The *Bacillus subtilis* AddAB Helicase/Nuclease is Regulated by its Cognate Chi Sequence *in Vitro*

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The AddAB enzyme is important to homologous DNA recombination in *Bacillus subtilis*, where it is thought to be the functional counterpart of the RecBCD enzyme of *Escherichia coli*. *In vivo*, AddAB responds to a specific five-nucleotide sequence (5'-AGCGG-3' or its complement) in a manner analogous to the response of the RecBCD enzyme to interaction with \( \chi \) sequences. Here, we show that purified AddAB enzyme is able to load at a double-stranded DNA end and is both a DNA helicase and nuclease, whose combined action results in the degradation of both strands of the DNA duplex. During translocation, recognition of the properly oriented sequence 5'-AGCGG-3' causes attenuation of the AddAB enzyme nuclease activity that is responsible for degradation of the strand 3'-terminal at the entry site. Therefore, we conclude that 5'-AGCGG-3' is the *B. subtilis* Chi site and it is hereafter referred to as \( \chi_{Bs} \). After encountering \( \chi_{Bs} \) both the degradation of the 5'-terminal strand and the helicase activity persist. Thus, processing of a double-stranded DNA end by the AddAB enzyme produces a duplex DNA molecule with a protruding 3'-terminated single-stranded tail, a universal intermediate of the recombination process.

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Introduction

The repair of genotoxic lesions is a central aspect of DNA metabolism in living cells, and many complex enzymatic processes are devoted to maintaining the integrity of the genome. Homologous recombination is one important pathway by which potentially lethal lesions, such as double-stranded DNA breaks, can be repaired. These lesions arise frequently during normal cell growth if DNA replication is impeded (Michel *et al*., 1997) or, in eukaryotes, at the onset of meiosis (Baudat & Nicolas, 1997; Dernburg *et al*., 1998; McKim & Hayashi-Hagihara, 1998; Sun *et al*., 1989). In *Escherichia coli*, double-stranded DNA (dsDNA) breaks are repaired by homologous recombination between the damaged DNA and an intact copy of the chromosome. Numerous genetic and biochemical data have shown that the RecBCD enzyme is critical for the initiation of this repair pathway (for a review, see Smith, 1988; Kowalczykowski *et al*., 1994). RecBCD enzyme will bind to dsDNA ends and proceed to unwind the DNA while extensively degrading the strand terminating 3' at the entry site (referred to as the “top-strand”; see Figure 1) (Dixon & Kowalczykowski, 1993). This destructive mode, however, is switched to a recombinogenic mode after the enzyme recognizes a properly oriented \( \chi \) sequence. The \( \chi \) octamer, 5'-GCTGGTGG-3', initially defined as a recBC-dependent recombination hotspot (Lam *et al*., 1974; Smith *et al*., 1981; Stahl *et al*., 1975), elicits a number of biochemical changes in RecBCD enzyme’s behavior: the enzyme briefly pauses at \( \chi \) before unwinding resumes, at which point the top-strand nuclease is down-regulated, and a weaker nuclease activity is activated on the opposite strand (Anderson & Kowalczykowski, 1997a; Dixon & Kowalczykowski, 1991, 1993). These modifications produce a processed dsDNA with a 3’ single-stranded DNA (ssDNA) tail terminating at \( \chi \) that can be bound by the RecA protein to initiate the homologous pairing phase of genetic recombination. The formation of a RecA filament on ssDNA
is a crucial step in this initiation reaction, and the χ-activated RecBCD enzyme is directly responsible for coordinating the loading of the RecA protein onto χ-containing ssDNA. This ensures maximal participation of this χ-containing ssDNA in the process of genetic recombination (Anderson & Kowalczykowski, 1997b).

The interaction between the *E. coli* RecBCD enzyme and χ has been extensively studied, and it serves as a model for understanding how homologous DNA recombination is initiated in bacteria. However, until very recently, evidence for conservation of an interaction between a helicase/exonuclease and a χ-like regulatory sequence as a means to initiate homologous recombination was strikingly sparse. RecBCD homologs have been found in several enterobacteria as well as in other, more distantly related bacteria, such as *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Neisseria meningitidis* and *Mycobacterium tuberculosis*. However, RecBCD-χ interactions, as defined by the production of a diagnostic χ-specific fragment by the *E. coli* enzyme, seem to be conserved only in enteric bacteria that are closely related to *E. coli* (McKittrick & Smith, 1989). This suggests that either the RecBCD-χ interaction is restricted to a small group of microbes or that sequences other than the canonical χ sequence might be used in different organisms. This latter view is supported by the recent identification of specific short DNA sequences (5-8 bp) that protect dsDNA from exonuclease degradation in the bacteria *Lactococcus lactis* (Biswas et al., 1995), *H. influenzae* (Source et al., 1998) and *Bacillus subtilis* (Chédin et al., 1998). In all cases, those putative χ sequences are believed to interact with an enzyme possessing both helicase and exonuclease activities. In *H. influenzae*, this enzyme is a clear homolog of RecBCD. However, for the two Gram-positive bacteria *L. lactis* and *B. subtilis*, the corresponding enzymes seem to belong to a novel class of enzymes that are functionally, but not structurally, homologous to the RecBCD class of enzymes.

