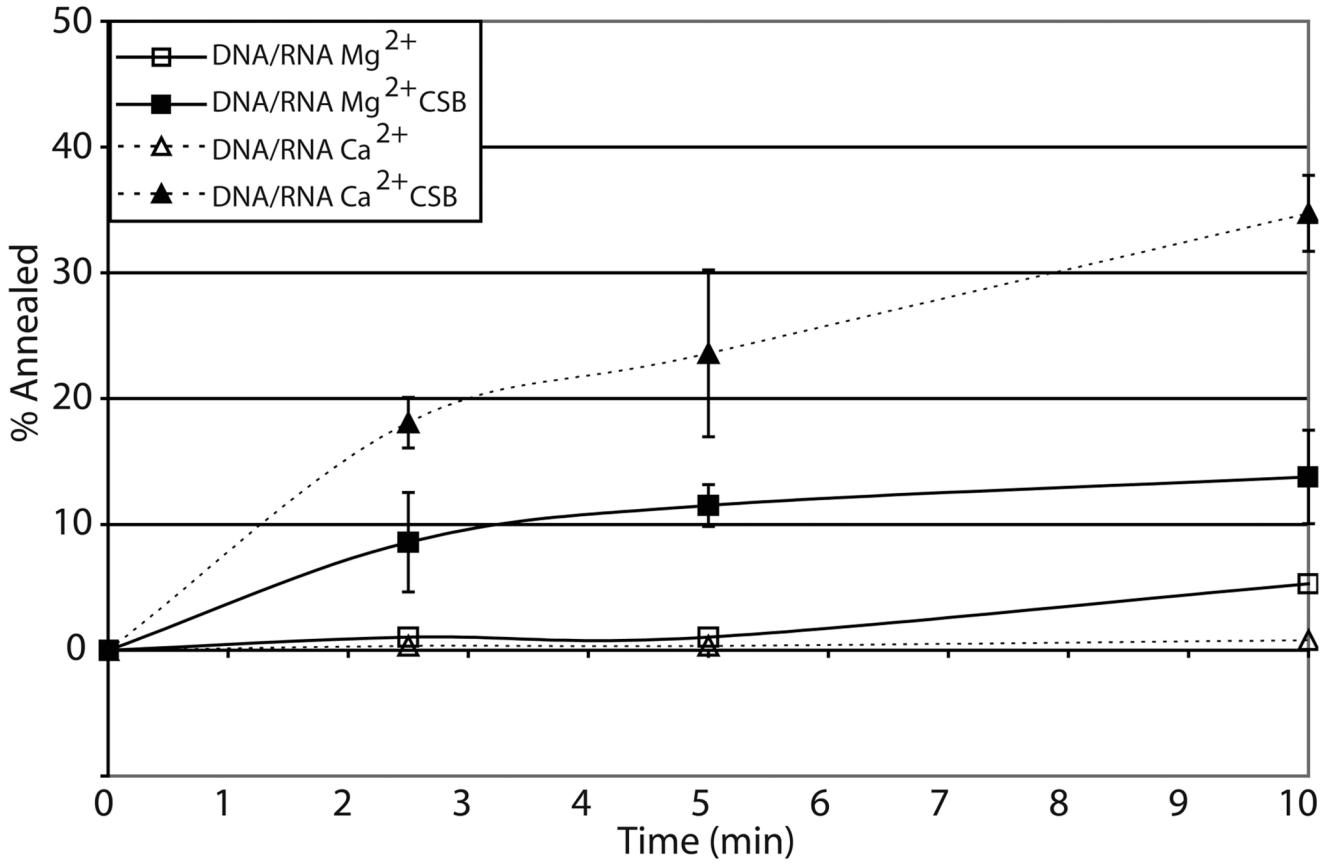

Fig. 3. Effects of Mg^{2+} or Ca^{2+} on CSB nucleic acid strand pairing

(a) Representative gel of CSB strand pairing activity for DNA50/DNA49 in the presence of no metal, 4 mM Mg^{2+}, or 1 mM Ca^{2+} (see Experimental Procedures). The positions of the initial single-stranded substrate and the duplex product are shown. The filled triangle represents labeled single-strand nucleic acid alone. (b) Graph of CSB dependent DNA/DNA strand pairing time course kinetics. (c) DNA/RNA strand pairing time course kinetics. In panels b and c, shown are the annealing reactions with and without CSB (see inset). Reactions were carried out with 450 fmol CSB protein with 100 fmol labeled and 100 fmol unlabeled oligonucleotides for the time indicated. Average values are plotted with standard deviations of at least 3 independent experiments.

