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A Domain of RecC Required for Assembly of the Regulatory RecD Subunit Into the *Escherichia coli* RecBCD Holoenzyme

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ABSTRACT

The heterotrimeric RecBCD enzyme of *Escherichia coli* is required for the major pathway of double-strand DNA break repair and genetic exchange. Assembled as a heterotrimer, the enzyme has potent nuclease and helicase activity. Analysis of *recC* nonsense and deletion mutations revealed that the C terminus of RecC is required for assembly of the RecD subunit into RecBCD holoenzyme but not for recombination proficiency; the phenotype of these mutations mimics that of *recD* deletion mutations. Partial proteolysis of purified RecC polypeptide yielded a C-terminal fragment that corresponds to the RecD-interaction domain. RecD is essential for nuclease activity, regulation by the recombination hotspot Chi, and high affinity for DNA ends. The RecC-RecD interface thus appears critical for the regulation of RecBCD enzyme via the assembly and, we propose, disassembly or conformational change of the RecD subunit.

DNA repair and recombination are highly regulated processes that require the modulation of degradative and recombinogenic activities of multiple enzymes that act on DNA. Such regulation can alter the distribution of recombination around special sites on the chromosome. For example, the RecBCD enzyme of *Escherichia coli* is regulated by complex interactions between its three subunits and a Chi site (GCTGGTGG), a DNA sequence near which recombination occurs at high frequency (Taylor 1988; Kowalczykowski *et al.* 1994; Smith 2001). The enzyme is a heterotrimer with a mass of 330 kD, composed of one copy of the products of the *recB*, *recC*, and *recD* genes (Taylor and Smith 1995), and is required for the major pathway of homologous recombination and DNA repair involving linear double-stranded (ds) DNA. RecBCD enzyme is an ATP-dependent ds and single-stranded (ss) exonuclease, an ss endonuclease, a DNA helicase, and a DNA-dependent ATPase. Full enzymatic activity requires all three enzyme subunits; in some cases one or two subunits containing identified domains demonstrate weaker enzymatic activity (Boehmer and Emmerson 1992; Korangy and Julin 1992, 1993; Yu *et al.* 1998b). ATPase domains have been identified in RecB and RecD (Hickson *et al.* 1985; Finch *et al.* 1986a; Julin and Lehman 1987), and at least part of the nuclease domain is in the C-terminal portion of RecB (Yu *et al.* 1998a,b; Wang *et al.* 2000).

The structure and function of RecBCD enzyme is regulated by Chi sites, hotspots of homologous recombination at which a 3′ ssDNA end is made (Ponticelli *et al.* 1985; Dixon and Kowalczykowski 1993) and loaded with RecA protein (Anderson and Kowalczykowski 1997). The RecA·ssDNA filament then undergoes pairing and strand exchange with a homologous chromosome (Kowalczykowski 2000). The RecD subunit plays at least two roles in the RecBCD enzyme-Chi interaction. First, the RecD subunit is required, along with a nuclease domain in RecB, for nuclease activity (Chaudhury and Smith 1984a; Yu *et al.* 1998a,b); this activity is required to form the recombinogenic 3′ ssDNA end at Chi. Second, RecD interferes with a RecA-loading domain in RecBCD enzyme (Amundsen *et al.* 2000). RecBC enzyme (without the RecD subunit) loads RecA on the DNA constitutively during DNA unwinding (Churchill *et al.* 1999), whereas RecBCD enzyme does not load RecA until the enzyme acts at Chi (Anderson and Kowalczykowski 1997). RecD and a nuclease-dependent signal thus play a role in the regulation of RecA loading (Anderson *et al.* 1999; Amundsen *et al.* 2000). As a consequence of RecBCD enzyme regulation, both the DNA substrate and RecBCD enzyme are changed by an interaction at Chi (Taylor and Smith 1992, 1999). A RecBCD enzyme molecule that acts at one Chi site cannot act at a second Chi site on the same DNA molecule, although DNA unwinding continues to the end of the molecule (Taylor and Smith 1992). After acting at Chi, RecBCD enzyme disassembles into its three subunits, rendering the enzyme inactive on another DNA molecule (Taylor and Smith 1999). Understanding the assembly and disassembly of RecBCD enzyme is thus important in understanding regulation of the enzyme.

Analysis of *recBCD* mutants has established the role of certain RecBCD enzyme subunits and activities in recombination proficiency. Null mutations in *recB* or *recC* eliminate recombination proficiency and all enzy-

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TABLE 1

E. coli **strains and plasmids**

Strain	Genotype	Reference or source
V67	*recB21*::IS*186 argA21 hisG4 recF143 rpsL31 galK2 xyl-5* λ^- F^-	Schultz *et al.* (1983)
V68	As V67, except *recC73* in place of *recB21*	Schultz *et al.* (1983)
V186	Δ*(thyA-argA)232* IN(*rrnD-rrnE*)1 λ^- F^-	Chaudhury and Smith (1984b)
V222	As V67, except *recD1013 argA*::Tn*10* in place of *recB21*	Amundsen *et al.* (1990)
V330	Δ*(recC-argA)234* λ^- F^- [a]	Chaudhury and Smith (1984b)
V794	As S927 except F′15 $recBC^{1041}D$	This work
V1306	*thi-1 relA1* λ^- (Hfr PO44)	Schultz *et al.* (1983)
S927	*thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 proA2 hisG4 argE3 rpsL31 tsx-33 mtl-1 supE44 thyA* λ^- (F′15 $thyA^+$ $recBCD^+$ $argA^+$)	Schultz *et al.* (1983)
Plasmid no.	***rec*** **genotype of insertion and vector**	**Reference or source**
pDWS2	*recBCD* in pBR322	Ponticelli *et al.* (1985)
pAC2	$recBC^{1010}D$ in pBR322	Amundsen *et al.* (1986)
pSA21	$recB^{21}CD$ in pBR322	Amundsen *et al.* (1986)
pSA160	$recBC^{1041}D$ in pBR322	This work
pSA161	*recC* (codons 1–1122) in pSE380	This work
pSA162	*recC2723* (codons 1–804) in pSE380	This work
pSA163	*recC2724* (codons 805–1122) in pSE380	This work
pSE380	None	Invitrogen
pSA125	*recC* in pBluescript II KS	This work
pSA130–pSA143	*recC2709*–*recC2722* in pBluescript II KS	This work[b]
pBluescript II KS	None	Stratagene

[a] One deletion endpoint falls within *recC* between 1830 and 1890 bp from the *recC* start codon (data not shown).