In *B. subtilis*, the model organism for Gram-positive bacteria, the counterpart of the RecBCD enzyme is thought to be the AddAB enzyme. Genetically, mutations in either *addA* or *addB* genes result in reduced cell viability, increased sensitivity to DNA-damaging agents, and reduced levels of DNA recombination in certain strains (Kooistra et al., 1988; Alonso et al., 1988), which are phenotypic characteristics of *recB* or *recC* mutations in *E. coli*. In addition, expression of AddAB enzyme in *E. coli* complements RecBC− phenotypes (Kooistra et al., 1993) and, in *vitro*, the AddAB enzyme possesses exonuclease and helicase activities (Haijema et al., 1996a). From previous *in vivo* work, we showed that a specific five-nucleotide sequence, 5’-AGCGG-3’ allowed formation of a high molecular weight form of plasmid DNA, presumably by protecting this DNA from degradation by AddAB (Chédin et al., 1998). Although hotspot activity was not demonstrated for this sequence, it was proposed that this pentamer was the *B. subtilis* analog of the well-described Chi site of *E. coli* (χEc).

Below, we establish that this supposition was correct, and 5’-AGCGG-3’ will therefore be referred to as χBs.

Despite these parallels with the *E. coli* system, the biochemical consequences of the interaction...
between AddAB enzyme and its putative \( \chi_{Bs} \) sequence are unclear. Although the *in vitro* observations suggest that the enzyme loses its ability to degrade double-stranded DNA after \( \chi_{Bs} \) is recognized (Chédin *et al.*, 1998), the exact nature of the changes elicited by this interaction could not be inferred. Here, we show that the purified AddAB enzyme is a potent helicase and exonuclease which, in contrast to the RecBCD enzyme, degrades both strands of the DNA duplex almost symmetrically. When a properly oriented \( \chi_{Bs} \) sequence is present, exonucleolytic degradation of the 3'-terminated strand at the entry point ceases, while degradation of the 5'-terminated strand continues. This alteration in the behavior of the enzyme results in the production of a dsDNA molecule carrying a long 3'-overhang, an intermediate that is universal for the initiation of DNA recombination. Our data also imply that the interaction between an exonuclease/helicase and a regulatory sequence is an evolutionarily conserved means to initiate DNA recombination, and that the AddAB enzyme defines a novel class of enzymes that are functionally, but not structurally, homologous to the RecBCD class of enzymes.

**Results**

**The AddAB enzyme degrades both strands of dsDNA during unwinding**

Before investigating the interaction between the AddAB enzyme and its putative Chi sequence, we wanted to examine the processing of DNA that is devoid of Chi sites (\( \chi_{0} \)). To this end, plasmid pADGF0 was linearized using ClaI and 5'-end labeled with \( [\gamma^{32}\text{P}]\text{ATP} \). Figure 2 shows a time course of processing of this substrate by the AddAB enzyme at two different magnesium ion concentrations (Mg(OAc)\(_2\)). As expected, the initial dsDNA is rapidly processed by the enzyme and is converted to full-length ssDNA and a variety of degradation products. The amount of full-length ssDNA, however, varied strongly with the concentration of magnesium ions. At 0.5 mM Mg(OAc)\(_2\), about 80% of the unwound DNA was full-length ssDNA after five minutes, whereas at 2 mM Mg(OAc)\(_2\), only 10% of the unwound DNA was full-length. Similar results were obtained with dsDNA that was linearized with other restriction endonucleases or with dsDNA that was 3'-end labeled (data not shown). These results show that, at low magnesium ion concentrations, the nuclease activity of AddAB enzyme is low, so that processing of linear dsDNA under these conditions mostly produces fully unwound ssDNA. However, at higher magnesium ion concentrations, AddAB both unwinds and degrades dsDNA, as evidenced by the decrease in production of full-length ssDNA. This behavior is similar to that of the RecBCD enzyme (Dixon & Kowalczykowski, 1993, 1995; Taylor & Smith, 1995), whereby the free magnesium ion concentration determines the overall level of nuclease activity, but has little effect on helicase activity (Roman & Kowalczykowski, 1989b).

