The GTP-Dependent Restriction Enzyme McrBC from *Escherichia coli* Forms High-Molecular Mass Complexes with DNA and Produces a Cleavage Pattern with a Characteristic 10-Base Pair Repeat†

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ABSTRACT: The GTP-dependent restriction enzyme McrBC consists of two polypeptides: one (McrB) that is responsible for GTP binding and hydrolysis as well as DNA binding and another (McrC) that is responsible for DNA cleavage. It recognizes two methylated or hemimethylated RC sites (R^mC) at a distance of approximately 30 to more than 2000 base pairs and cleaves the DNA close to one of the two R^mC sites. This process is strictly coupled to GTP hydrolysis and involves the formation of high-molecular mass complexes. We show here using footprinting techniques, surface plasmon resonance, and scanning force microscopy experiments that in the absence of McrC, McrB binds to a single R^mC site. If a second R^mC site is present on the DNA, it is occupied independently by McrB. Whereas the DNA-binding domain of McrB forms 1:1 complexes with each R^mC site and shows a clear footprint on both R^mC sites, full-length McrB forms complexes with a stoichiometry of at least 4:1 at each R^mC site, resulting in a slightly more extended footprint. In the presence of McrC, McrB forms high-molecular mass complexes of unknown stoichiometry, which are considerably larger than the complexes formed with McrB alone. In these complexes and when GTP is present, the DNA is cleaved next to one of the R^mC sites at distances differing by one to five helical turns, suggesting that in the McrBC−DNA complex only a few topologically well-defined phosphodiester bonds of the DNA are accessible for the nucleolytic center of McrC.

Restriction enzymes are a diverse group of enzymes with a common function, highly specific DNA cleavage, which is an effective measure for protecting the cell from foreign DNA without threatening the integrity of the cellular DNA (reviews in refs *1*−*3*). Three basic types of restriction enzymes have been discovered so far: the ATP-dependent type I and type III restriction enzymes which have an associated DNA methyltransferase activity (reviews in refs *4*−*6*) and the type II enzymes which do not require nucleoside triphosphates as cofactors and do not have an associated DNA methyltransferase activity but rather, in most cases, a separate companion methyltransferase (reviews in refs *7*−*9*).

Restriction enzymes that do not fall into these categories exist, among them the McrBC restriction endonuclease of *Escherichia coli* [putative homologues are present in some other eubacteria (*62*)], whose activity was first described and genetically mapped by Luria and Human (*10*) and later genetically analyzed in detail by the groups of Raleigh (*11*−*15*), Noyer-Weidner (*16*, *17*), and Braymer (*18*−*21*). McrBC shares with type I and type III restriction enzymes the requirement for nucleoside triphosphates but, as opposed to those, does not have an associated DNA methyltransferase activity. Furthermore, its cofactor is GTP rather than ATP (*22*). As a matter of fact, McrBC is the only known GTP-dependent restriction enzyme.

McrBC recognizes two methylated or hemimethylated RC sites (R^mC), located on one and/or the other strand of the duplex, approximately 30 to more than 2000 base pairs apart (*22*, *23*), and cleaves the DNA in both strands close to one of the R^mC sites (*24*). McrB is the subunit responsible for GTP binding and hydrolysis (*25*) as well as specific DNA binding (*26*, *27*), whereas McrC harbors the catalytic center for DNA cleavage (*62*). DNA cleavage is strictly coupled to the hydrolysis of GTP (or XTP or ITP, but not ATP) (*22*,

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28). On the other hand, McrB by itself is an active GTPase, which can be stimulated by McrC, but does not require DNA for its GTPase activity (*25*, *28*). Depending on the experimental conditions (presence or absence of Mg^{2+}, presence or absence of GTP or GTPγS), McrB forms different high-molecular mass complexes with DNA having at least one R^mC site (*26*, *27*). Complexes with bona fide DNA substrates (having two R^mC sites in the required spacing) increase in size in the presence of McrC, Mg^{2+}, and GTP (*27*) or GTPγS (*29*), as shown by gel electrophoretic mobility shift experiments. McrB leaves a clear footprint on DNA containing one and two R^mC sites, with one and two regions, respectively, protected around the R^mC site. This footprint is not dependent on the presence of GTP and is not changed by the addition of McrC (*29*). GTP, however, increases the affinity of McrB for its substrate by almost 10-fold as shown by quantitative footprint experiments using GTPγS (*29*). A more important function of GTP, however, seems to be to "fuel" DNA translocation by its hydrolysis. According to ref *24*, two McrBC molecules bound to two different R^mC sites on the same DNA translocate the DNA, dependent on GTP hydrolysis, while remaining bound to the R^mC sites, until they collide, which triggers DNA cleavage, which is similar to the process described for the type I and type III restriction enzymes (*30*−*32*). Cleavage is also observed when a DNA substrate is used with only one R^mC site, but only when another protein is firmly bound adjacent to the recognition site or when the substrate is covalently closed circular DNA (*24*), similar to the process described for type IC restriction enzymes (*33*).

Neuwald et al. (*34*) were the first to detect sequence homology between McrB and the AAA^+ family of motor proteins, chaperone-like ATPases associated with various cellular activities, among them the DnaA, RuvB, and NtrC proteins involved in replication, recombination, and transcription, respectively, as well as other proteins not interacting with DNA, but involved in the assembly and disassembly of protein complexes. This is reminiscent of a suggestion that we had made for McrBC with reference to the assembly and disassembly of microtubules which is also accompanied by GTP hydrolysis (*25*). Several members of the AAA^+ family form oligomeric ring structures. Intriguingly, in the presence of GTPγS, McrB forms ring-shaped heptamers or dimers of heptamers with a central channel; the presence of McrC seems to stabilize the dimer of heptamers of McrB, as shown by gel filtration and scanning transmission electron microscopy (*35*). It is unclear whether the DNA-binding domain of McrB resides in the central channel or on the exterior, and whether the DNA is threaded through the channel. It is also not known where McrC is located and how many McrC molecules are bound when associating with the McrB ring structure. A major open question is how the structure of McrBC changes upon complex formation with DNA and how the structural information that begins to emerge relates to the proposals made concerning the mechanism of DNA binding, translocation, and cleavage. In the paper presented here, we have addressed some of these questions, namely, the size of the McrBC−DNA complex, what it looks like, and where it exactly cleaves the DNA. On the basis of these and previously published results on McrBC as well as other restriction endonucleases, a model for the mechanism of action of McrBC is proposed.