[b] See Figure 4.

matic functions (Rec$^-$ Nuc$^-$; Howard-Flanders and Theriot 1966; Willetts *et al.* 1969; Willetts and Mount 1969), whereas null mutations in *recD* do not affect recombination proficiency but do eliminate nuclease activity and Chi activity (Rec$^+$ Nuc$^-$; *i.e.*, the ‡ phenotype; Chaudhury and Smith 1984a; Amundsen *et al.* 1986). The ‡ phenotype is consistent with the DNA-unwinding and RecA-loading activity of RecBC enzyme as described above. Two mutations in *recC* also have the ‡ phenotype (Chaudhury and Smith 1984a; this work). These special *recC* mutants could be explained by a failure to assemble RecD into RecBCD holoenzyme. We show here that the C-terminal portion of RecC is altered in these mutants and is indeed required for RecD assembly into holoenzyme. Collectively, our data identify a 35-kD domain of RecC that is required for RecD assembly and consequently is essential for the regulation of RecBCD enzyme by Chi.

MATERIALS AND METHODS

Bacterial strains, plasmids, and genotype designations: All strains and plasmids are listed in Table 1 with their genotypes and sources. For visual clarity, allele numbers are expressed as superscripts when more than one *recBCD* gene is designated. The corresponding polypeptide designation is also expressed as a superscript.

Mutant isolation: The isolation of the *recC1010* mutant has been described (Chaudhury and Smith 1984a). The *recC1041* mutant was produced by treating strain S927 (F′15 $thyA^+$ $recBCD^+$ $argA^+$; Schultz *et al.* 1983) with nitrosoguanidine (Sigma, St. Louis) as described by Amundsen *et al.* (1990). The mutagenized F′ factors were transferred to strain V186, and *rec1041* was identified by altered phage sensitivity (Schultz *et al.* 1983), indicating an alteration in RecBCD enzyme activity. Complementation analysis, performed as described by Amundsen *et al.* (1990), showed that the *rec1041* mutation was in *recC* (data not shown). An 18.5-kb *Bam*HI fragment containing the $recBC^{1041}D$ genes was cloned into the *Bam*HI site of pBR322 as described by Ponticelli *et al.* (1985), to create pSA160.

RecBCD enzyme purification and detection: $RecBC^{1041}$ enzyme was purified from 3 liters of V68 (*recC73*) carrying pSA160 ($recBC^{1041}D$) as described by Amundsen *et al.* (2000). Purification was through HiTrapQ, Sephacryl S-300, and HiTrap heparin (all from Amersham Pharmacia). Enzymes were analyzed by native (5% polyacrylamide in 50 mm MOPS-KOH, pH 7.0, and 1 mm EDTA) or SDS polyacrylamide (3–8% polyacrylamide in 50 mm Tris-acetate buffer, pH 8.25, with 0.1% SDS) gel electrophoresis as described by Taylor and Smith (1999). The concentration of $RecBC^{1041}$ enzyme was determined by comparing the amount of Coomassie-stained protein material on native and SDS polyacrylamide gels to known amounts of RecBC and RecBCD enzyme. Western blots were probed with mouse monoclonal antibodies specific for RecB, RecC, or RecD (Taylor and Smith 1999). RecBC enzyme was prepared by mixing purified RecB and RecC polypeptides (Boehmer and Emmerson 1991) and further purified on a HiPrep Sephacryl S-300HR column (Amersham Pharmacia). RecBCD mutant enzymes were also analyzed in cell extracts prepared as described by Tomizawa and Ogawa (1972) except that cultures were grown in Terrific broth (Sigma).

RecBCD enzyme reaction conditions and DNA substrates:

RecBCD enzyme was assayed for ATP-dependent solubilization of ^{3}H-labeled dsDNA (Eichler and Lehman 1977; Amundsen *et al.* 1990). Assays for DNA unwinding and RecA loading (Churchill *et al.* 1999) used plasmid pBR322 DNA digested with *Hin*dIII. Substrate preparation and reaction conditions were as reported by Amundsen *et al.* (2000).

Protease cleavage of RecC polypeptide: Purified RecC polypeptide (20 μg) in 80 μl of protease digestion buffer [20 mM KPO_4, pH 6.8, 5 mM dithiothreitol (DTT), 10% glycerol] was digested at 25° with 0.9 ng of subtilisin (Sigma) or 1.9 ng of trypsin (Sigma) for the times indicated. At each time point an 8-μl sample was removed and phenylmethylsulfonyl fluoride was added to 10 mM. Samples were analyzed by electrophoresis through a 4–12% Bis-Tris SDS gel (Invitrogen, San Diego) in 50 mM MOPS-SDS buffer (Invitrogen). The digestion products were visualized by staining (Fast stain, Zoion Research, Allston, MA). The 35-kD product of trypsin cleavage was transferred to a PVDF membrane (Millipore, Bedford, MA) by electroblotting in CAPS buffer (Matsudaira 1987) for N-terminal sequence analysis. Proteins were sequenced on an Applied Biosystems (Foster City, CA) model 492 N-terminal protein sequencer using Edman degradation reactions.