When dsDNA is processed by the RecBCD enzyme, most of the full-length ssDNA produced originates from the 5'-terminated strand at the entry point of the enzyme (i.e. “bottom-strand”; see Figure 1). This behavior is due to the intrinsic asymmetry of DNA degradation by the RecBCD enzyme: the top-strand of the duplex is degraded more vigorously than the bottom-strand (Dixon & Kowalczykowski, 1993). To determine if exonucleolytic degradation by the AddAB enzyme is also asymmetric, we designed DNA substrates that would restrict entry of the AddAB enzyme to only...
one end of the linear dsDNA. For this, we generated dsDNA with 5′-ssDNA overhangs (approximately 50 nt long) using exonuclease III, and then treated the DNA with a restriction enzyme to create a nearly blunt end at one end of the molecule to permit entry of the enzyme. As with RecBCD enzyme, overhangs block the entry of AddAB enzyme to the dsDNA at all magnesium ion concentrations tested (data not shown and see Figure 5 below). Either the top-strand or the bottom-strand was labeled as described in Materials and Methods, and the action of AddAB enzyme was assayed at different magnesium ion concentrations, since this parameter controls the overall level of nuclease activity. Any strand asymmetry in the degradation process would be manifest as a differential loss of one of the strands during unwinding. Figure 3 shows that, as the Mg(OAc)₂ concentration increases, the amount of remaining full-length ssDNA strongly decreases for both top-strand and bottom-strand labeled substrates. Moreover, it is apparent that both strands of the duplex are degraded with similar efficiencies, with a sharp transition occurring around 1 mM Mg(OAc)₂. Although the bottom-strand is slightly more sensitive to degradation than the top-strand for this particular substrate, we conclude that, unlike the RecBCD enzyme, which shows a strong strand asymmetry in the same assay (F.C. & S.C.K., unpublished data), the AddAB enzyme degrades both strands of the duplex nearly symmetrically.

Interaction with $\chi_{Bs}$ results in the production of a 5′-terminated $\chi_{Bs}$-specific fragment

When the RecBCD enzyme encounters a properly oriented $\chi$ site, the nuclease activity on the top-strand is attenuated, thus allowing for the preservation of ssDNA downstream of $\chi$ on the top-strand. With plasmid-size DNA substrates, a 5′-terminated, $\chi$-specific, top-strand, downstream fragment is produced (Figure 1). It was recently shown that the sequence 5′-AGCGG-3′, referred to as $\chi_{Bs}$, allows double-stranded DNA to resist exonucleolytic degradation by the AddAB enzyme in B. subtilis, thus suggesting that at least the nuclease activity of the complex is regulated (Chédin et al., 1998). To investigate the effect of $\chi_{Bs}$ on the processing of dsDNA by the AddAB enzyme, two different plasmids were used. pADG6406-1 (Chédin et al., 1998) carries a single $\chi_{Bs}$ sequence, designated as $\chi_N$. Its derivative, pADGF1, carries two additional tandem $\chi_{Bs}$ sequences (collectively designated as $\chi_F$) present in the opposite orientation (Figure 4(a)). When these two DNA molecules are linearized and labeled at their 5′ ends, processing by AddAB enzyme produces novel DNA bands (Figure 4(b)). Native agarose gel electrophoresis, however, does not permit an accurate assessment of the number, size, or single-stranded nature of these new bands. For this reason, the same samples were analyzed by alkaline gel electrophoresis (Figure 4(c)). For pADG6406-1, only one 5′-end labeled fragment is produced in response to $\chi_N$. This fragment migrates as ssDNA with an approximate size of 1900 nt. For pADGF1, a second, $\chi_F$-specific band is produced, in addition to the $\chi_N$-specific fragment. The size of this fragment, ~1700 nt, is expected due to the position of $\chi_F$ and the fact that $\chi_F$ has an opposite orientation compared to $\chi_N$. Although the two $\chi_{Bs}$-specific fragments observed with pADGF1 could not be resolved on the native gel, they can clearly be distinguished on the alkaline gel. Under the conditions used here, the yield of $\chi_F$-specific fragments produced relative to the amount of ssDNA processed was close to 40%. The yield of $\chi_F$-specific fragments was higher than with $\chi_N$, in agreement with $\chi_F$ being composed of two tandem $\chi_{Bs}$ sites. When other restriction endonucleases were used to linearize either pADG6406-1 or pADGF1, the size of the $\chi_{Bs}$-specific fragments shifted accordingly (data not shown).

Although in vivo data show that the $\chi_{Bs}$ sequence is active in only one orientation (Chédin et al., 1998), the exact polarity of recognition by AddAB was not known. Because of this uncertainty and because the AddAB enzyme could enter these DNA substrates from either end, we could not
attribute the observed $\chi_{Bs}$-specific fragments to the portion of DNA that was processed before or after $\chi_{Bs}$. However, if the analogy to RecBCD enzyme applies, and because $\chi_{Bs}$ protects linear dsDNA from degradation by AddAB enzyme in vivo (Chédin et al., 1998), we expect that the observed $\chi_{Bs}$-specific fragments correspond to a DNA fragment located downstream of $\chi_{Bs}$ with respect to the enzyme’s entry point.