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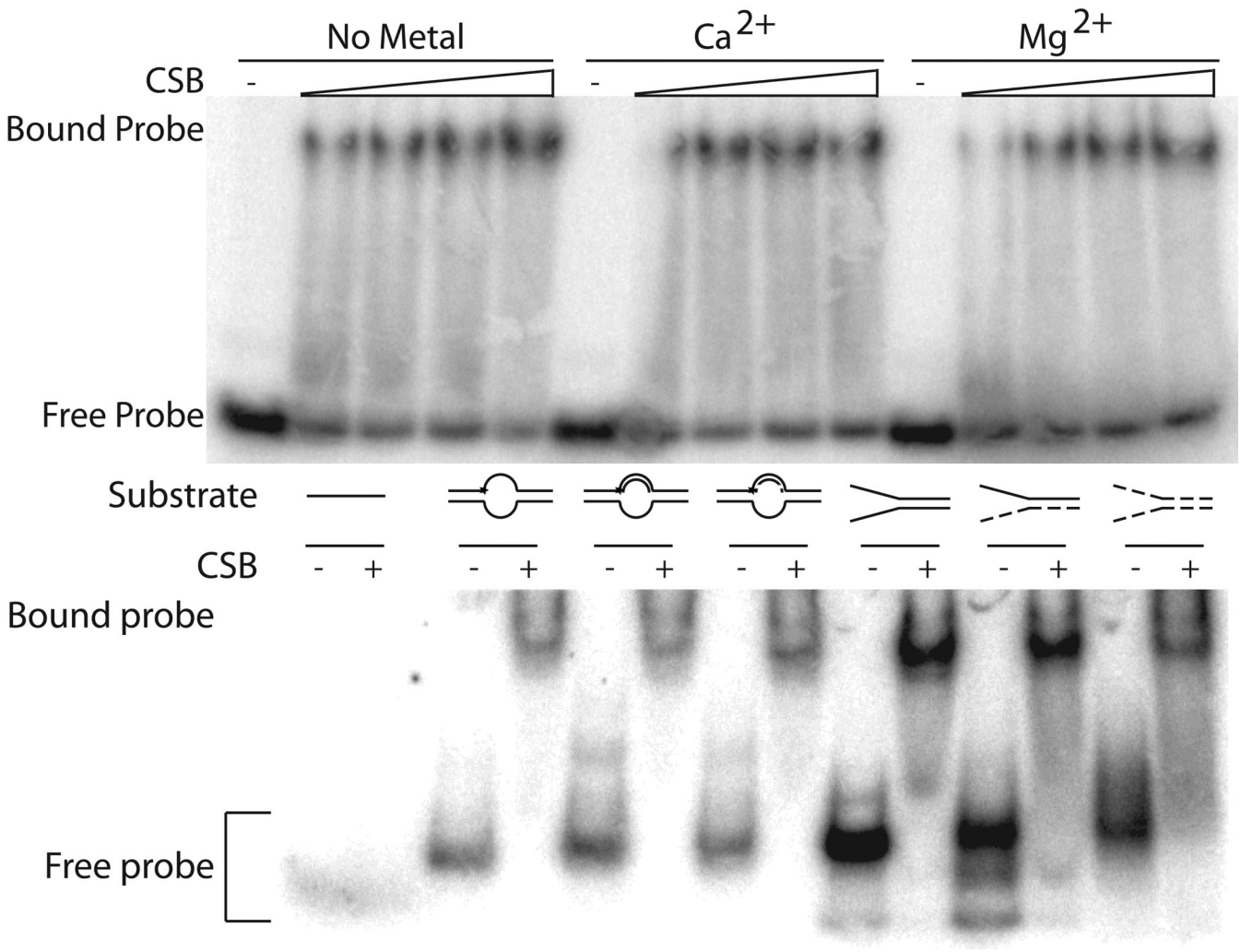