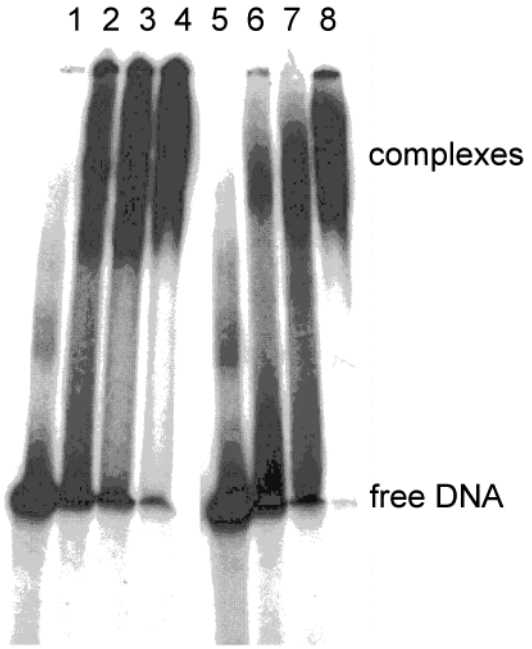

FIGURE 1: Electrophoretic mobility shift experiments with a DNA substrate containing two McrBC recognition elements to illustrate the DNA binding activity of McrB with and without the GST tag. McrB with the GST tag (lanes 1−4, 0, 0.035, 0.12, and 0.35 μM McrB, respectively) and McrB without the GST tag (lanes 5−8, 0, 0.051, 0.17, and 0.51 μM McrB, respectively) were incubated with 25 nM ^{32}P-labeled Sb61 (see Figure 2) at room temperature in 20 mM Hepes-KOH (pH 7.6), 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 mM GTP and then loaded onto a 20 cm × 20 cm × 0.15 cm 6% polyacrylamide gel, which was run for 3 h in the cold room and then subjected to autoradiography (*27*). High-molecular mass complexes are formed with essentially the same concentration dependence in the two sets of experiments (see the decrease in Sb61 concentration upon addition of increasing amounts of McrB). The size of these complexes is slightly larger for the GST-tagged McrB, as expected because of the size of the GST tag.

MATERIALS AND METHODS

Proteins. $McrB^{1-162}$, McrB, and McrC were produced as N-terminal GST fusion proteins and His_6-tagged proteins, respectively. Their expression and purification was described by Pieper et al. (*25*, *36*, *62*). It must be emphasized that the large GST tag, which has a propensity to dimerize, does not affect the formation of high-molecular mass complexes between McrB and DNA containing R^mC sites. As can be seen in Figure 1, which shows the results of gel electrophoretic mobility shift experiments with McrB (with and without a GST tag) and a DNA substrate with two R^mC sites, similar high-molecular mass complexes are formed with a similar concentration dependence, i.e., affinity.

DNA Substrates. The DNA substrates used in the present study are listed in Figure 2.

Sb61 is a fully synthetic double-stranded oligodeoxynucleotide obtained from Interactiva (Ulm, Germany). It contains 5-methylcytosine and 5-iodocytosine residues at defined positions introduced during synthesis. The sequence is given in the Results.

Sb73 is a PCR product derived from pBR322 (positions 2056−2128), modified such that it contains three *Msp*I sites, two of which generate an R^mC site, when methylated primers are used.

Sb82a and -b are PCR products derived from pBR322 (positions 2054−2135), modified such that it contains only

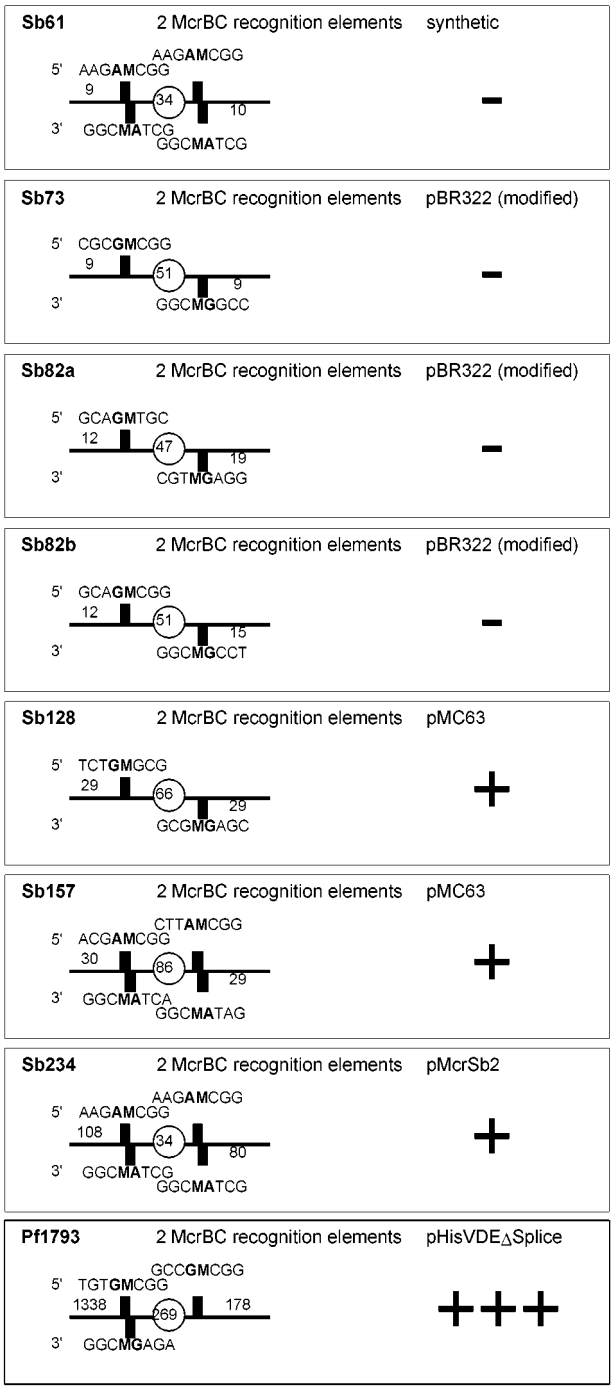

FIGURE 2: Overview of the DNA substrates used in this study. DNA substrates were synthesized chemically or by PCR (denoted Sb), or obtained by restriction digestion (denoted Pf). The length of the substrate is indicated in the name; e.g., Sb61 is a 61mer. The distances of the R^mC sites from the 5′- and 3′-ends are given, as well as the distances between R^mC sites. When two sites are vis à vis, the shortest distance is given. The flanking sequences (^mC abbreviated as M) of each R^mC site are shown. R^mC sites are indicated by a vertical black bar. Note that throughout the paper two R^mC sites are considered as one McrBC recognition element when located vis à vis and, therefore, not separate from each other. − denotes that the DNA is not detectably cleaved by McrBC, + a fair substrate, and +++ a very good substrate, under the conditions of our experiments.

two *Alu*I sites, both of which generate an R^mC site, when methylated primers are used. Sb82b differs from Sb82a in a replacement of the *Alu*I sites with *Msp*I sites.

Sb128 is a PCR product derived from pMC63 (*23*) (positions 1811−1907 and 1−3), which generate one or two R^mC sites, when one or two methylated primers are used.

Sb157 is a PCR product derived from pMC63 (*23*) (positions 236−392) which was methylated in vitro using M.*Msp*I.

Sb234 is a PCR product derived from pMcrSb2 (*37*) (positions 5093−5190 and 1−136), a derivative of pBW201 and pBW5 (*26*), which was methylated in vitro using M.*Msp*I.

Pf1793 is a restriction fragment produced by *Eco*88I and *Pvu*II codigestion of pHisVDEΔSplice (W. Wende, Giessen, Germany), a derivative of pHisPI-SceI (*38*), which was methylated in vitro using M.*Msp*I.