Subcloning and exonuclease III deletion analysis of *recC*: The *recC* gene was cloned by DNA amplification with Expand DNA polymerase (Roche, Indianapolis) from pDWS2 (*recBCD*$^+$; Ponticelli *et al.* 1985) template DNA. The upstream primer (CGCGGATCCGCGGGGTACCAACAGCTCT GGCGGCATGGCTGG), beginning 195 nucleotides upstream of the *recC* start codon, and the downstream primer (CGC GGATCCCCGGAATTCGCGGATAGATTGCGCAATTTTTAT ACAG), with 14 nucleotides following the *recC* stop codon, both contained a *Bam*HI recognition sequence. The product of DNA amplification was digested with *Bam*HI and ligated into the *Bam*HI site of Bluescript II KS (Stratagene, La Jolla, CA), yielding pSA125. Exonuclease III deletions were made with the Erase-a-Base system (Promega, Madison, WI) after digesting pSA125 with *Apa*I and *Xho*I. The extent of each deletion was determined by sequencing plasmid DNA. The partially deleted plasmids were introduced into strain V68 (*recC73* null mutant) and assayed for recombination proficiency, nuclease activity, and enzyme subunit assembly (Figures 4 and 5).

Fragments of RecC flanking the trypsin-hypersensitive site (see results) were expressed from the *trc* promoter of pSE380 (Invitrogen). *recC*$^+$ (codons 1–1122, pSA161), *recC2723* (codons 1–804, pSA162), and *recC2724* (codons 805–1122, pSA163) were cloned by DNA amplification with Expand DNA polymerase (Roche) from pDWS2 template DNA (details available upon request).

RecBCD enzyme assembly following subunit mixing: Purified RecB and RecC subunits were prepared as described by Boehmer and Emmerson (1991). Other subunits [RecD from pB100 and pNH52 (Boehmer and Emmerson 1991) and RecC from pSA125 and deletion derivatives] were provided in extracts of V330 [Δ*(recC-argA)234*] carrying appropriate plasmids. The approximate concentration of enzyme subunits in cell extracts was determined by fractionating extracts and known amounts of RecBCD enzyme on denaturing polyacrylamide gels and comparing the signal intensities following Western blot analysis using RecD or RecC monoclonal antibodies. For the enzyme assembly assay, RecBCD enzyme subunits (8 nM RecD, 32 nM RecC, 32 nM RecB) were combined in R buffer (20 mM KPO_4, pH 7.6, 0.1 mM EDTA, 50 mM DTT, 100 mM NaCl, 10% glycerol, 1 mg/ml BSA) and incubated overnight at room temperature. Samples were analyzed by electrophoresis on native polyacrylamide gels (see above) to assess the state of enzyme assembly.

RESULTS

***recC*‡ mutants, like *recD* nonsense mutants, are recombination proficient but lack nuclease activity:** During a search for novel *recBCD* mutants we found a candidate, *recC1041*, that appeared to have the ‡ phenotype (Rec$^+$ Nuc$^-$; Chaudhury and Smith 1984a; Amundsen *et al.* 1986; materials and methods). To determine if the candidate was indeed a ‡ mutant, we first measured recombination following Hfr conjugation (Table 2). We compared recombinant frequencies of *recC1041* with those of two known ‡ mutants, *recC1010* and *recD1013*, a nonsense mutation in the fourth codon of *recD* (data not shown; Chaudhury and Smith 1984a). The two special *recC* mutants and the *recD1013* nonsense mutant were almost as recombination proficient as the wild-type strain (Table 2) and produced 100 times more recombinants than did the null (*recC73*) mutant control. Recombination proficiency was also reflected by the resistance to the DNA-damaging agent mitomycin C manifested by the *recC*, *recD*, and wild-type strains relative to the *recB21* null mutant (Table 2; as noted above *recB* and *recC* null mutants have identical phenotypes).

To determine if the *recC1041* mutant lacked nuclease activity, we measured this activity in mutant and wild-type strains directly in extracts and indirectly in cells. Extracts of the *recC1010* or *recC1041* mutants, like the *recD1013* mutant, had <2% of the ATP-dependent exonuclease activity of the wild-type (*recBCD*$^+$) strain (Table 2). An assay that detects RecBCD enzyme nuclease activity inside the cell confirmed these observations. The phage T4 gene *2* product binds to the ends of T4 DNA, thereby protecting the injected DNA from degradation by RecBCD enzyme (Oliver and Goldberg 1977). Consequently, T4 gene *2*$^-$ mutants make plaques only on strains lacking RecBCD enzyme nuclease activity. The titer of T4 *2*$^-$ phage on the *recC1010* and *recC1041* strains, like that on the *recD* nonsense mutant, was 10^6 times higher than that on the wild-type strain, reflecting the absence of dsDNA exonuclease activity in both *recC* mutants and the *recD* mutant. These data show that *recC1010* and *recC1041* are ‡ mutants: they are recombination proficient but lack nuclease activity (Rec$^+$ Nuc$^-$).

The *recC*‡ mutants have the same phenotype as a *recD* nonsense mutant (Table 2), suggesting that RecD is not active in the *recC*‡ mutants. If a region of RecC interacts with RecD, the *recC*‡ mutations might cluster close together and eliminate RecD association with RecBCD enzyme. We sequenced the *recC* genes from these ‡ mutants and found that the two mutations were 191 bp apart near the C terminus of *recC* (Figure 1; data not shown). *recC1010* is a missense mutation at codon 905 of 1122 *recC* codons, and *recC1041* is a nonsense mutation at codon 841. These results indicate that the region of RecC missing in RecC1041 (amino acids 841–1122) is not required for recombination proficiency but, as shown next, is needed for assembly of RecD into holoenzyme.