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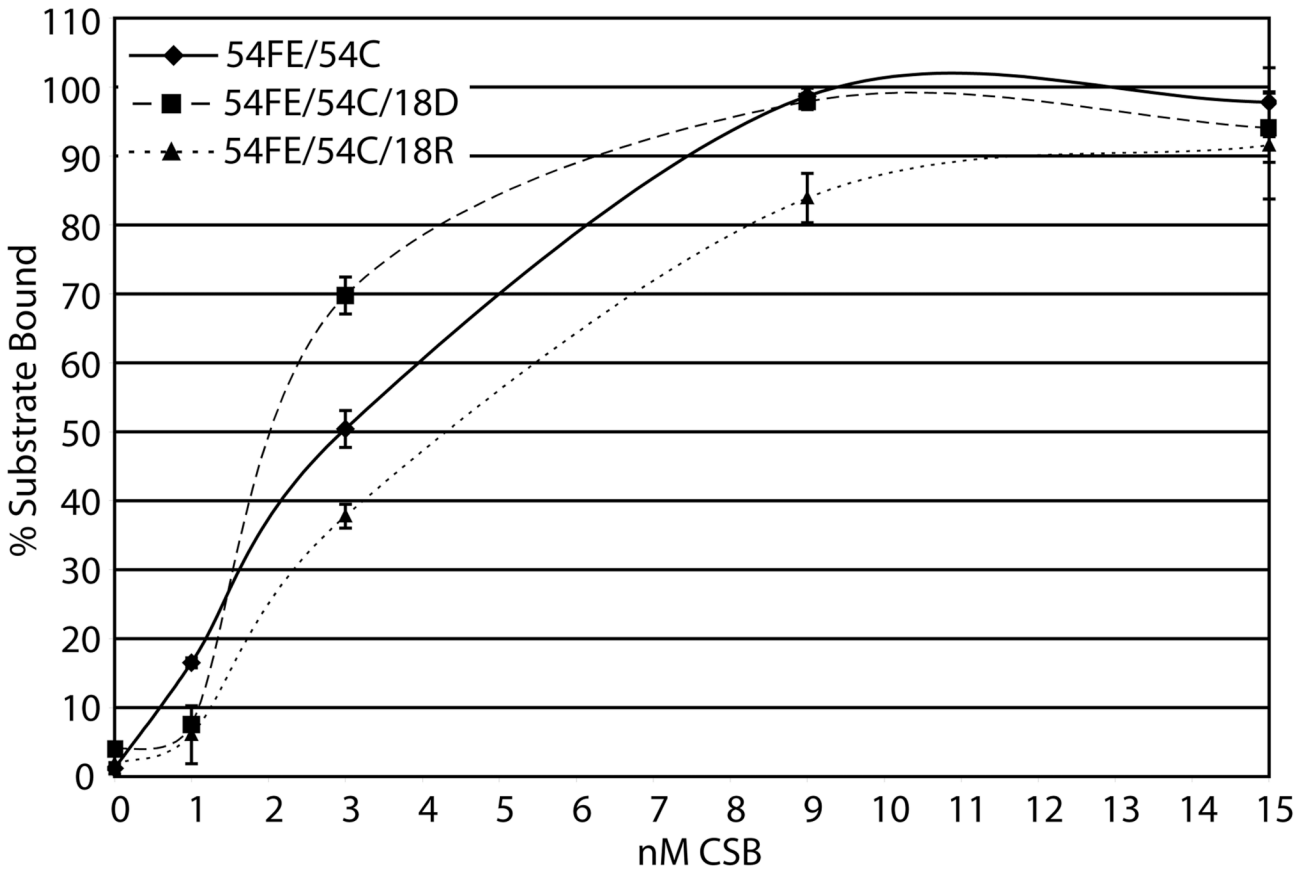