All DNA substrates produced by PCR or restriction digestion were purified by preparative agarose gel electrophoresis, followed by extraction of the DNA from the gel using NucleoSpin Extract (Macherey-Nagel, Düren, Germany).

Footprinting. Protein ($McrB^{1-162}$, McrB, McrC, or McrBC at 4 μM) and 500 ng of DNA (Sb234), radioactively labeled at the 5′-end of the upper strand, were preincubated in 25 mM Hepes-KOH (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/mg bovine serum albumin for 30 min at room temperature. After transfer on ice, 1.5 milliunits/μL DNase I or 0.4 nM *Serratia marcescens* nuclease was added and incubation continued for 2 min. The reaction was stopped by adding the same volume of 200 mM NaCl, 20 mM EDTA, 1% SDS, and 250 μg/mL tRNA. The DNA was precipitated with 3 volumes of ice-cold ethanol. After 30 min at −20 °C, the precipitate was collected by centrifugation, dissolved in 90% formamide, 25 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol, and loaded onto an 8% polyacrylamide gel containing 7 M urea. After electrophoreses, the gels were subjected to autoradiography.

Photo-Cross-Linking. McrB (1 μM) and radioactively labeled ^{5I}C-substituted oligodeoxynucleotide Sb61 (1 μM) were preincubated on ice for 10 min in 100 mM Hepes-KOH (pH 7.6) in a volume of 25 μL. Photo-cross-linking was carried out on ice with a 40 mW HeCd laser emitting at 325 nm (OMI-3074-10-M-R, Laser 2000, Bischofswerder, Germany). Samples of 2.5 μL were withdrawn after defined time intervals and analyzed on 15% SDS−polyacrylamide gels. Gels were silver-stained and dried, and radioactive bands were visualized by autoradiography (Instant Imager, Packard, Meriden, CT).

Surface Plasmon Resonance Experiments. Surface plasmon resonance experiments were carried out on a BIAcore 2000 instrument (BIAcore, Freiburg, Germany) using type SA sensor chips coated with a carboxymethyl dextran matrix with covalently bound streptavidin. DNA (Sb128, without and with one or two R^mC sites) was attached via its biotin label, introduced during PCR using 5′-biotinylated primers, to three of the four channels. Then, 200 pg of DNA/flow cell was bound, giving rise to a signal of 200 RU. For the binding experiments, a buffer consisting of 10 mM Hepes-KOH (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20, supplemented with 10 mM $MgCl_2$, 1 mM GTP, 100 ng/μL bovine serum albumin, and 2.5 ng/μL poly[dI-dC] or poly[dA-dT] was used. $McrB^{1-162}$, McrB, McrC, and McrB plus McrC at the given concentration were

dissolved in binding buffer and injected into the flow cell. The flow rate was typically 10−20 μL/min. All experiments were carried out at ambient temperature. Surfaces were regenerated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 300 mM NaCl, and 0.02% SDS.

Scanning Force Microscopy. Imaging was performed with a Nanoscope III instrument (Digital Instruments, Santa Barbara, CA) according to published procedures (*39*, *40*). Samples contained 2.5−3 nM DNA (Pf1793), varying amounts of protein, and 1 mM GTP in filtered buffer [20 mM Hepes-KOH (pH 7.6), 50 mM KCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol]. They were incubated on ice for 10−20 min and then applied to freshly cleaved mica (Plano GmbH, Wetzlar, Germany), gently washed with 10−20 mL of H_2O, dried under a nitrogen flow, and imaged in air (at ambient temperature and humidity) using the tapping mode. Etched silicon cantilevers, the Nanosensor type (L.O.T. Oriel, Darmstadt, Germany) with a tip curvature of ∼10 nm, a thickness of 3.5−5 μm, a specified force constant of 17−64 N/m, and a resonance frequency between 250 and 400 kHz (specifications as given by the manufacturer), were employed. Contour lengths were measured by drawing a curved line along the DNA and determining the pixel distance using the program NIH Image 1.62.

DNA Cleavage Experiments. DNA cleavage experiments were carried out with 100−200 ng of DNA radioactively labeled at the 5′-end of the lower strand, with varying concentrations of McrB (2−6 μM) in 20 mM Hepes-KOH (pH 7.6), 50 nM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM GTP, and 100 ng/μL bovine serum albumin at 30 °C. Aliquots (10 μL) were withdrawn after defined time intervals, and the reaction was stopped by addition of an equal volume of 250 mM EDTA, 25% sucrose, 1.2% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol (pH 8.0) (NaOH). Product analysis was carried out by electrophoresis on 6% polyacrylamide gels in the presence of 7 M urea. Radioactive bands were visualized by autoradiography.

RESULTS

We have analyzed the interaction of McrB, McrC, and their macromolecular substrates by a variety of techniques. McrB, and its variants, were produced and used as N-terminal GST fusion proteins, which means that the possibility that the GST tag (which is needed for overproduction, purification, and stability of McrB) to a certain extent could influence the results cannot be excluded. From early work of Noyer-Weidner and colleagues (from whom we had obtained the fusion constructs), however, it is clear that the DNA binding and cleavage activities of McrBC are unaffected by the GST fusion [Krüger, T. (1992) Dissertation, Freie Universität Berlin, Berlin]; we assume that this is also true for the partial reactions, as was shown for the McrB−DNA interaction (27).

Footprint Experiments with McrB$^{1-162}$, McrB, and McrBC. The DNA binding site of McrB resides in the N-terminal domain (*27*, *36*). To find out whether and to what extent DNA binding by the N-terminal domain, McrB$^{1-162}$, differs from that by McrB and McrBC, we have performed footprint experiments with bovine DNase I and *Serratia marcescens* nuclease (*Sma*Nuc) using a DNA substrate (Sb234, cf. Figure 2) of 234 base pairs with two R^mC sites located at positions 81 and 121. Sb234 was produced by PCR with appropriate primers to introduce two R^mC sites by subsequent methylation using M.*Msp*I. In agreement with results reported by Stewart et al. (*29*), DNase I footprints of McrB showed two major sites of protection around the R^mC sites, regardless of the absence or presence of McrC (data not shown). *Sma*Nuc footprint experiments were also carried out for McrB$^{1-162}$. The comparison with the results obtained for McrB and McrBC (Figure 3) demonstrates that McrB$^{1-162}$ protects the two R^mC sites but, as opposed to full-length McrB, does not protect the short stretch of DNA between the two R^mC sites, which are separated by 34 base pairs in the substrate chosen for the footprint experiments. McrC does not produce a footprint on DNA and does not change or extend the footprint produced by McrB, confirming the results of Stewart et al., who had carried out DNase I footprint experiments with substrates containing one or two RmC sites, in the absence and presence of McrC (*29*). The larger footprint of McrB compared to that of McrB$^{1-162}$, which comprises the DNA binding domain of McrB (*36*), could be due to the fact that the C-terminal domain of McrB prevents access of the nuclease to the stretch of DNA between the two R^mC sites. The fact that McrC which interacts with McrB, presumably via the C-terminal domain of McrB (*28*), does not have an effect on the footprint by McrB suggests that its association with McrB does not prevent access of DNase I or *Sma*Nuc to the specifically bound DNA in the McrBC−DNA complex. Although McrC does not produce a footprint on DNA, it seems to interact weakly and nonspecifically with DNA (see the legend of Figure 3), which is not unexpected, as it must interact with DNA during cleavage. Similar observations have been made with the cleavage domain of *Fok*I (*41*) and PI-*Sce*I (*42*).