TABLE 2

***recC1010* and *recC1041* mutants have the same phenotype (Rec$^+$ Nuc$^-$, ‡) as *recD* nonsense mutants**

rec allele[a]	Hfr recombination (% His$^+$[Str^r])[b]	Mitomycin C sensitivity (eop)[c]	Relative ds exonuclease activity[d]	Efficiency of plating phage T4 *2*$^{-e}$
recBCD	4.6, 3.0	0.27, 0.38	1	9.9×10^{-7}, 5.3×10^{-7}
—	0.01, 0.02	2.8×10^{-4}, 5.6×10^{-4}	<0.02	1, 1
recBC^{1010}D	2.6, 1.3	0.66, 0.68	<0.02	1.8, 1.9
recBC^{1041}D	2.2, 1.8	0.13, 0.20	<0.02	2.5, 2.3
recBCD^{1013}[f]	1.3, 1.9	0.60, 0.42	<0.02	0.88, 0.93

[a] Strains are transformants of V68 (*recC73*), except for *recBCD^{1013}* which was in V222 (*recD1013*), for Hfr recombination or transformants of V186 [Δ*(argA recDBC thyA)232*] for mitomycin C sensitivity, ds exonuclease activity, and T4 *2*$^-$ plating. The indicated *rec* alleles were present on derivatives of pDWS2 (Ponticelli *et al.* 1985). —, indicates that strain V68 carried pBR322 (the vector portion of pDWS2) and strain V186 carried pSA21 (*recB^{21}CD*).

[b] The number of His$^+$[Str^R] recombinants per Hfr donor cell (Hfr PO44), corrected for the viability of the recipient culture (Schultz *et al.* 1983). Results of two experiments are shown.

[c] eop, efficiency of plating: titer of culture on LB agar with 0.25 μg of mitomycin C/ml divided by the titer on LB agar. Results of two experiments are shown.

[d] Specific activity in units of ATP-dependent dsDNA exonuclease activity/mg of extract protein (Eichler and Lehman 1977), relative to *recBCD*, which was 850 units/mg of protein.

[e] Phage titer on the indicated strain divided by the titer on V186 (with *recB^{21}CD*). Results of two experiments are shown. Phage T4 gene *2 amN51* were grown on a *recB21* nonsuppressing strain (V67).

[f] *recD1013* is a nonsense mutation (C → T at nucleotide 10) in the fourth codon of the gene (data not shown).

***recC*‡ mutants fail to assemble RecBCD holoenzyme:** We hypothesized that the *recC*‡ phenotype could be explained by the failure to assemble RecD into holoenzyme, functionally yielding RecBC enzyme inside the cell. We therefore examined the state of RecBCD enzyme in extracts of wild-type and *recC*‡ mutant strains. Native polyacrylamide gel electrophoresis resolved purified RecC, RecBC, and RecBCD holoenzyme, as revealed by Western blots probed with a monoclonal antibody specific for RecC (Figure 2A, lanes 1–3). The wild-type *recBCD*$^+$ extract contained RecBCD holoenzyme, free RecC, and a small amount of RecBC (lanes 4–5). In contrast, extracts of *recC1010* and *recC1041* contained RecBC and RecC but no detectable RecBCD holoenzyme (lanes 8–11). We show below that these extracts contained all three polypeptides. These results show that the *recC*‡ mutants fail to assemble RecD into the enzyme complex.

Analysis of RecC polypeptides on denaturing (SDS) polyacrylamide gels confirmed that the *recC1041* nonsense mutation resulted in the production of a truncated form of RecC (Figure 2B, lanes 11–12; data not shown). The RecC1041 polypeptide had a molecular mass of ~95 kD, as predicted by the position of the nonsense mutation in the gene (at codon 841; see above). Wild-type (*recBCD*$^+$) and *recC1010* extracts (lanes 7–10) contained full-length RecC polypeptide that comigrated with purified RecC polypeptide (lane 4), or RecC in RecBC enzyme and RecBCD holoenzyme (lanes 1 and 2). The purified RecC polypeptide in lane 3 of Figure

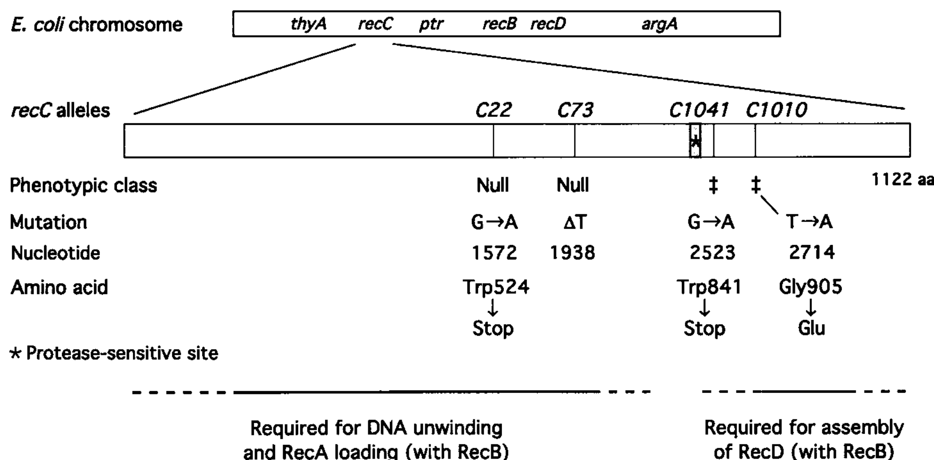

Figure 1.—The *recC*‡ mutations map near the C terminus of RecC. The genetic map of the *thyA-argA* region of the *E. coli* chromosome shows the position and type of certain *recC* mutations and the accompanying predicted codon changes (data not shown; *recC73* was reported by Arnold *et al.* 2000). The region containing a protease-hypersensitive cleavage site (codons 805–819) is indicated (*). Nucleotide numbering begins with the A of the ATG start codon (Finch *et al.* 1986b).