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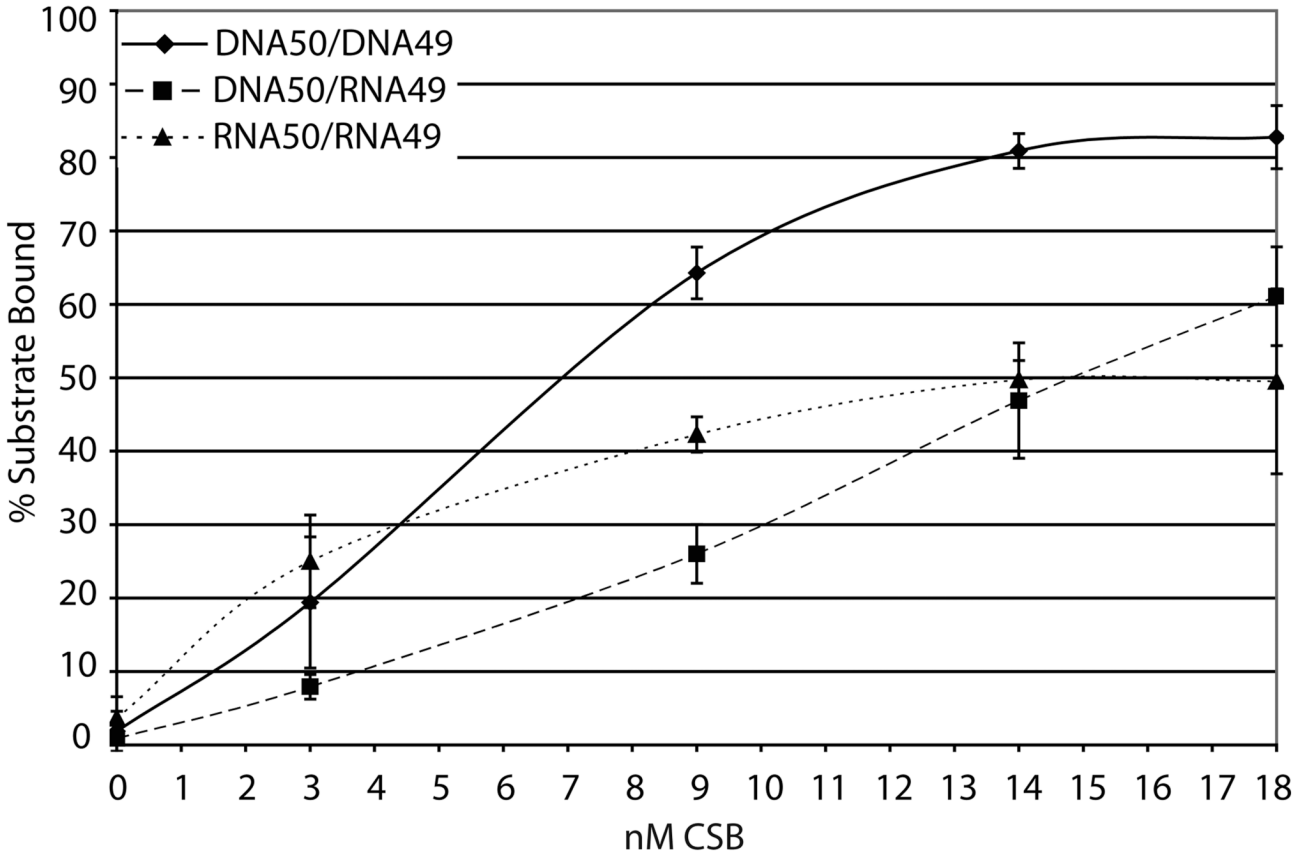