Photo-Cross-Linking Experiments with McrB- and 5-Iodocytosine-Substituted Oligodeoxynucleotides. The footprint experiments by Stewart et al. (*29*) and the ones reported here show that the R^mC site is within the footprint of McrB. This is not unexpected as the methyl group must be recognized by McrB. In accordance with these results, McrB can be specifically cross-linked to a synthetic oligodeoxynucleotide (Sb61; cf. Figure 2) carrying in one strand two R^mC sites in the M.*Msp*I recognition sequence (CCGG) and in the complementary strand (the bottom strand of Sb61; cf. Figure 2) in addition to an R^mC site, an $R^{5I}C$ site, in which one 5-methylcytosine is replaced by 5-iodocytosine (^{5I}C), viz., d(AATTCTCGCT A^{5I}CCGGTCTTA CCGATTAATA TGATATCATA CGAGCTCGCT A^{m}CCGGTCTTA G).

Figure 4 shows the kinetics of the photo-cross-linking reaction for this oligodeoxynucleotide. After irradiation for 90 min, a cross-linking yield of approximately 5% was obtained. An oligodeoxynucleotide in which the 3′-adjacent cytosine (position 13; cf. the sequence above) is replaced with 5-iodocytosine is cross-linked in an even better yield (approximately 20%, data not shown). These findings demonstrate that the cytosine residues within and 3′-adjacent to the R^mC site are in direct contact with McrB. Three other oligodeoxynucleotides with ^{5I}C in positions 7, 9, and 17 (cf. the sequence above) could not be cross-linked to McrB. This does not necessarily mean that these positions are not in direct contact with McrB, but that no reactive amino acid residues are nearby, as 5-iodopyrimidines give rise to "zero-length" cross-links (*43*, *44*).

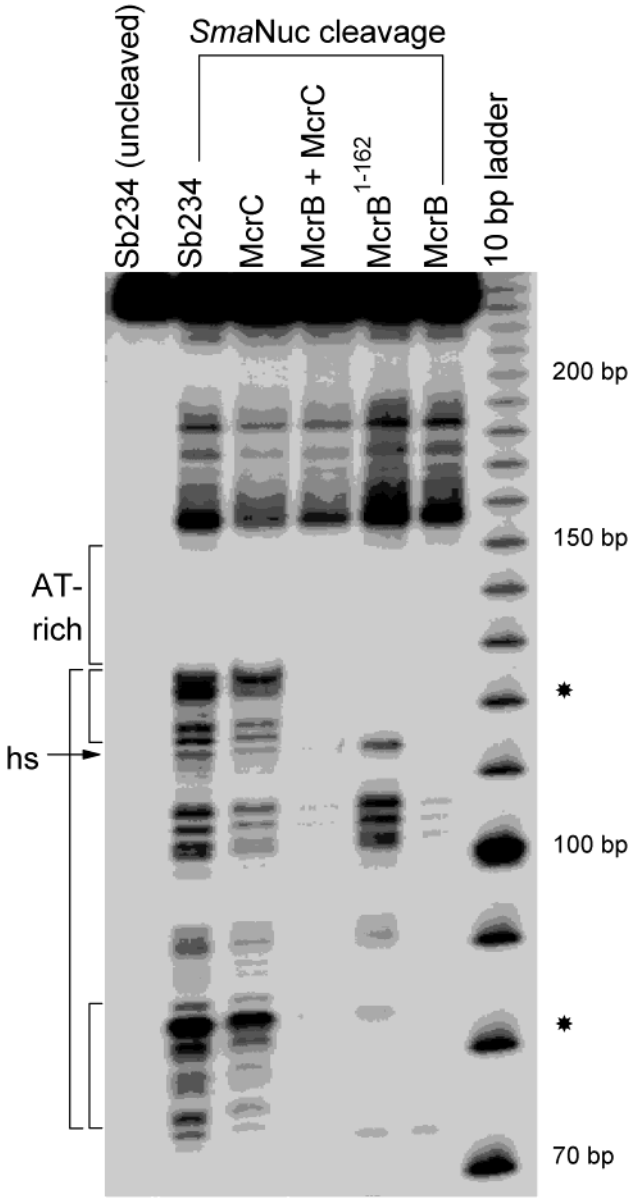

FIGURE 3: *S. marcescens* nuclease footprints of McrB$^{1-162}$, McrB, and McrBC on a DNA substrate containing two McrBC recognition elements. McrB$^{1-162}$ (or McrB, McrC, and McrBC at 4 μM) was preincubated with 500 ng of radioactively labeled Sb234 (cf. Figure 2) in the presence of 10 mM $MgCl_2$, before *Sma*Nuc was added. After a 2 min incubation with 0.4 nM *Sma*Nuc, the reaction products were analyzed by denaturing polyacrylamide gel electrophoresis. Diminished susceptibility to *Sma*Nuc is indicated by brackets, and a hypersensitive band is denoted with an arrow. An AT-rich region not attacked by *Sma*Nuc in the DNA substrate (*61*) and, therefore, not informative, is indicated. The asterisk marks the positions of the methylated cytosine base. McrB$^{1-162}$ protects the two regions around the two R^{m}C sites, whereas McrB also protects the region between the two R^{m}C sites. In this region, McrB$^{1-162}$ produces a hypersensitive band (indicated hs) compared to the free DNA. In the presence of McrC, all bands are slightly less intense. This could be the result of weak nonspecific binding of McrC to DNA.

Surface Plasmon Resonance Experiments with McrB$^{1-162}$, McrB, and McrBC. Formation of a complex between McrB and DNA was analyzed previously by gel electrophoretic mobility shift experiments. Depending on the conditions and the DNA used, complexes of different electrophoretic mobilities were detected (*27*, *29*). Of concern for Stewart et al. (*29*) was the observation that in the presence of GTP or GTPγS the gel shift experiments show essentially the same