The active orientation of $\chi_{Bs}$ is 5'-AGCGG-3'

To unambiguously determine in which orientation $\chi_{Bs}$ is recognized by the translocating AddAB enzyme, we used the strategy shown in Figure 3 to limit entry of AddAB to one end of the DNA substrate. Plasmid pADG6406-1 was linearized using Clal, treated with exonuclease III as described in Materials and Methods, and 5'-end labeled. Figure 5(b) (left panel) shows that a DNA molecule carrying two recessed ends is almost completely resistant to processing by the AddAB enzyme (the slight amount of full length ssDNA and $\chi_{Bs}$-specific fragment produced at the later time points probably results from slow degradation of the ssDNA tails by the ssDNA exonuclease activity of AddAB enzyme or from incomplete tailing of a small fraction of the molecules). In contrast, substrates with one entry site (Figure 5(b), middle and right panels) are rapidly processed. A $\chi_{Bs}$-specific fragment is seen only for the DNA substrate that allows entry of the AddAB enzyme from the right side (Spel-treated). In this orientation, the 3'-terminated strand at the entry point (top-strand) carries the sequence 5'-AGCGG-3'. Hence, for recognition to occur, the AddAB enzyme must approach the sequence 5'-AGCGG-3' from the 3' side. According to the E. coli nomenclature, 5'-AGCGG-3' is therefore the equivalent of the E. coli $\chi$ site. The size of the $\chi_{Bs}$-specific fragment produced in this experiment was confirmed using alkaline gel electrophoresis, demonstrating that it corresponds, as expected, to the top-strand downstream $\chi_{Bs}$-specific fragment (data not shown). Interaction between AddAB enzyme and its cognate $\chi_{Bs}$ sequence therefore produces a top-strand ssDNA fragment downstream of $\chi_{Bs}$.

Interaction with $\chi_{Bs}$ causes degradation to attenuate at the last base on the 3' side of $\chi_{Bs}$

To map precisely the 3'-end of the $\chi_{Bs}$-specific fragment, we produced DNA substrates carrying
about 100 bp from the 5’-end (Figure 6(a)) and analyzed the products of a reaction with AddAB enzyme using denaturing 8% polyacrylamide gels. The mobility of the various products was then compared to a sequence ladder. As can be seen from Figure 6(b), the χ_0 control DNA displays a nonspecific pattern of ssDNA fragments (although the banding pattern suggests that the enzyme preferentially cuts DNA before A residues and, to a lesser extent, T residues). For the χ_F-containing DNA, one major band is observed for each χ_F site contained within χ_Bs. Each χ_Bs-specific fragment terminates at the last base to the 3’-side of χ_Bs (Figure 6(b)). Since magnesium ion concentration strongly influences the location of the 3’ extremity of the χ-containing ssDNA fragment produced by RecBCD enzyme (Taylor & Smith, 1995), we repeated this experiment over a range of Mg(OAc)_2 concentrations (1 mM to 10 mM) and found that the location of the 3’-end of the χ_Bs-specific fragment was invariant for AddAB (data not shown).

To examine the effect of DNA sequence context on the interaction of χ_Bs with AddAB enzyme, other DNA substrates were generated (see Materials and Methods; Figure 6(c)). In all cases, processing by AddAB enzyme produced a χ_Bs-specific fragment that terminated one nucleotide to the 3’-side of χ_Bs. In some instances, however, the AddAB enzyme generated DNA fragments whose 3’ ends were located in a G-rich stretch of DNA (Figure 6(c), FSK1516). Interestingly, these G-rich stretches contained a χ_Bs-like sequence with four matches out of five positions (underlined with broken line), suggesting the AddAB enzyme retained some ability to recognize χ_Bs variants, as was described for the RecBCD enzyme (Cheng & Smith, 1984, 1987).