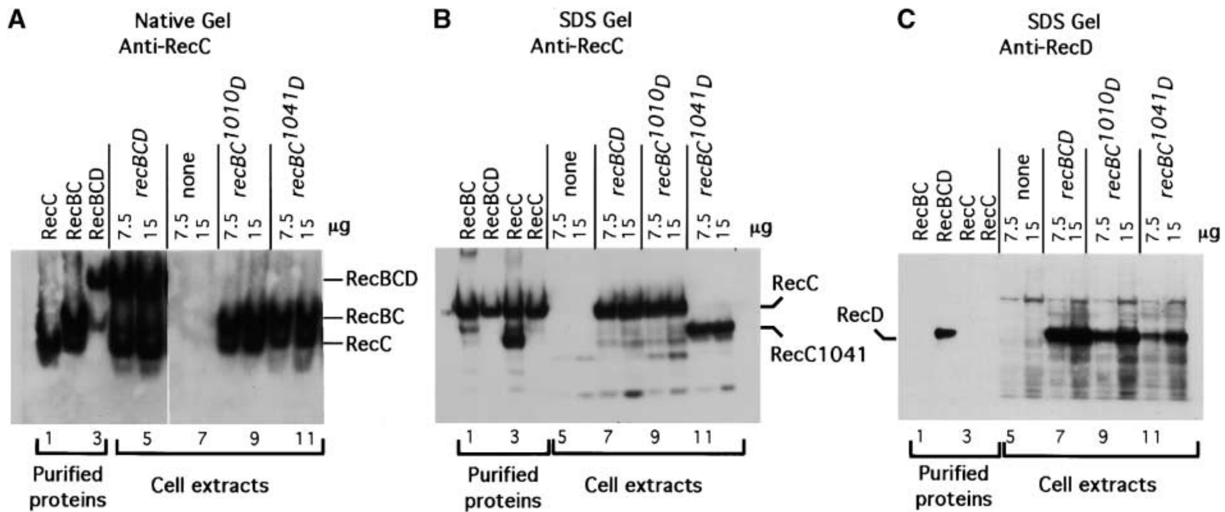

FIGURE 2.—RecC‡ mutants do not assemble RecD into RecBCD enzyme heterotrimer. Extracts were from late log phase cultures of strain V330 [Δ*(recC-argA)234*] containing derivatives of plasmid pDWS2 *(recBCD)*, pAC2 (*recBC^{1010}D*; AMUNDSEN *et al.* 1986), or pSA160 (*recBC^{1041}D*). Purified RecC, RecBC, and RecBCD enzyme (40 ng each) were run as markers. Forms of RecBCD enzyme contained in 7.5 or 15 μg of extract protein were separated on native or SDS polyacrylamide gels and detected by Western analysis using the indicated monoclonal antibodies. The RecC polypeptide shown in lane 3 of part B had been stored at 4° for several months and contained an apparent protease cleavage product (see RESULTS).

2B had been stored at 4° for several months and contained an apparent protease cleavage product whose size was also ∼95 kD (see also Figure 6; this cleavage product is discussed further below).

We determined that extracts of the *recC‡* mutants contained RecD polypeptide even though it was not assembled into RecBCD holoenzyme. Western blots of SDS polyacrylamide gels were probed with a monoclonal antibody specific for RecD (Figure 2C). RecD polypeptide was detected in purified RecBCD enzyme (lane 2) and extracts of *recBCD*$^+$, *recC1010*, and *recC1041* strains (lanes 7–12). A similar analysis performed with a RecB monoclonal antibody demonstrated that all extracts contained RecB polypeptide as well (data not shown). These data show that the *recC‡* mutants produced RecD but failed to assemble it into holoenzyme and suggest that a C-terminal domain of RecC is essential for this process.

Our view of RecC polypeptide now includes two general regions (Figure 1). The first, the N-terminal region, is apparently sufficient, with RecB polypeptide, to mediate recombination proficiency (Table 2). The second, the C-terminal region, missing in RecC1041 and altered by a single amino acid change in RecC1010, is essential for RecBCD holoenzyme assembly (Figure 2).

Purified RecBC^{1041} enzyme is a heterodimer, unwinds DNA, and loads RecA protein: RecBCD enzyme must facilitate the loading of RecA protein to support recombination (AMUNDSEN *et al.* 2000; ARNOLD and KOWALCZYKOWSKI 2000). The recombination proficiency of *recD* mutants has been attributed to the ability of the RecBC enzyme (missing the RecD subunit) to unwind DNA and facilitate the loading of RecA protein onto ssDNA that is produced during DNA unwinding (CHURCHILL *et al.* 1999). Because *recC1041* is recombination-proficient (Table 2), we expected purified RecBC^{1041} enzyme to have the enzymatic activities associated with RecBC enzyme.

We first examined the state of assembly of purified enzyme from the *recC1041* strain. After electrophoresis on a native polyacrylamide gel, a Western blot was probed with a RecC monoclonal antibody and revealed that the preparation contained RecBC^{1041} heterodimers but not RecBC^{1041}D heterotrimers (data not shown). Thus, RecBC^{1041} enzyme was a heterodimer in crude extracts (Figure 2A) and after purification, indicating a defect in enzyme assembly in both situations.

We next compared the DNA-unwinding and RecA-loading activities of purified RecBC enzyme and RecBC^{1041} enzyme (Figure 3). As shown previously (CHURCHILL *et al.* 1999), RecBC enzyme unwound the dsDNA substrate, producing ssDNA (lanes 3, 6, 9, and 12). Loading of RecA protein on the 3′ end of ssDNA protected the DNA from exonuclease I digestion (lanes 4, 5, 10, and 11); this protection required RecA to be present in the reaction (lanes 7, 8, 13, and 14). DNA produced by boiling was not protected in the presence of subsequently added RecA protein and RecBC (data not shown), as expected from the requirement that RecBC enzyme actively unwind the DNA to load RecA (CHURCHILL *et al.* 1999). As expected from the recombination proficiency of *recC1041*, we detected DNA-unwinding and RecA-loading activity in purified RecBC^{1041} enzyme (lanes 15, 18, 21, and 24). Unwound ssDNA was protected from exonuclease I only in the presence of RecA (compare lanes 16 and 17 and 22 and 23 with lanes 19