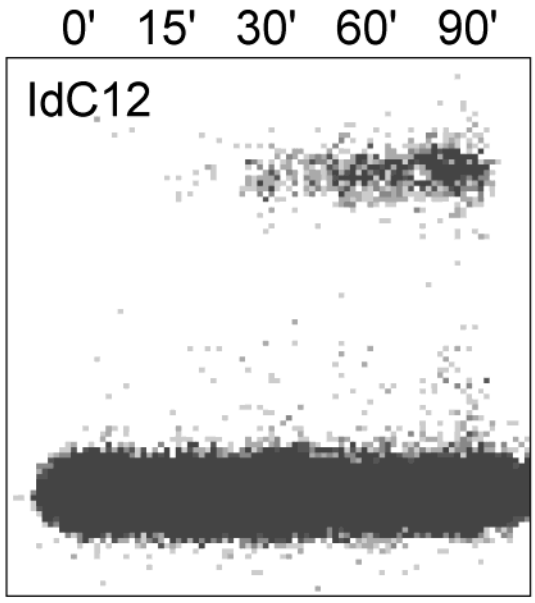

FIGURE 4: Photo-cross-linking of McrB to 5-iodopyrimidine-substituted oligodeoxynucleotides. McrB (1 μM) was preincubated with radioactively labeled Sb61 (1 μM) (cf. Figure 2) containing a 5-iododeoxycytidine residue in position 12 (IdC 12) and then irradiated at 325 nM with a HeCd laser. Aliquots were taken after 15, 30, 60, and 90 min and analyzed by SDS−PAGE. The gel was silver-stained (not shown), and radioactive bands were visualized using an Instant Imager. The upper band represents the McrB−Sb61 adduct and the lower band the free DNA. The cross-link yield that was obtained was estimated to be approximately 5% for IdC 12.

fast-migrating species regardless of the presence of one or two R^{m}C sites. This is not consistent with the DNase I footprint results which clearly showed a distinct difference between DNA substrates with one or two R^{m}C sites (*29*), indicating occupation of one or two sites, respectively. It was argued that the apparent discrepancy is due to the fact that during a gel shift experiment labile complexes may partially dissociate. To analyze binding of McrB as well as McrBC to DNA by an equilibrium method that is sensitive to the size of the complex formed, we have carried out surface plasmon resonance experiments, which measure the change in mass at the surface of a chip. The major advantage of this technique is that protein−DNA interactions can be monitored in real time, very accurately, and very sensitively. A response of 1 RU (resonance unit) corresponds to 1 pg of protein/mm^2. Since the BIAcore measures mass differences, one can calculate the mean stoichiometry of the complexes at any given protein concentration.

For these experiments, a biotinylated DNA substrate (Sb128; cf. Figure 2) of 128 base pairs was used, which was produced by PCR with appropriate primers to introduce one or two R^{m}C sites located at positions 31 and 98, respectively. For control, an unmethylated 128mer was also produced. These three DNA substrates were coupled to the same streptavidin-coated sensor chip using three of the four available flow cells. McrB binding to these substrates was assessed in parallel. For the evaluation of the sensorgrams, the signal due to nonspecific binding of McrB to unmethylated DNA was subtracted from the signal obtained for specific binding to the DNA with one or two R^{m}C sites. Figure 5 shows the binding curves obtained with McrB$^{1-162}$, at different concentrations, and the DNA substrate with one

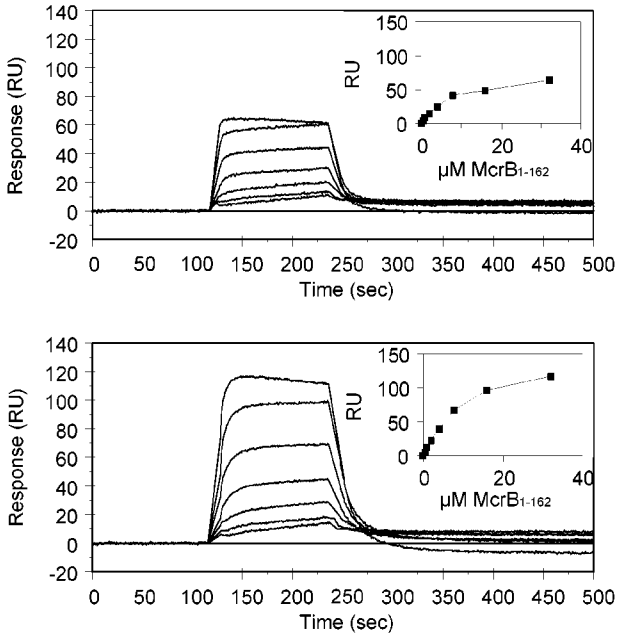

FIGURE 5: Surface plasmon resonance experiments with McrB$^{1-162}$ and DNA containing one or two R^{m}C sites. Shown are sensorgrams obtained with 0.5, 1, 2, 4, 8, 16, and 32 μM McrB$^{1-162}$ interacting with immobilized Sb128 (cf. Figure 2) with one R^{m}C site (top) and Sb128 with two R^{m}C sites (bottom). The association phase begins at approximately 120 s and the dissociation phase at approximately 240 s. Show in the insets are the binding isotherms derived from these experiments.

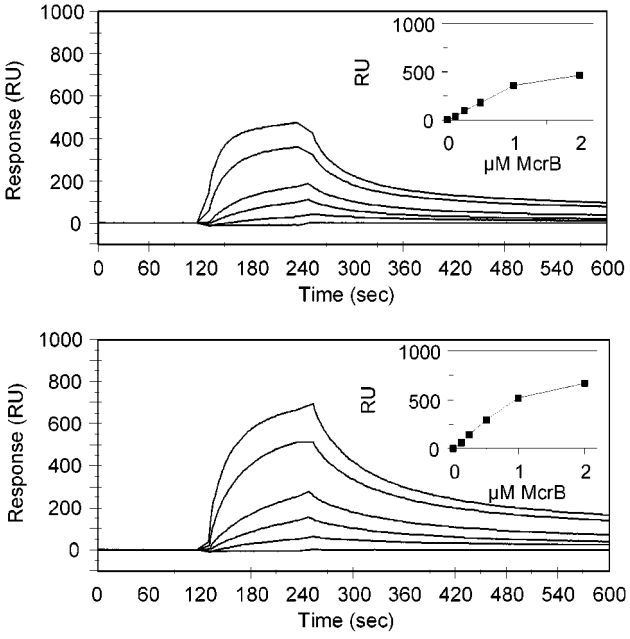

FIGURE 6: Surface plasmon resonance experiments with McrB and DNA containing one or two R^{m}C sites. Shown are the sensorgrams obtained with 0, 0.125, 0.25, 0.5, 1, and 2 μM McrB interacting with immobilized Sb128 (cf. Figure 2) with one (top) or two R^{m}C sites (bottom) in the presence of 1 mM GTP. The association phase begins at approximately 120 s and the dissociation phase at approximately 250 s.

or two R^{m}C sites. It is evident that the two-site substrate binds approximately twice as much McrB$^{1-162}$ as the one-site substrate. The comparison of the amplitude (in resonance units, RU) obtained after binding of near-saturating amounts of McrB$^{1-162}$ to the DNA (at 32 μM McrB$^{1-162}$, 65 RU for the one-site substrate and 120 RU for the two-site substrate) with that obtained after coupling of the DNA to the sensor chip (200 RU) allows us to calculate the stoichiometry of the complex to be approximately 1:1 for the one-site substrate and 2:1 for the two-site substrate [considering the molecular mass of the DNA (84 kDa) and the protein (GST−McrB$^{1-162}$, 45.5 kDa) as well as the amount of DNA immobilized on the chip (200 pg)]. This means that one molecule of McrB$^{1-162}$ is bound per R^{m}C site. The half-life of the McrB$^{1-162}$−DNA complex is on the order of a few seconds.