A second χ_Bs-specific fragment is observed with 3’-end labeled substrates

In principle, four potential χ_Bs-specific fragments can be produced upon interaction of the AddAB enzyme with its cognate Chi sequence. As shown in Figure 1, two of these putative fragments have the original 5’ terminus (top-strand downstream and bottom-strand upstream), while the remaining two have the original 3’ terminus (top-strand upstream and bottom-strand downstream). So far, we showed that interaction between the AddAB enzyme and χ_Bs results in the production of a single 5’-terminated, top-strand downstream χ_Bs-specific fragment (Figure 4). To investigate whether additional χ_Bs-specific fragments are produced, we compared the reaction products obtained upon processing of χ_Bs-containing substrates that were either 5’-end or 3’-end labeled (Figure 7). As
expected, a top-strand downstream χBs-specific fragment is observed when the substrates are 5'-end labeled. However, χBs-specific fragments are also detected with 3'-end labeled substrates. With pADG6406-1, one novel fragment is produced. Its size (~1900 nt), as judged from alkaline gel electrophoresis (data not shown), indicates that this fragment corresponds to the top-strand upstream, χBs-specific fragment. With pADGF1, a second novel fragment is detected, in addition to the one described. Its size (~600 nt) is also consistent with it corresponding to the top-strand upstream fragment. Note, however, that the yield of the shorter 600 nt fragment is greater than that of the longer 1900 nt fragment. This result is expected if the top-strand is subjected to infrequent non-specific cleavages, such that a small fragment has a high probability of escaping cleavage, while a longer fragment is more likely to be cleaved (see Discussion). We therefore conclude that, under the conditions used here, the AddAB enzyme does not fully degrade the DNA upstream of χBs and that interaction with this site results in a high probability of cleavage on the top-strand at χBs.

The yield of the upstream χBs-specific fragment depends strongly upon the level of AddAB enzyme nuclease activity

To further characterize the conditions under which this 3'-terminated, upstream χBs-specific fragment is produced, we examined the effect of magnesium ion concentration on the yield of this fragment relative to the 5'-terminated downstream χBs-specific fragment. Figure 8 shows that the downstream χBs-specific fragment is detected with a similar yield over a wide range of magnesium ion concentrations (0.5 to 15 mM, Figure 8 and data not shown). In contrast, the upstream χBs-specific fragment behaves similarly to the full length ssDNA and is no longer detected when the magnesium concentration is raised above 2-3 mM (Figure 8 and data not shown). From these results, we conclude that under conditions which promote higher nuclease activity, the AddAB enzyme vigorously degrades the top-strand before encountering a correctly oriented χBs site, after which its nuclease activity on the top-strand is turned off, thus producing a ssDNA fragment downstream of χBs. However, at lower nuclease conditions, the probability of a non-specific cleavage before χBs is low, leaving the top-strand upstream of χBs intact. In agreement, we observed that the yield of the top-strand upstream fragment was inversely proportional to the length of DNA before χBs, suggesting that longer DNAs have an increased probability of receiving at least an endonucleolytic cut even under low nuclease conditions (Figures 7, 8 and data not shown). Similar magnesium ion concentration-dependent appearance of the top-strand upstream χ-specific fragment was observed for the RecBCD enzyme (Dixon & Kowalczykowski, 1995). The interaction with χBs is presumably associated with a pause, resulting in a high probability of cleavage at this site, as suggested for the RecBCD enzyme (Dixon & Kowalczykowski, 1995).

Discussion

Here, we show that the B. subtilis AddAB enzyme interacts with the five-nucleotide sequence, 5'-AGCGG-3', which we define as χBs. We further demonstrate that this interaction results in the regulation of AddAB nuclease activities in a way that allows for the formation of a DNA substrate suitable for the initiation of homologous recombination.

B. subtilis AddAB defines a novel class of enzymes that are functional, yet distinct, homologs of RecBCD enzymes

Evidence gathered from the complete genome sequences of various organisms indicates that the E. coli RecBCD enzyme is the prototypic member of a large family of enzymes that are found throughout the eubacteria. Close homologs of RecBCD enzyme are found in genera such as Spirochaetales, Green sulfur bacteria, Chlamydiales, and Proteobacteria. Although Chi recognition by RecBCD-type enzymes has only been demonstrated for E. coli and H. influenzae, it is reasonable to assume that this interaction is conserved in these other organisms. Our results show that the AddAB enzyme from B. subtilis is a functional homolog of the RecBCD enzyme. However, the AddAB enzyme possesses only two subunits and, while the AddA subunit shows homology to the RecB subunit, the AddB subunit has only limited homology to the RecC subunit and no homology to the RecD subunit. It therefore appears that AddAB is not strictly homologous to RecBCD. Rather, we propose that AddAB defines a novel class of enzymes.

Our proposal follows from the fact that homologs of AddAB have been discovered in several other bacteria. The RexAB enzyme of L. lactis is a two subunit complex showing extensive sequence similarity to AddAB. This enzyme was shown genetically to interact with a specific seven-nucleotide sequence (Biswas et al., 1995; el Karoui et al., 1998). Furthermore, an analysis of all publicly available eubacterial genomes permits identification of AddAB homologs in Clostridium acetobutylicum, Enterococcus faecalis, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus mutans and Streptococcus pneumoniae. All of those organisms belong to the Bacillus/Clostridium subdivision of the Gram-positive bacteria, which therefore seems to be a specialized “niche” where the AddAB-class of enzymes evolved.