Fig. 4. Nucleic acid binding by CSB

(a) Mg^{2+} and Ca^{2+} have little effect on CSB dsDNA binding. Reactions were carried out with 50 fmol labeled duplex 34G/34C DNA (probe) and 150 to 500 fmol CSB in the conditions indicated (see Experimental Procedures). Shown is a representative native polyacrylamide gel. (b) CSB binds to a wide variety of alternate model nucleic acid substrates. Substrate depictions are displayed on top: from left to right, 19T, 54FE/54C, 54FE/54C/18D, 54FE/54C/18R, DNA50/DNA49, DNA50/RNA49, and RNA50/RNA49. Solid lines represent DNA and dashed lines represent RNA. Reactions with (+) or without (−) CSB are shown. Note: free probe migrates differently depending on nucleic acid substrate. (c) Graph of CSB bubble substrate and pseudo-triplex nucleic acid binding. (d) Graph of CSB forked duplex nucleic acid substrate binding. Saturating binding of DNA50/RNA49 was observed at 25 nM CSB concentration with 67.5 ± 3.6% substrate binding (data not shown). Average values are plotted with standard deviations of at least 3 independent experiments.

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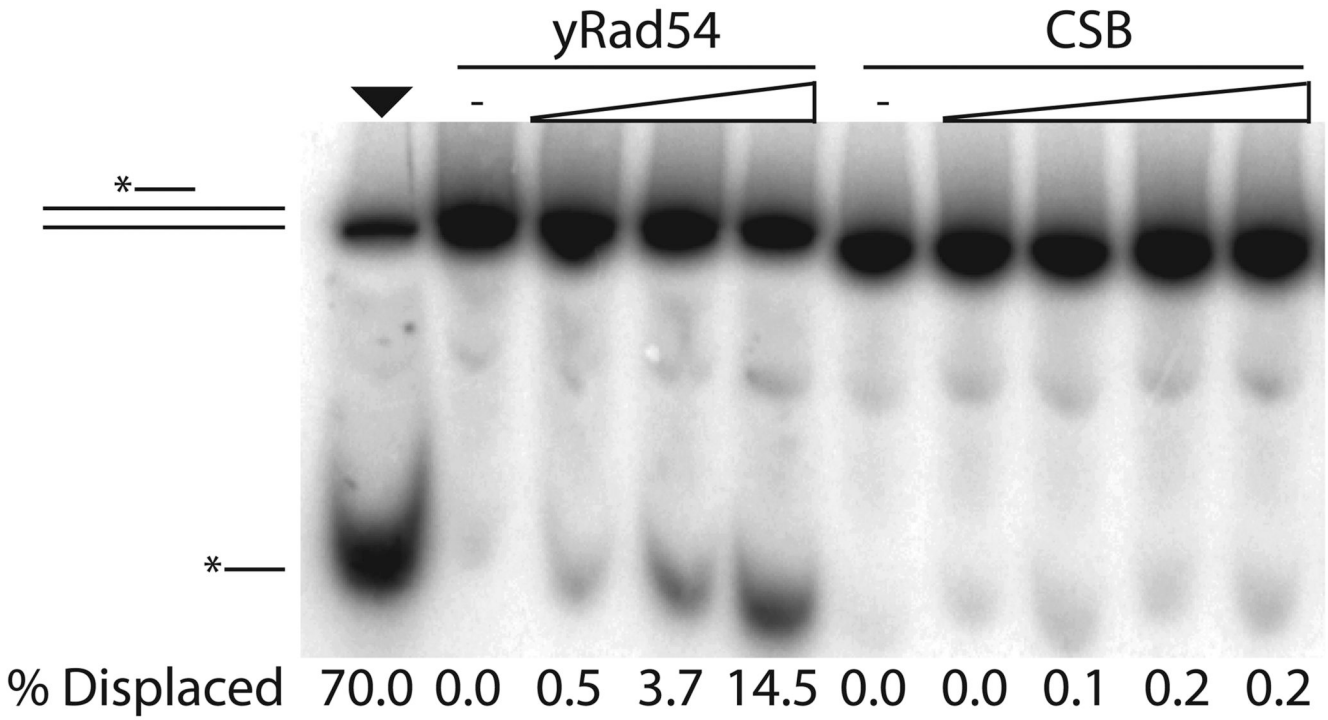

Fig. 5. CSB is unable to use ATP hydrolysis for dsDNA translocation

Representative gel of triplex displacement assay with yRad54 or CSB in the presence of 4 mM Mg^{2+}. Triplex displacement by increasing concentrations of yRad54 (180 fmol to 1.8 pmol) is shown as a control (left). Reactions with increasing CSB (60 fmol to 1.8 pmol) were performed as described in Experimental Procedures. % triplex strand displacement is indicated below each lane.