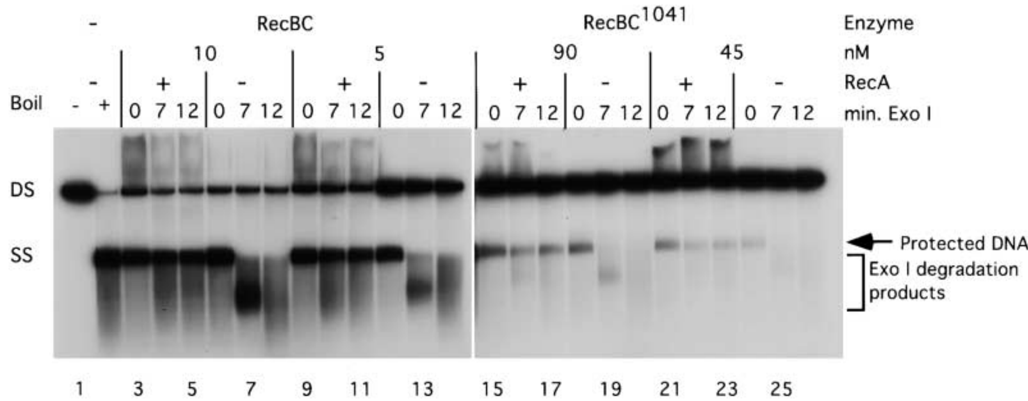

FIGURE 3.—RecBC^{1041} enzyme unwinds DNA and loads RecA protein. RecBC and RecBC^{1041} enzyme were assayed using 5′-^{32}P-labeled pBR322 DNA as described (CHURCHILL *et al.* 1999; AMUNDSEN *et al.* 2000). The DNA substrate (4.7 nM) and the indicated amount of RecBC or mutant enzyme were incubated at 37° for 5 min with or without RecA protein as indicated. An aliquot was removed for analysis (0 min Exo I). Exonuclease I was added to the remaining sample and incubation continued for 7 and 12 min. After addition of SDS to dislodge RecA protein, the products of the reaction were analyzed by electrophoresis in a 1% agarose gel. The positions of dsDNA substrate (DS, lane 1) and unwound ssDNA (SS, boiled, lane 2) are shown.

and 20 and 25 and 26). The activity of purified RecBC^{1041} enzyme in this assay, although weaker than that of RecBC enzyme, is qualitatively consistent with the recombination proficiency observed in genetic assays of *recC*‡ mutants. There is a similar quantitative difference in activity between RecBCD enzyme and RecBC enzyme (CHURCHILL *et al.* 1999; AMUNDSEN *et al.* 2000). RecBC enzyme has much less affinity for DNA than does RecBCD enzyme (data not shown) yet is sufficiently active to support recombination proficiency in the cell (CHAUDHURY and SMITH 1984a).

We next tested whether purified RecBC^{1041} enzyme had nuclease activity. In accord with the assays of unfractionated extracts (Table 2), purified RecBC^{1041} enzyme had <0.02% of the nuclease activity of RecBCD enzyme (1.9×10^5 ds exonuclease units per milligram of RecBCD polypeptides). Thus, the truncated RecC1041 polypeptide, combined with RecB, had DNA-unwinding and RecA-loading activities but not dsDNA exonuclease activity. These activities are qualitatively the same as those of RecBC enzyme with full-length RecC polypeptide.

C-terminal deletions of *recC* also have the ‡ phenotype and fail to assemble RecD: Since *recC1041* (‡) is a nonsense mutation, we reasoned that C-terminal *recC* deletion mutations would also have the ‡ phenotype (Rec$^+$ Nuc$^-$) and would further define regions of RecC required for recombination proficiency and RecBCD enzyme assembly. As predicted, exonuclease III-generated deletions of the C terminus of *recC*, ranging from 38 to 332 codons, produced the ‡ phenotype (Figure 4). Thus, 50 codons toward the N terminus of RecC beyond *recC1041* could be deleted without affecting recombination proficiency. In contrast, deletion of >444 *recC* codons produced a null phenotype (Rec$^-$ Nuc$^-$), indicating the loss of an essential function such as DNA binding, DNA unwinding, or RecA loading when ≤678 codons of *recC* remain.

In summary, the analysis of *recC* C-terminal deletions identified mutations with three distinct phenotypes: wild-type (expressing codons from 1 to 1118), ‡ (expressing codons from 1 to 790–1084), and null (expressing codons from 1 to 456–678). Taken together, these results show that codons 1–790 of RecC are sufficient to confer a recombination-proficient phenotype: the C terminus of RecC is not required for recombination proficiency.

We next determined whether the RecC deletion polypeptides, like the RecC1041 nonsense fragment, failed to assemble RecD into holoenzyme. We monitored RecD assembly into holoenzyme after mixing purified RecB polypeptide with extracts containing RecD and selected RecC deletion polypeptides (Figure 5). Subunit monomers, heterodimers, and heterotrimers were detected on Western blots of native polyacrylamide gels probed with a RecC monoclonal antibody. RecBCD enzyme heterotrimers were detected only in the presence of RecB, RecD, and full-length RecC polypeptide. Expression of 1084, 981, or 889 codons of *recC* allowed RecB and RecC association but not RecD assembly into holoenzyme (Figure 5A). The absence of RecD in the enzymes indicates that the missing C termini of RecC were required for enzyme assembly. The extent of RecBC heterodimer formation varied, being greater for more nearly full-length polypeptides than for the shorter versions. Nevertheless, the corresponding *recC* deletion strains were recombination proficient (Figure 4), indicating that these RecBC enzyme derivatives were able to unwind DNA and load RecA protein.

RecC polypeptides with amino acids 1–790 or 1–804 did not form detectable amounts of RecBC heterodimers in extracts (Figure 5), although strains carrying the corresponding *recC* deletion alleles, *recC2717* and *recC2723*, were recombination proficient (Figure 4). The absence of stable RecBC^{2717} and RecBC^{2723} heterodimer formation may reflect inefficient assembly in cell-free extracts. We infer from these data that at least some of the amino acids in the region from 790 to 840 of RecC are important for stable assembly of RecB with RecC.