Processing of DNA substrates devoid of χBs

We show here that the AddAB enzyme is both a powerful DNA helicase, able to unwind a linear
double-stranded DNA fragment of at least three kilobases, and a powerful exonuclease. Both activities require the hydrolysis of ATP as a source of energy and the presence of magnesium as a divalent cation. The intensity of the nuclease activity is strongly dependent on the concentration of magnesium ion (Figures 2, 3 and 8). This dependence on the magnesium ion concentration was
also observed for the RecBCD enzyme in *E. coli* (Dixon & Kowalczykowski, 1995). However, in contrast to the RecBCD enzyme, AddAB degrades both strands of the duplex symmetrically even under conditions where the RecBCD enzyme shows a marked preference for degrading the 3’-terminated strand at the entry point (Figure 2; Dixon & Kowalczykowski, 1993; F.C. & S.C.K., unpublished observations).

Recently, a domain required for all of RecBCD nucleolytic activities was identified (Yu et al., 1998b). This domain, present at the C terminus of the RecB subunit, is conserved at a similar position in the AddA subunit (Hajjema et al., 1996b). Interestingly, we were able to identify a second nuclease domain in the C-terminal portion of the AddB subunit by the virtue of its strong homology to the previously defined RecB nuclease domain and its counterpart in AddA. This domain is highly conserved among members of the AddAB family of enzymes (data not shown). A similar search, however, failed to reveal any additional nuclease domain in the RecB, RecC or RecD subunits of the *E. coli* enzyme. The AddAB enzyme therefore carries two putative nuclease domains, while RecBCD carries only one; this distinction could explain the ability of the AddAB enzyme to degrade both strands of dsDNA, where RecBCD enzyme degrades only one or the other.

**Interaction of AddAB enzyme with χ<sub>Bs</sub>**

From *in vivo* experiments, it was proposed that AddAB was able to interact with a specific five-nucleotide sequence, 5’-AGCGG-3’, its complement, or both, referred to as χ<sub>Bs</sub> (Chédin et al., 1998). Here, using purified components, we demonstrated that AddAB is indeed able to interact with, and to be regulated by, χ<sub>Bs</sub>. Furthermore, we defined the active orientation of χ<sub>Bs</sub> as the sequence 5’-AGCGG-3’, which must be approached by an AddAB enzyme travelling from the 3’ side of the DNA for recognition to occur (Figure 5). This observation represents the first biochemical analysis of a system other than the RecBCD system of *E. coli*, and provides direct support to the notion that the interaction between a DNA helicase/nuclease and a regulatory DNA sequence has been evolutionarily conserved. The outcome of this interaction depends on the initial strength of the nuclease activity. Under low nuclease conditions, interaction with χ<sub>Bs</sub> results in the production of two χ<sub>Bs</sub>-specific fragments derived from the cleavage of the top-strand at χ<sub>Bs</sub> (Figures 7 and 8). However, as the strength of the nucleolytic degradation is increased, the top-strand upstream fragment rapidly disappears while the top-strand downstream fragment remains protected (Figure 8). This indicates that interaction results in the attenuation of the exonuclease activity on the top-strand, a behavior which parallels that of the RecBCD enzyme (Dixon & Kowalczykowski, 1993). Under all conditions tested, no discrete fragment was recovered from the bottom-strand, which indicates that the enzyme resumes unwinding after χ<sub>Bs</sub> and that the 5’ to 3’ degradation of that strand is unaffected by χ<sub>Bs</sub>. Hence, interaction with χ<sub>Bs</sub> transforms the AddAB enzyme from a helicase which degrades both DNA strands to one with only 5’ to 3’ exonuclease.

Mapping of the χ<sub>Bs</sub>-specific nuclelease-attenuation sites to nucleotide resolution revealed that the AddAB enzyme predominantly stops degrading DNA one nucleotide to the 3’ side of χ<sub>Bs</sub> (Figure 6). Varying the sequence context in which χ<sub>Bs</sub> is located did not lead to a significant modification of the distribution of the cleavage sites (Figure 6(c)) and the pattern did not change over a wide range of conditions. This behavior is in contrast to that of the RecBCD enzyme, which shows an array of attenuation sites distributed from four to six nucleotides before χ at low nuclease conditions, to four nucleotides inside χ at high nuclease conditions (Taylor & Smith, 1995). This difference may suggest that the recognition of χ<sub>Bs</sub> by AddAB enzyme results in a longer pause than for the RecBCD enzyme, producing a more specific cleavage event, or alternatively, that the exonuclease domain of AddAB is more tightly tethered than the equivalent “swing” domain of the RecBCD enzyme (Yu et al., 1998a). In some instances, however, strong attenuation sites were detected in χ<sub>Bs</sub>-
like sequences, especially if those were embedded in stretches of G residues (Figure 6(c)).