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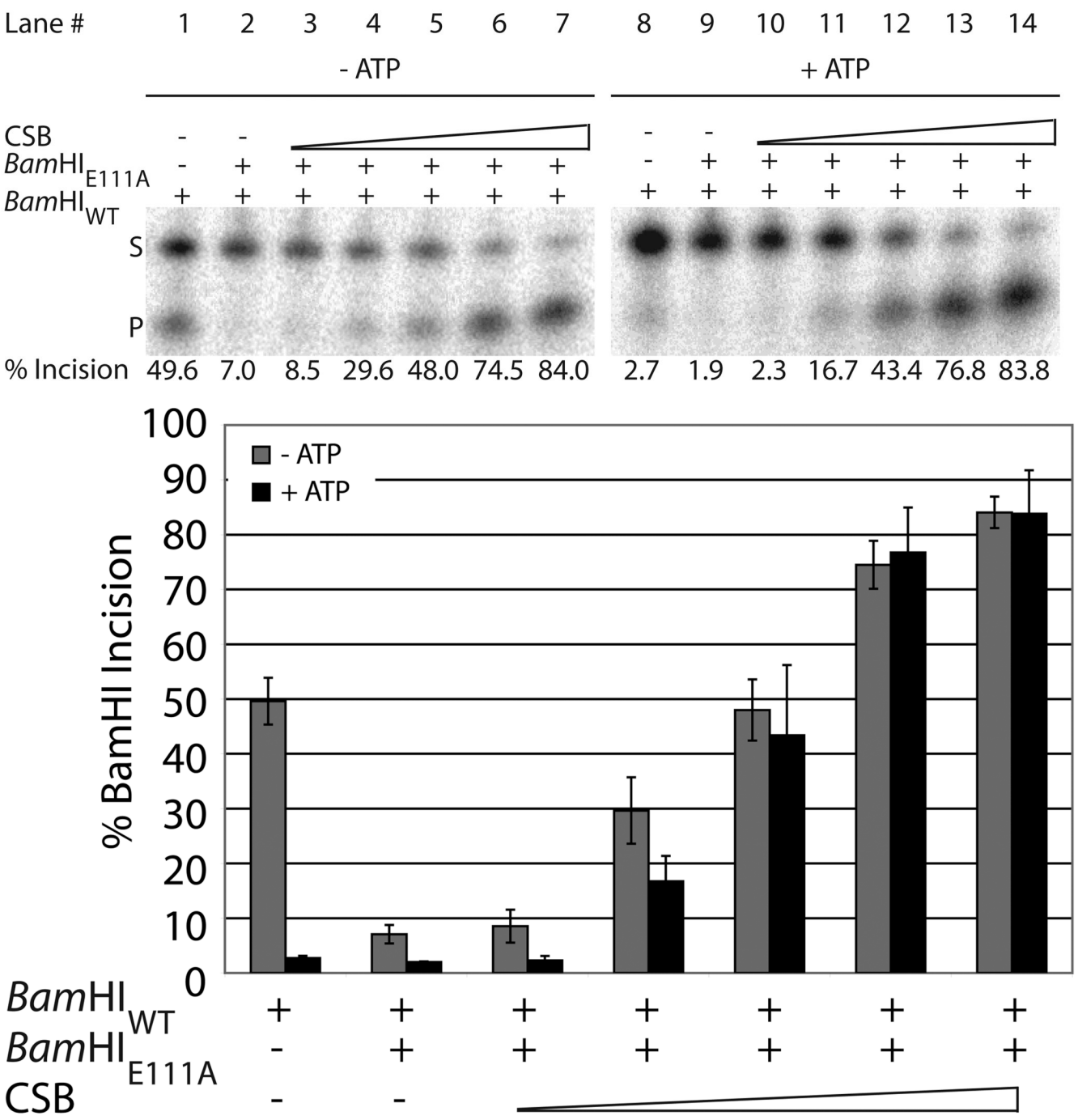