Partial proteolysis identifies two domains in RecC:

RecC polypeptide encoded (N — C)	*recC* allele	Codons remaining	Recombination	Nuclease	Phenotype
	+	1122	2.6, 3.8	+	WT
	2709	1118	2.3, 1.6	+	WT
	2710	1084	2.2, 1.4	-	‡
	2711	1037	1.4, 0.7	-	‡
	2712	1035	1.4, 0.6	-	‡
	2713	1029	0.8, 0.2	-	‡
	2714	981	0.6, 0.2	-	‡
	2715	922	1.7, 0.6	-	‡
	2716	889	2.4, 0.9	-	‡
	1041	840	1.1, 1.4	-	‡
	2723	804	2.1, 1.3	-	‡
	2717	790	0.9, 0.2	-	‡
	2718	678	0.01, 0.009	-	Null
	2719	618	0.01, 0.007	-	Null
	2720	592	0.04, 0.004	-	Null
	2721	581	0.002, 0.005	-	Null
	2722	456	0.002, 0.009	-	Null
None	-	0	0.001, 0.003	-	Null

FIGURE 4.—Deletions of the C terminus of *recC* produce the ‡ (RecD⁻) phenotype. Open bars indicate the extent of the RecC polypeptide remaining in the exonuclease III-generated deletions. The extents of RecC polypeptide expressed by *recC1041* (nonsense) and *recC2723* (N-terminal expression subclone) are shown for comparison. The phenotype of each derivative was determined in genetic assays as described in Table 2 for recombination proficiency (Hfr recombination) and intracellular nuclease activity (efficiency of plating of phage T4 gene 2^- mutants). (+) The presence of nuclease activity, which resulted in an efficiency of plating of phage T4 2^- of $<1 \times 10^{-4}$. (−) The lack of nuclease activity resulting in an efficiency of plating of >0.8.

The preceding genetic results indicate that the C terminus of RecC contains a domain that is required to assemble RecD into RecBCD holoenzyme. We obtained physical evidence for such a domain by protease digestion of purified RecC polypeptide. Partial digestion of purified RecC with trypsin or subtilisin yielded two fragments of ~95 and ~35 kD (Figure 6, lanes 6, 7, 9, and 10). As noted previously, fragments of similar sizes were observed in purified RecC polypeptide that had been stored at 4° for several months and was cleaved as a result of trace contamination (Figure 2B, lane 3; Figure 6, lane 8). The two fragments of RecC produced by partial digestion with trypsin were physically dissociated, as indicated by analysis on a native polyacrylamide gel (data not shown).

We determined the positions of protease cleavage by N-terminal protein sequencing and mass spectroscopy. The N terminus of the ~35-kD trypsin-generated fragment began with amino acid 805 (data not shown). The N terminus of the ~35-kD fragment of RecC cleaved during storage began with amino acid 819 and had a molecular mass of 35,153 D (MALDI analysis, data not shown; predicted to be 35,157 D). These results locate a protease-sensitive cleavage site that corresponds to a position 22 to 35 codons from the *recC1041* stop codon toward the N terminus of RecC (Figure 1). The 35-kD domain thus corresponds to the region shown above to be essential for RecD assembly into holoenzyme.

If the C terminus of RecC is required for assembly of RecD, it or full-length RecC might bind to RecD. We searched for such an interaction with the subunit assembly assay using Western blots of native polyacrylamide gels (see above and Figure 5). For these experiments we mixed extracts containing RecD polypeptide, purified RecB polypeptide, and full-length RecC polypeptide or truncated RecC polypeptides from extracts. In these assays we routinely detected RecBCD enzyme heterotrimer and RecBC heterodimer when using full-length RecC polypeptide (Figure 5B, lanes 2, 4, 6, and 8), but we failed to see any evidence of a RecC-RecD interaction in the absence of RecB polypeptide (lanes 3 and 7). Full-length RecC, the N terminus of RecC (RecC2723), or the C terminus of RecC (RecC2724) all failed to form a detectable complex with RecD (lanes 3, 7, 11, and data not shown). The almost neutral 67-kD RecD polypeptide would be expected to markedly retard the migration of the RecC polypeptide: see for example the considerable retardation of RecBCD enzyme *vs.* RecBC enzyme in Figure 5A. These data suggest that RecC interacts with RecD to form a heterotrimer only in the presence of RecB. We cannot exclude, however, the possibility that a RecCD complex was not detectable in this assay or that the experimental conditions were not appropriate for an interaction that occurs inside the cell. Further experiments on enzyme assembly may identify conditions that allow a RecC-RecD interaction.

DISCUSSION

We have used genetic and physical assays of *recBCD* wild-type and *recC*‡ mutant strains to identify a domain of RecC that is required to assemble the regulatory RecD subunit into RecBCD holoenzyme. This assembly into RecBCD enzyme heterotrimer is critical because the

Figure 5.—C-terminal deletions of *recC* fail to assemble RecD into holoenzyme. Extracts were prepared as described in Figure 2 from V330 [Δ*(recC-argA)234*] containing plasmids expressing full-length RecC (A, pSA125; B, pSA161) or the indicated C-terminal deletions. Purified RecB polypeptide or extract containing RecD polypeptide, or both, were added to the RecC extract as indicated, and assembly of heterodimer and heterotrimer forms of RecBCD enzyme were monitored on native polyacrylamide gels as in Figure 2A.

RecD subunit is required for nuclease activity, high affinity for DNA ends, and the regulation of RecBCD enzyme activities by Chi sites. Thus, the RecC-RecD interface is likely to be a major target of the RecBCD enzyme-Chi regulatory mechanism. Our work also demonstrates that the region required for assembly of RecD into holoenzyme is dispensable for recombination proficiency (Table 2); deletion of the C terminus of RecC leaves the RecBC^{1041} enzyme able to promote recombination through DNA unwinding and RecA loading (Figure 3). This result further defines the minimal region of RecC required for RecBC enzyme activity and reveals the role of two regions of RecC in enzyme activity.