The mechanism by which Chi sequence recognition is transformed into a change of the enzyme’s activity remains elusive. For the RecBCD enzyme, the changes resulting from the interaction with χ are thought to occur through the modification of the RecD subunit. One model hypothesizes that the RecD subunit is ejected from the complex, a proposal which is consistent with various in vivo observations (Koppen et al., 1995; Myers et al., 1995; Stahl et al., 1990; Thaler et al., 1988), whereas another simply proposes that the RecD subunit is modified in an as yet unknown way (Anderson et al., 1997; Churchill et al., 1999; Dixon et al., 1994). For the AddAB enzyme, however, physical ejection is impossible since the complex comprises two subunits, both of which are strictly essential to the enzyme’s function (Kooistra et al., 1993). Therefore, for AddAB, it is more likely that the interaction with χBs results in an as yet undefined conformational change.

Regulation by χBs allows the production of a universal recombination intermediate

As discussed above, interaction of the AddAB enzyme with χBs transforms AddAB from a double-stranded exonuclease to a 5’ to 3’ exonuclease. The net result of this modification is, therefore, the production of dsDNA with a 3’-ssDNA tail. Studies of the relative invasiveness of various types of ssDNA substrates in RecA protein-promoted reactions showed that 3’ ends are more invasive than 5’ ends (Konforti & Davis, 1987, 1990). Similarly, physical analysis of recombination intermediates showed that dsDNA with a protruding 3’-terminated ssDNA is produced during the initiation phase of recombination both in Bacteria and Eucarya (Anderson & Kowalczykowski, 1997a; Friedman-Ohana & Cohen, 1998; Sun et al., 1991; White & Haber, 1990). These DNA intermediates, therefore, are universally used to initiate homologous DNA recombination. In all cases, the processing of dsDNA is followed by the binding of a DNA strand exchange protein to the ssDNA intermediate. Interestingly, the translocating RecBCD enzyme also functions to load the RecA protein onto the downstream χ-containing ssDNA (Anderson & Kowalczykowski, 1997b). Whether this level of coordination between the AddAB enzyme and the RecA protein is observed in B. subtilis is now under investigation.

Materials and Methods

Purification of AddAB

Plasmid pWSK2978 (gift from J. Kooistra; Kooistra et al., 1993), carrying both the addA and addB genes under the control of the natural promoter for the operon, was introduced into E. coli strain AB1157 recB::Tn10 (gift from B. Michel; U zest et al., 1995). The protocol for purification was based on a previously published protocol
for purification of the RecBCD enzyme (Roman & Kowalczykowski, 1989a; Eggleston & Kowalczykowski, 1993). The presence of AddAB enzyme was followed using ATP-dependent exonuclease activity, as described below. AddAB enzyme concentration was determined using a molar extinction coefficient of $2.7 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ at 280 nm (derived from Mach et al., 1992) and a molecular mass of 275,531 Da as determined from its nucleotide sequence. The $A_{280}/A_{260}$ ratio was 1.35. The preparation’s purity appeared to be greater than 90% as judged from Coomassie-stained polyacrylamide gels. Two other bands could be detected and probably correspond to proteolytic degradation fragments of AddB. Because of this, the subunit ratio was found to be slightly in favor of AddA.

**ATP-dependent exonuclease assays**

The specific activity of the enzyme in nuclease units was determined as described by Eichler & Lehman (1977) using sonicated $^3\text{H}$-labeled *E. coli* chromosomal DNA. The reaction mixture (300 µl) consisted of 100 mM glycine-NaOH (pH 9.2), 50 mM MgCl$_2$, 0.1 mg/ml BSA, 0.67 mM DTT, 20 µM [H]DNA and 75 µM ATP, when present. Incubation was for 20 minutes at 37°C, and the reaction was stopped by addition of 15 µl of sonicated calf thymus DNA (2.5 mg/ml) and 300 µl of 15% TCA. After mixing, the tubes were placed on ice, and spun at 12,000 rpm for 20 minutes; 500 µl of the supernatant was added to 5 ml of scintillation cocktail, and then counted using a Beckman scintillation counter.

**DNA substrates**

Plasmid pADG6406-1 was described previously (Chédin et al., 1998). This 3 kb plasmid carries a $\chi_{\text{Bi}}$ sequence at position 1805. Plasmid pADGF1 is a derivative of pADG6406-1 containing two additional $\chi_{\text{Bi}}$ sequences cloned in tandem, in the opposite orientation, at the unique EcoRI site (the $\chi_{\text{Bi}}$-containing linker cloned here was described in Figure 2 of Chédin et al., 1998). Plasmid pADGF0 is a derivative of pADG6406-1 in which two targeted silent mutations were introduced in the natural $\chi_{\text{Bi}}$ sequence (from 5'-AGCGG-3' to 5'-AATGG-3'), thus inactivating the site (this study and Chédin et al., 1998). All plasmid DNA was purified by two rounds of cesium chloride density-gradient ultracentrifugation (Sambrook et al., 1989). The molar concentration of the dsDNA in nucleotides was determined spectrophotometrically at 260 nm using an extinction coefficient of 6290 M$^{-1}$ cm$^{-1}$ at 260 nm. Plasmid DNA was cut with various restriction enzymes as recommended by the suppliers and radiolabeled at either the 3'-end using the Klenow fragment of DNA polymerase.