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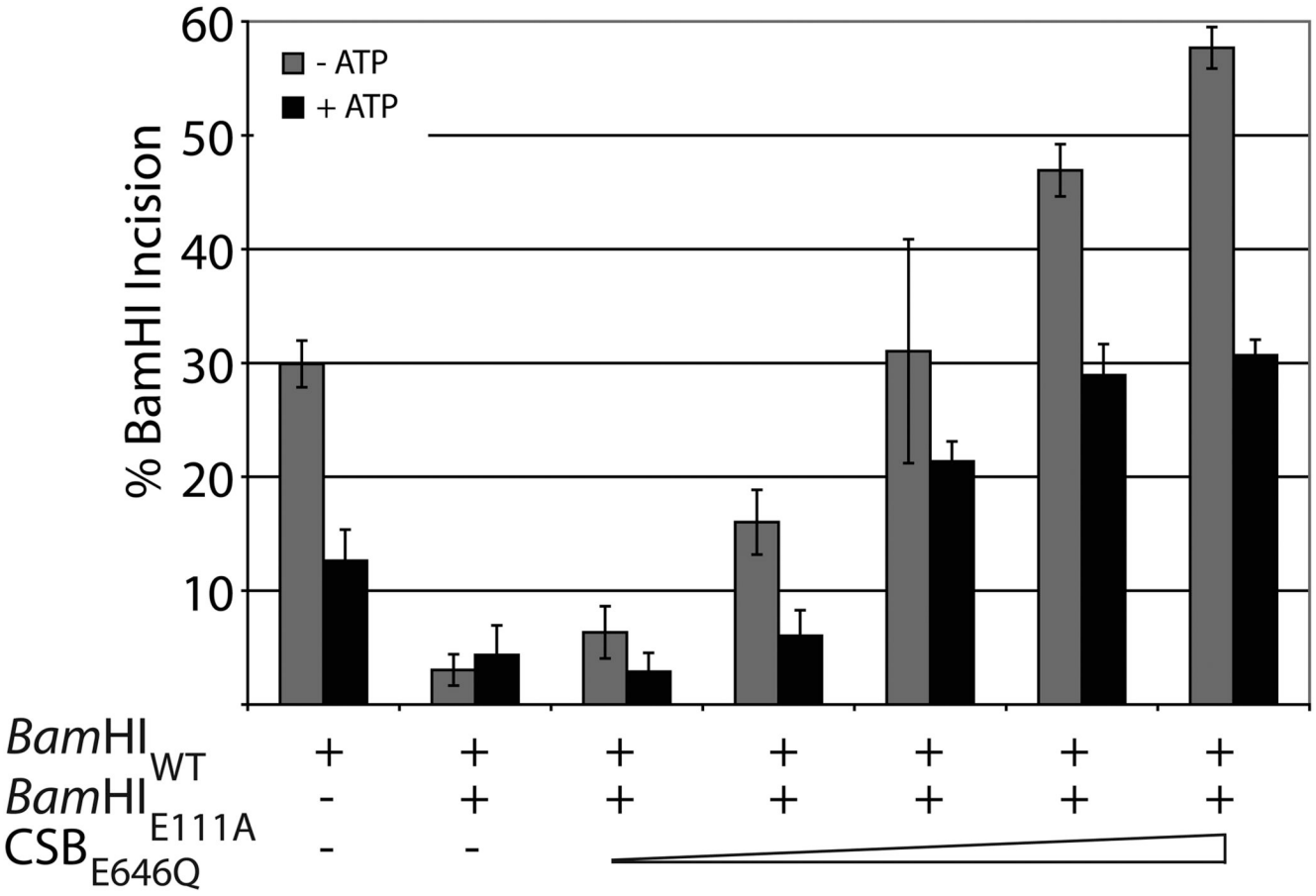