Similar experiments were carried out with full-length McrB in the presence of a large excess of GTP. Figure 6 shows that with regard the occupation of one or two sites qualitatively similar results were obtained for McrB and McrB$^{1-162}$, with considerably more McrB bound to the two-site substrate than to the one-site substrate. However, there is a quantitative difference; for a 1:1 complex, we would expect approximately 140 RU for the one-site and 280 RU for the two-site substrate (considering the moleculear mass of 81 kDa for the GST−McrB fusion protein). The values obtained at the highest McrB concentration of 2 μM are 450 and 700 for the one- and two-site substrates, respectively, implying that at saturation which was not reached in the experiment, at least four McrB molecules are bound per R^{m}C site. This means that McrB molecules interact with each other, not only in the absence of DNA as shown recently by Panne et al. (*35*) but also in its presence. The fact that McrB$^{1-162}$ molecules do not interact with each other suggests that the C-terminal domain is responsible for the interaction. This is in agreement with results of Panne et al. (*35*), which show that McrB$_S$ (i.e., McrB$^{162-459}$) as well as McrB$_L$ (i.e., full-length McrB) forms heptameric oligomers.

The half-life of the McrB−DNA complex is on the order of 0.5 min, which means that this complex is approximately 10 times more stable than the McrB$^{1-162}$−DNA complex.

In the absence of GTP, less binding of McrB was observed (data not shown), in agreement with the results of Stewart et al. (*29*), who demonstrated that GTP increases the affinity of McrB for DNA.

Different from McrB$^{1-162}$, McrB shows a slight negative cooperativity in binding to DNA with two R^{m}C sites (compare Figures 5 and 6); we attribute this to the narrow spacing (66 base pairs) of the two R^{m}C sites, which could lead to some steric interference of two McrB molecules (possibly due to the GST tag) when bound side by side on the DNA.

Surface plasmon resonance experiments were also carried out to analyze the effect of McrC on McrB binding to DNA. The sensorgrams in Figure 7 were obtained at a single McrB concentration. The signals were recorded for the DNA substrates with no, one, or two R^{m}C sites and corrected for nonspecific effects of protein binding to the streptavidin-coated sensor chip as well as nonspecific binding of McrC to the methylated DNA substrate. The first phase (1) characterizes the binding of 100 nM McrB in the presence of GTP to the DNA substrates; there is negligible binding to the DNA without R^{m}C sites, and more binding to the DNA with two sites than to the DNA with one site. The second phase (2) is started with the injection of 100 nM McrB and 200 nM McrC, which leads to a very large increase in the amplitude of the signal. The complex formed by McrBC and the two-site substrate is much larger than the complex formed

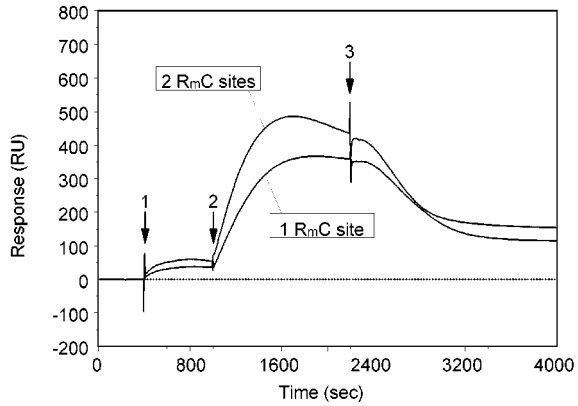

FIGURE 7: Surface plasmon resonance experiments with McrBC and DNA containing one or two R^mC sites. Shown are the sensorgrams obtained with McrB and McrC with immobilized Sb128 (cf. Figure 2) with one (lower trace) or two R^mC sites (upper trace) in the presence of 1 mM GTP. Marked with 1 is the beginning of the association phase with McrB alone (0.1 μM), with 2 the beginning of the association phase with McrB (0.1 μM) and McrC (0.2 μM), and with 3 the dissociation phase. The decrease in the magnitude of the signal observed with the substrate containing two R^mC sites after the plateau is reached could be due to the DNA cleavage activity of McrBC in the presence of GTP.

by McrB and this substrate (compare 450 and 50 RU). Furthermore, the McrBC−DNA complex is also more stable, with an estimated half-life of several minutes. As the surface plasmon resonance experiment only allows measurement of differences in mass, nothing can be said about the stoichiometry of the complexes. It is noteworthy that for McrBC, there is again a difference in size between the complexes formed with the one- and two-site substrates, but the relative difference is smaller than that between the corresponding McrB and $McrB^{1-162}$ complexes.

Scanning Force Microscopy of McrB− and McrBC−DNA Complexes. To obtain an idea of what the complexes formed by McrB and McrBC with their DNA substrate look like, scanning force microscopy (SFM) experiments were carried out. As a substrate, a restriction fragment (Pf1793; cf. Figure 2) of 1793 base pairs with two potential R^mC sites at a distance of 269 base pairs was used, which was methylated in vitro with M.*Msp*I. These R^mC sites are located at positions 1339 and 1612; their asymmetric location, close to one end of the DNA, should facilitate the identification of specifically bound proteins. Figure 8 shows in panels A and B images of the restriction fragment (2.5 nM) obtained in the presence of 3 nM McrB and 1 mM GTP. DNA molecules with one protein bound at a position corresponding to one of the two R^mC sites and DNA molecules with both R^mC sites occupied were seen. Only a few nonspecifically bound McrB molecules were observed. Figure 8 shows in panel C an SFM image of the restriction fragment (3 nM) in the presence of both 9 nM McrB and 9 nM McrC as well as 1 mM GTP. Although Pf1793 is a good substrate for McrBC, no significant cleavage was expected under the conditions of sample preparation (mixing on ice, immediate drying after deposition of the sample on the mica surface), and almost no small DNA molecules (i.e., fragments) were seen on the micrographs. Instead, DNA molecules with large "blobs" located at one end were observed, which we interpret to correspond to the high-molecular mass complexes detected by surface plasmon resonance (see above) and by gel

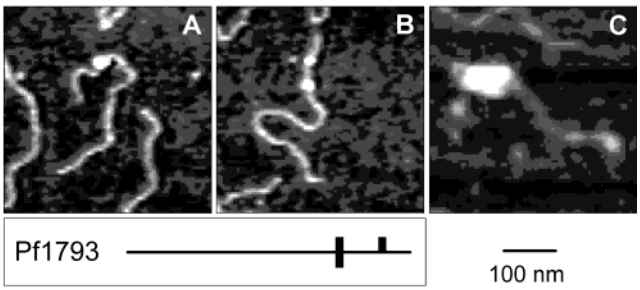

FIGURE 8: Scanning force microscopy of McrB− and McrBC−DNA complexes. The images shown [(A) and (B) 2.5 nM Pf1793 and 3 nM McrB and (C) 2.5 nM Pf1793, 9 nM McrB, and 9 nM McrC] were obtained using the tapping mode in air. On the bottom of the figure a map of the substrate used, Pf1793 (cf. Figure 2) which contains two R^mC sites, is shown with the location of the two R^mC sites. Note that in the McrB−DNA complex (B) both R^mC sites are occupied and that the DNA between the R^mC sites is free of protein. In contrast, in the McrBC−DNA complex (C), the DNA between and around the two R^mC sites is not free. The "blob", therefore, represents McrBC and associated DNA.

electrophoretic mobility shift experiments (*26*, *27*, *29*). In these complexes, both R^mC sites are involved and no free DNA was seen between these sites (269 base pairs) and next to the distal site (178 base pairs away from the end of the DNA), indicating that these parts of the DNA are covered by protein in these complexes.