The C-terminal region of the RecC subunit is required for enzyme assembly as demonstrated by deletion mutations in *recC* that remove codons 790–1122 of *recC* and eliminate RecD assembly into RecBCD enzyme (Figures

Figure 6.—Physical evidence for two RecC domains. Purified RecC polypeptide (2 μg per lane) was digested with subtilisin (lanes 2–7) or trypsin (lanes 9–14) for the times indicated. Lanes 1 and 15 contained size markers (Bio-Rad) and lane 8 contained RecC polypeptide that was stored for several months at 4°. The digestion products were analyzed by gel electrophoresis and visualized by staining (Fast stain, Zoion Research).

1, 2, and 5). The *recC1010* point mutation in codon 905 has the same nonassembly phenotype, indicating that a single amino acid change in this region of RecC can also disrupt RecD assembly (Figure 2). RecC polypeptide contains a protease-sensitive cleavage site in the region of amino acids 805–819 (see results and Figures 1 and 6). After cleavage at this site, the two fragments were physically dissociated. These results suggest that the C-terminal ~35-kD portion of RecC corresponds to a separate physical domain essential for interaction with RecD. This region was dispensable for recombination proficiency, indicating that the enzyme functions of DNA unwinding and RecA loading do not require the C terminus of RecC.

The ~95-kD N-terminal domain of RecC contains functions essential for recombination. The N terminus of RecC, with RecB, was sufficient to confer recombination proficiency by its DNA-unwinding and RecA-loading activities (Figure 3; Table 2). Deletions leaving fewer than 679 codons of the *recC* N terminus conferred a null phenotype, indicating that this region is required for some combination of DNA binding, DNA unwinding, and RecA loading (Figure 4). The N terminus of RecC is also required for Chi activity: *recC** mutants, which alter codons 646–653, lack Chi genetic activity but retain nuclease activity and DNA-unwinding activity (Schultz *et al.* 1983; Handa *et al.* 1997; Arnold *et al.* 1998, 2000).

We have shown that the C-terminal domain of RecC is required for assembly of RecD into holoenzyme. We searched for, but were unable to demonstrate, a direct interaction between RecD and full-length RecC or the ~35-kD C-terminal domain (Figure 5 and data not shown). Evidence for a RecC-RecD interaction was ob-

tained, however, in a genetic study detailing the effects of overproduction of individual RecBCD enzyme subunits on Chi activity (Rinken and Wackernagel 1992). Chi hotspot activity in phage lambda vegetative crosses requires RecBCD holoenzyme and is detected when more recombination events occur in an interval with Chi than in the same interval without Chi (Stahl and Stahl 1977). Rinken and Wackernagel (1992) noted that overproduction of the RecC subunit reduced but did not eliminate Chi activity and suggested that free RecC binds RecD, thereby reducing the amount of RecD subunit available for assembly into RecBCD holoenzyme. RecBC enzyme (lacking the RecD subunit) does not act at Chi, so hotspot activity was predicted to decline, as observed (Chaudhury and Smith 1984a; Amundsen *et al.* 1986; Rinken and Wackernagel 1992). This result supports the hypothesis that free RecC and RecD subunits interact inside the cell through, we infer, the C terminus of RecC. Our failure to detect a RecC-RecD interaction could be due to the lack of appropriate conditions used in the subunit assembly assay. In cells, partially folded RecC and RecD might interact without RecB, but only during translation. It is also possible that the RecC subunit forms a complex with RecB, onto which RecD is then assembled; when RecB was present in our assays, RecBCD holoenzyme was routinely assembled (Figure 5).

RecBCD enzyme is inactivated by interaction with Chi in an apparent two-step process that involves the RecD subunit (Taylor and Smith 1992, 1999). The interface between the RecC and RecD subunits identified here may be a target for the Chi-dependent signals that change enzyme activity. The first step of inactivation involves a change in RecBCD enzyme activity at Chi. RecD plays a part in this step because it is required (Chaudhury and Smith 1984a; Amundsen *et al.* 1986), with the C terminus of RecB, which contains at least part of the nuclease domain (Yu *et al.* 1998a,b; Wang *et al.* 2000), for full nuclease activity and action at Chi sites. Changes in RecBCD enzyme activity occur as the enzyme interacts with a DNA substrate containing a Chi site in the following way. RecBCD enzyme binds to the end of DNA and initiates unwinding (Taylor and Smith 1980). During the ensuing unwinding, RecBCD enzyme recognizes Chi and makes a 3′-end for RecA loading (Dixon and Kowalczykowski 1993; Anderson and Kowalczykowski 1997) by nicking the DNA (Ponticelli *et al.* 1985; Taylor *et al.* 1985) or reducing exonuclease activity (Dixon and Kowalczykowski 1991). The RecD subunit inhibits RecA loading until RecBCD enzyme interacts with Chi (Churchill *et al.* 1999; Amundsen *et al.* 2000). The change in nuclease activity or a signal coordinated with nuclease activity appears to cause an as-yet-undetermined change in RecD that exposes a domain of RecB for RecA loading (Amundsen *et al.* 2000). As proposed previously, RecD may undergo a conformational change or be released from the holoenzyme at Chi (Thaler *et al.* 1988; Stahl *et al.* 1990). In either case the interface with RecC is likely to play a role in signaling this change, allowing a conformational change or disassembly of RecD. The second step of inactivation occurs after RecBCD enzyme has moved through the DNA substrate and is disassembled into its three individual subunits but only after encountering a Chi site (Taylor and Smith 1999). The RecC-RecD interface must be disrupted at this stage of Chi-dependent inactivation of RecBCD enzyme.

Within a RecBCD enzyme molecule, separate domains may transmit signals to modulate enzyme activity. As noted above, Chi recognition changes the activity of RecBCD enzyme by as-yet-undetermined changes in the RecD subunit. The N terminus of RecC, which contains a domain for Chi recognition (Arnold *et al.* 2000), may transmit a signal to the C terminus of RecC, which then causes a change in RecD. Further analysis of enzyme assembly and the critical RecC-RecD interface may define more precisely how RecBCD enzyme is regulated by Chi.

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