Fig. 6. CSB is able to displace or rearrange a pre-bound protein:dsDNA complex in an ATP independent manner

(a) Representative gels of CSB concentration-dependent protein displacement/rearrangement monitored by *Bam*HI digestion (Left panel, 0 mM ATP; Right panel, 2.5 mM ATP). See reaction condition details in Experimental Procedures. Shown above are respective lane numbers. Shown below is the average percent *Bam*HI_{WT} incision, following pre-binding of *Bam*HI_{E111A}, calculated from at least 3 independent experiments. (b) Graph quantitating percent *Bam*HI_{WT} incision in the presence of *Bam*HI_{E111A} and increasing concentrations of CSB (Grey, 0 mM ATP; Black, 2.5 mM ATP). (c) Graph quantitating percent *Bam*HI_{WT} incision in the presence of *Bam*HI_{E111A} and increasing concentrations of CSB_{E646Q} (Grey, 0 mM ATP; Black, 2.5 mM ATP). Average values are plotted with standard deviations of at least 3 independent experiments. Note inhibition of *Bam*HI_{WT} activity upon ATP addition alone.

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Table 1

Oligonucleotides used in this study

Name	Sequence 5'-3'	Reference
19T	TTTTTTTTTTTTTTTTTT	This work
34G	GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG	62
34C	CTGCAGCTGATGCGCCGTACGGATCCCCGGGTAC	62
54FE	CCGCTGCCGCTGAATTGCFCCCTCGATCTAGGTCGATGATCCTAAGCATAAGCA	63
54C	TGCTTATGCTTAGGATCAAGCTGGATCAAGCTCCCAGCAATTCAGCGGCAGCGG	63
18D	TCGACCTAGATCGAGGGT	63
18R	rUrCrGrArCrCrUrArGrArUrCrGrArGrGrGrU	63
DNA50	GGGACGCGTCGGCCTGGCACGTCGGCCGCTGCGGCCAGGCACCCGATGGC	61
DNA49	TTTGTTTGTTTGTTTGTTTGTTTGCCGACGTGCCAGGCCGACGCGTCCC rGrGrGrArCrGrCrGrUrCrGrGrCrCrUrGrGrCrArCrGrUrCrGrGrCrCrGrCrUrGrCrGrGrCrCr	61
RNA50	ArGrGrCrArCrCrCrGrArUrGrGrC rUrUrUrGrUrUrUrGrUrUrUrGrUrUrUrGrUrUrUrGrUrUrUrGrCrCrGrArCrGrUrGrCrCrArG	61
RNA49	rGrCrCrGrArCrGrCrGrUrCrCrC	61
DNA25F	CCGACGTGCCAGGCCGACGCGTCCC	61
DNA25	GCCATCGGGTGCCTGGCCGCAGCGG	61
RNA25F	rCrCrGrArCrGrUrGrCrCrArGrGrCrCrGrArCrGrCrGrUrCrCrC	61
RNA25	rGrCrCrArUrCrGrGrGrUrGrCrCrUrGrGrCrCrGrCrArGrCrGrG GGGACGCGTCGGCCTGGCACGTCGGrCrCrGrArCrGrUrGrCrCrArGrGrCrCrGrArCrGr	61
D25R25	CrGrUrCrCrC rUrUrUrGrUrUrUrGrUrUrUrGrUrUrUrGrUrUrUrGrUrUrUrGCCGACGTGCCAGGCCGA	61
R24D25	CGCGTCCC rGrCrCrArUrCrGrGrGrUrGrCrCrUrGrGrCrCrGrCrArGrCrGrGCCGCTGCGGCCAGGCAC	61
R25D25	CCGATGGC	61
(TC)20	TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC	52
42BamHI	CCGCTGAATTGCACCGGATCCCTAGGTCGATGATCCTAAGCA	This work
42Comp	TGCTTAGGATCATCGACCTAGGGATCCGGTGCAATTCAGCGG	This work