DNA Cleavage Experiments with McrBC. To analyze the kinetics of DNA cleavage by McrBC, it is advantageous to have a simple substrate. For McrBC, this was originally proposed to be a DNA with two R^mC sites at a distance of 40−80 base pairs (*22*). Later it was shown that the optimal spacing is approximately 55−103 base pairs, with detectable cleavage also observed at a spacing of 32−2000 base pairs; no cleavage was seen with a spacing of ≤22 base pairs or with 3000 base pairs between the two R^mC sites (*23*). We have prepared several such substrates (Figure 2) conforming to this definition (having 34−51 base pairs between two McrBC recognition elements), which, however, were not cleaved by McrBC (viz. Sb61, Sb73, and Sb82a and -b; cf. Figure 2), presumably because the DNA flanking the R^mC sites is too short and not because the spanning is not sufficient (Sb234 with a spacing of 34 base pairs is cleaved). A substrate that is cleaved (Sb128; cf. Figure 2) has 29 base pairs outside of the two R^mC sites which are separated by 66 base pairs.

To analyze the kinetics of cleavage of Sb128 by McrBC, the DNA was radioactively labeled at the 5′-end of the lower strand (using a labeled primer during PCR) and incubated with McrB and McrC in the presence of GTP. Reaction products were analyzed by electrophoresis on denaturing 6% polyacrylamide gels. Figure 9 shows the results of the product analysis. Five groups of fragments are produced in a time-dependent manner, differing in length by 10 nucleotides. Each group comprises two major products and several minor ones differing in length by one nucleotide. The group with the smallest fragments (comprising 39 and 40 nucleotides) results from cleavage eight and nine nucleotides downstream of the ^{5m}C at position 31. The group with the largest fragments (comprising 81 and 82 nucleotides) results from cleavage 50 and 51 nucleotides downstream of ^{5m}C at this position. The major cleavage sites in the Sb128 substrate are 29 and 30 nucleotides downstream of ^{5m}C, followed by

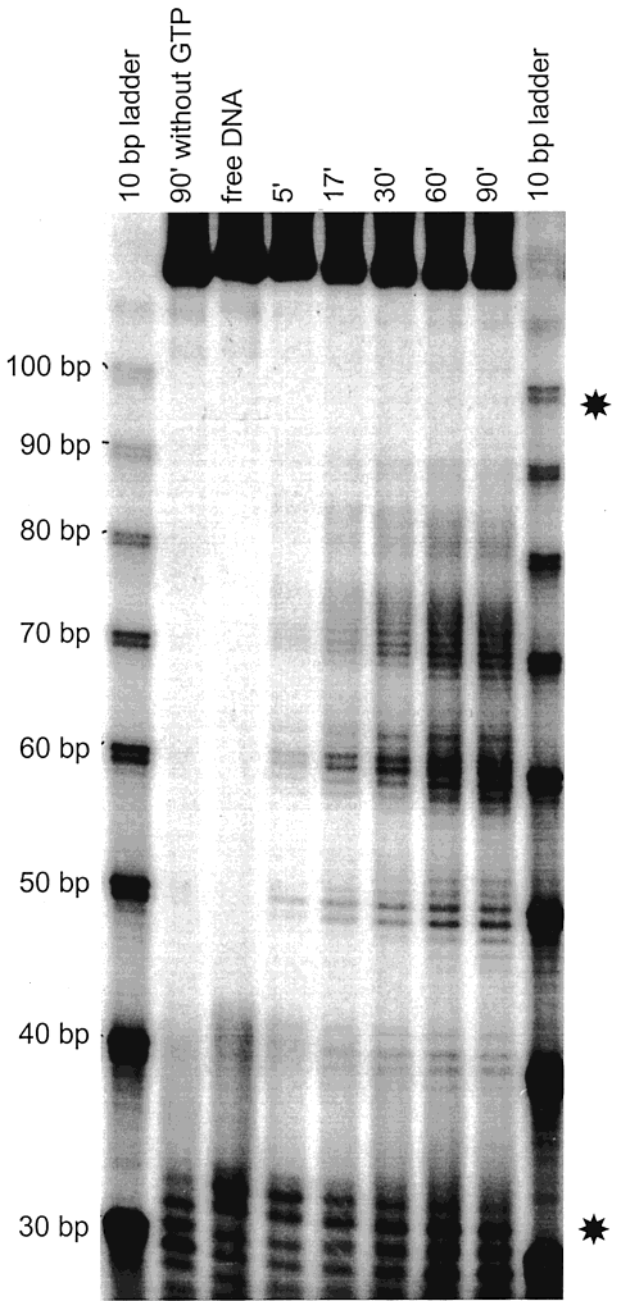

FIGURE 9: Product analysis after cleavage of a minimal DNA substrate by McrBC. A "minimal" substrate, Sb128 (cf. Figure 2), radioactively labeled on the 5′-end of the lower strand only, was incubated with 4 μM McrB and 1 μM McrC in the presence of 1 mM GTP. After 5, 15, 30, 60, and 90 min, aliquots were withdrawn and the product mixture was analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. The first and last lanes show a 10-base pair ladder; the second lane shows the DNA substrate after incubation for 90 min with McrBC in the absence of GTP, and the third lane shows the DNA substrate. The positions of the methylated cytosine bases are marked with asterisks.

sites 39 and 40 nucleotides away. Very faint bands are also seen 60 nucleotides away. We have also observed the 10-base pair periodicity for other substrates with two R^mC sites, viz., Sb157 and Sb234 (cf. Figure 2). This periodicity corresponds to the double-helix repeat length. This may be a coincidence, but may also reflect the fact that McrC attacks phosphodiester bonds presented on the surface of a large complex, in which several McrC molecules are organized in a defined manner.

DISCUSSION

The restriction endonuclease McrBC has three activities acting in concert to achieve DNA cleavage: (1) GTP hydrolysis, (2) DNA recognition, and (3) DNA translocation. GTP hydrolysis and DNA recognition are activities inherent to McrB and take place also in the absence of McrC. DNA translocation is needed to bring the two R^mC sites occupied by McrB together and like DNA cleavage seems to require the cooperation of McrB and McrC. Although recent studies have helped to elucidate details of the mechanism of action of McrBC (*24*, *29*, *35*), several aspects remained unclear. The major unresolved issue concerns the assembly, structure, and function of the *active* McrBC−DNA complex.

We have studied the interaction of the DNA-binding domain of McrB (i.e., $McrB^{1-162}$), full-length McrB, and McrC with DNA having one or two R^mC sites by several techniques, viz., DNase footprinting, photo-cross-linking, surface plasmon resonance, and scanning force microscopy. On the basis of these studies, we arrive at the following conclusions, which confirm and extend previously published notions concerning the mechanism of action of McrBC.