![Figure 8. Differential sensitivity of the upstream and downstream top-strand $\chi_{\text{Bi}}$-specific fragments to conditions that affect the AddAB nuclease activity. (a) Structure of the substrates used. pADGF1 was cut with BamHI and either 5'-end (left panel) or 3'-end labeled (right panel). Reactions were performed at varying magnesium ion concentrations and aliquots were withdrawn three minutes after addition of the enzyme. Reaction products were analyzed after electrophoresis through native 1% agarose gels, as depicted in (b). The major reaction products are indicated by arrows.](image-url)
ase I and appropriate [γ-32P]dNTPs or the 5'-end by sequential action of shrimp alkaline phosphatase and phage T4 polynucleotide kinase in the presence of [γ-32P]ATP. Labeled DNA was further purified from unincorporated radionucleotides by passage through a S-200 MicroSpin column (Pharmacia). Tailed substrates unincorporated radionucleotides by passage through a Kowalczykowski, 1997a). The 5'-end labeling of these resected substrates was achieved using T4 polynucleotide kinase as described above. 3'-End labeling was performed using Klenow polymerase and only three of the four deoxynucleotides, including [γ-32P]dATP. Unincorporated radionucleotides were removed as described above.

PCR fragments were generated using plasmids pADG6406-1 or pADGF1 as templates and oligonucleotides FSK7 (5'-ACACATTGACTGTTT-3') and FSK8 (5'-GGGGATCTCITAGCTAGCA-3') as primers, or plasmid pBR322 and oligonucleotides FSK9 (5'-TTTGGATCCACCGATTTAGCTTG-3') and FSK10 (5'-ATATTCCATGTAATCCCTC-3'), FSK11 (5'-TGCC GCAAAAAAGGGA ATAAG-3') and FSK12 (5'-ACA GGATCCGAAGAAGTGCAGCC-3'), FSK15 (5'-GGTC GGATCCCTGCTCCTGTCGT-3') and FSK16 (5'-AAG CTCATGCAGTGCAGTCGT-3') as primers. Reactions were performed in 100 µl volume using the Pfu DNA polymerase (Stratagene). The resulting fragments were purified by agarose gel electrophoresis, 5'-end labeled as described above, treated with BamHI, which recognizes a unique site in the χ-redistal primer (bold letters), and passed through a S-200 microspin column. The 1 kb DNA ladder was purchased from Gibco-BRL and labeled using T4 polynucleotide kinase as described above.

**Reaction conditions and electrophoresis**

Standard reaction conditions corresponded to 25 mM Tris-acetate (pH 7.5), 2 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, 10 µM nucleotides DNA (equivalent to 1.6 nM DNA molecules), 2 µM E. coli SSBI protein, and 0.5 mM functional AddAB enzyme (the amount of functional enzyme was determined from a titration of enzyme helicase activity against a fixed concentration of DNA (Roman & Kowalczykowski, 1989b); the preparation used here was determined to be ~50% active). In certain instances, the magnesium acetate concentration was varied and is indicated accordingly. Assays were performed at 37 °C and were initiated by addition of the AddAB enzyme after a two-minute incubation of all other components at 37 °C. Aliquots of the reaction mixture (20-25 µl) were withdrawn at the indicated times and added to an equal volume of stop buffer (0.1 M EDTA, 2.5% (w/v) SDS, 10% (v/v) Ficoll, 0.125% (w/v) bromophenol blue, and 0.125% (v/v) xylene cyanol). Samples were subjected to electrophoresis in 1% native agarose gels for approximately 15 hours at 1.4 V/cm in TAE buffer. Gels were dried and analyzed directly using a Storm 840 PhosphorImager and the Image-QuaNT software.

**Analysis of reaction products on sequencing gels**

Aliquots (25 µl) were taken at the indicated timepoints and added to a mixture of phenol and chloroform (24:1) to stop the reaction and deproteinize DNA. The aqueous phase was withdrawn (10 µl), added to 4 µl of 4× loading buffer containing formamide, heated to 95 °C for three minutes, and loaded onto an 8% sequencing gel. The sequence ladder was generated using the Sequenase kit II from USB as recommended by the supplier. Electrophoresis was performed at 2000 V for four to five hours and the gel was analyzed directly using a Storm 840 PhosphorImager and the Image-QuaNT software.

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**References**


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