Our *Serratia* nuclease footprinting and surface plasmon resonance experiments show that the N-terminal domain of McrB, which harbors the DNA binding site but lacks the region involved in GTP binding and hydrolysis, like McrB (*29*) binds specifically to DNA having only one R^mC site. A second R^mC site, if present on the same DNA, is occupied independently. With full-length McrB, occupation of the second site seems to be slightly hindered, when the sites are not far apart (in our experiments this could also be the consequence of the GST tag). In the McrB−DNA complex, there is a close contact between the protein and the 5-methylcytosine of the R^mC site, mimicked by 5-iodocytosine in our photo-cross-linking experiments, and its 3′-neighbor.

Whereas only one molecule of the N-terminal domain of McrB is bound per R^mC site, at least four molecules of full-length McrB occupy one R^mC site. This is a lower estimate, based on the results of surface plasmon resonance experiments. It is compatible with the results of Panne et al. (*35*), who found that McrB in the absence of DNA and not dependent on GTP hydrolysis forms ringlike structures consisting of seven McrB molecules. The complex between full-length McrB and DNA is considerably more stable than the complex between the N-terminal domain and DNA; it becomes even more stable when GTP is present. A similar observation was made by Stewart et al. (*29*) with GTPγS, based on the results of quantitative DNase footprint experiments.

In the presence of GTP and McrC, McrB forms high-molecular mass complexes with DNA, which are considerably larger than the complexes with McrB and DNA, as demonstrated by surface plasmon resonance. This finding is consistent with the results of electrophoretic mobility shift experiments in which McrBC−DNA complexes of very low electrophoretic mobility were detected (*26*, *27*, *29*). Their size can be explained in part by the dimerization of the heptameric rings of McrB which is promoted by McrC even in the absence of DNA, as seen by scanning transmission electron microscopy (*35*), and in part by the association of McrC with McrB. Thus, a dimerization of heptameric McrB

rings (*35*) and the stoichiometric binding of approximately one McrC molecule to one McrB molecule (*25*) could explain the formation of the high-molecular mass complexes and the mass increase.

Scanning force microscopy confirms the formation of high-molecular mass complexes between McrB, McrC, and DNA, in the presence of GTP. Whereas McrB in the absence of McrC occupies two R^mC sites 269 base pairs apart independently, in the presence of GTP and McrC the McrB-occupied R^mC sites are "pulled" together to form a high-molecular mass complex of unknown stoichiometry.

We suggest that this complex is enzymatically active and cleaves the DNA, which appears to be tightly associated with the protein in the complex, because no free DNA is seen in the micrographs between and around the two R^mC sites. In this tight association, the DNA around the R^mC sites must be ordered, because cleavage occurs between the two R^mC sites (as demonstrated for a 128mer substrate with two R^mC sites 66 base pairs apart) approximately 10, 20, 30 (preferentially), 40, and 50 base pairs away from the R^mC site, i.e., in a periodicity representing the helical repeat. This is reminiscent of the cleavage pattern a nonspecific nuclease produces with a DNA spread out on a surface (*45*).

On the basis of the new results reported here and recent results from the laboratories of Bickle and Raleigh (*24*, *29*, *35*) a model for the mechanism of action of McrBC can be proposed. This model relies on analogies with two other families of nucleoside triphosphate-dependent restriction enzymes, the type I and type III restriction enzymes. Like McrBC, they require binding to two recognition sequences on linear DNA substrates which can be thousands of base pairs apart and are occupied independently. In a process requiring ATP, the DNA is translocated, while the proteins remain bound to their recognition sites, producing loops (*46*, *47*). Cleavage occurs when the two proteins collide or when the DNA cannot be translocated any further. A stalling translocation complex is also obtained when there is only one recognition site on a circular DNA or when a firmly bound protein is present on a linear DNA with only one recognition site (*30*, *32*, *33*, *48*−*53*). Type III enzymes, similar to McrBC, cleave the DNA close to one of the recognition sites, whereas type I enzymes can cleave the DNA far away from any of the recognition sites.

For the type I enzyme *Eco*KI, it was recently shown that it dimerizes on the DNA, thereby looping out the DNA (*54*, *55*), similar to the results described also for some type II restriction enzymes [F*ok*I (*56*, *57*) and S*au*3AI (*58*)]. *Eco*KI dimerization, however, is followed by translocation which requires ATP hydrolysis. For McrBC, a similar scenario might apply, considering that in the absence of McrC it predominantly forms heptameric rings which dimerize in the presence of McrC as investigated by scanning transmission electron microscopy (*35*). Our surface plasmon resonance results suggest that dimerization occurs also in the presence of a DNA substrate with two recognition sites. Dimerization and GTP-dependent translocation could be independent events, but could also happen sequentially, i.e., dimerization preceding DNA translocation.

The details of the translocation process remain to be analyzed, for example, whether the DNA is threaded through the hole in the McrB ring. One could speculate that DNA cleavage occurs after translocation has come to a stop, presumably because upon stalling the DNA is pulled into the active site of McrC. A straightforward explanation for our observation that the cleavage sites are regularly spaced approximately 10, 20, 30, 40, and 50 base pairs away from an R^mC site could be due to the association of DNA with the surface of the McrBC complex. Depending on which McrC subunit associated with individual McrB molecules organized in a ringlike structure (*35*) is the first to make the required tight contact for cleavage, a phosphodiester bond approximately 10, 20, 30, 40, and 50 base pairs away from the R^mC site is attacked. The size of the McrBC ring, with a diameter of approximately 10−15 nm (*35*), could explain the observed periodicity of 10 base pairs (3.4 nm), if one assumes that McrC can be associated with each individual McrB subunit.

There is yet another problem that needs to be addressed. McrBC cleaves both strands of the DNA. If this is happening within one binding event, this necessitates that two McrC molecules, each with one active center (*62*) must cooperate. We suggest that this is done in the same manner as described for the type IIs restriction enzyme *Fok*I, which has a DNA-binding domain (corresponding to McrB) and a separate cleavage domain (corresponding to McrC) (*59*). *Fok*I, as mentioned above, requires two recognition sites for cleavage and dimerizes on the DNA via its cleavage domain (*56*). Concerted double-strand cleavage occurs a few base pairs away from the recognition site (*60*). It is known from the work of Panne et al. (*35*) that the heptameric rings of McrB can dimerize to produce a tetradecameric double ring, and that in the absence of McrC this equilibrium is on the side of the heptamer. In the presence of McrC, the equilibrium is shifted toward the tetradecamer. This finding could be interpreted to mean that McrC is directly involved in the dimerization process, thereby arranging two catalytic centers such that a concerted double-strand cleavage can occur, as observed for *Fok*I. This is a plausible model, but further experiments are required to reveal the precise stoichiometry and organization of the enzyme complex on the DNA.

In conclusion, McrBC is a restriction enzyme with unique features, namely, being dependent on GTP and forming high-molecular mass oligomers. On the other hand, it shares with type I and III restriction enzymes the functional organization (having separate subunits for DNA binding and cleavage), the requirement for nucleoside triphosphate-fueled DNA translocation, and the amino acid composition of the active site for DNA cleavage.

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