Bacteriophage T4 endonuclease II, a promiscuous GIY-YIG nuclease, binds as a tetramer to two DNA substrates

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ABSTRACT

The oligomerization state and mode of binding to DNA of the GIY-YIG endonuclease II (EndoII) from bacteriophage T4 was studied using gel filtration and electrophoretic mobility shift assays with a set of mutants previously found to have altered enzyme activity. At low enzyme/DNA ratios all mutants except one bound to DNA only as tetramers to two DNA substrates. The putatively catalytic E118 residue actually interfered with DNA binding (possibly due to steric hindrance or repulsion between the glutamate side chain and DNA), as shown by the ability of E118A to bind stably also as monomer or dimer to a single substrate. The tetrameric structure of EndoII in the DNA–protein complex is surprising considering the asymmetry of the recognized sequence and the predominantly single-stranded nicking. Combining the results obtained here with those from our previous *in vivo* studies and the recently obtained crystal structure of EndoII E118A, we suggest a model where EndoII translocates DNA between two adjacent binding sites and either nicks one strand of one or both substrates bound by the tetramer, or nicks both strands of one substrate. Thus, only one or two of the four active sites in the tetramer is catalytically active at any time.

INTRODUCTION

The GIY-YIG endonuclease II (EndoII) of coliphage T4, encoded by gene *denA*, catalyzes the initial step in host DNA degradation which causes irreversible host-shutoff and also initiates a nucleotide scavenge pathway that provides precursors for phage DNA synthesis (1). Phage DNA is protected from EndoII by substituting hydroxymethylated cytosines for cytosines in its own DNA (2,3). EndoII nicks DNA lacking cytosine modification at sites where only a CG base pair three positions away from the scissile bond is strongly conserved (4). Double-stranded cleavage by EndoII is the result of concerted single-strand nicks (5), but even at cleavage sites most often only one strand is nicked (4). EndoII shares the sequence elements defining the GIY-YIG family of proteins (6,7) and residues previously implied in catalysis by GIY-YIG enzymes as well as residues conserved in the N-terminal region (NTR) and middle region (MR) of EndoII are important in binding as well as catalysis by this enzyme (4).

Two members of the GIY-YIG family of endonucleases, each containing one copy of the GIY-YIG motif (6), have been extensively characterized. The homing endonuclease I-TevI binds as a monomer (8,9); the nucleotide excision repair endonuclease UvrC is a monomer in solution (10) and binds as a monomer at a $UvrB_2$ complex (11) at damaged DNA, though it can also bind as a tetramer to any double-stranded DNA (12). In addition to the GIY-YIG domain, these enzymes have separate domains that confer most of the binding strength and specificity. The GIY-YIG restriction endonucleases Eco29kI and Cfr42I, like EndoII, are likely to lack separate DNA-binding domains. Eco29kI is a monomer in solution (13) while its isoschizomer Cfr42I (32% sequence identity) is a tetramer in solution, suggesting that the enzyme also binds as a tetramer (14). These two restriction endonucleases make double-stranded cuts within their recognition sequence CCGC↓GG. Hence, GIY-YIG enzymes appear to apply a number of different strategies to bind to their recognition sequence, depending on additional modules but also on variations in the GIY-YIG module itself.

Analysis of a set of 13 EndoII mutants with reduced catalytic capacity, permitting their overexpression and purification, has shown that all but one form strongly retarded complexes with DNA in electrophoretic mobility shift assays [EMSA (4)]. The exception is a mutant where the conserved magnesium-coordinating and putatively

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catalytic glutamate residue (E118) is replaced by alanine, yielding quite fast-moving complexes (4). We present here an analysis of the oligomeric structure of EndoII and its mode of binding to its substrate using some of these mutants. The mutation K12A, located in the NTR, is primarily important for stable binding to DNA, while the mutation K76A, located in the MR, is important for recognition as well as stable binding. Mutations of strictly conserved GIY-YIG glycine and arginine residues, G49A and R57A, cause altered sequence recognition and poor binding, while mutation of the conserved glutamate residue E118A in addition to permitting formation of fast-moving enzyme-substrate complexes abolishes catalysis (4).

Our analysis showed that all mutants investigated form dimers and tetramers in solution and that the primary enzyme–DNA complex most likely consists of a tetramer binding to two DNA molecules. The catalytically inactive E118A mutant additionally forms stable complexes containing monomers and dimers bound to single DNA molecules, suggesting that the wildtype glutamate residue actually interferes with substrate binding.

MATERIALS AND METHODS

Strains and plasmids

EndoII was encoded by plasmids carrying the *denA* gene in-frame with a PelB leader peptide (altogether 31-amino acids) and six His residues at its N-terminus (15) (total molecular mass 19.8 kDa); constructs expressing E118A and R57A were prepared also without the PelB leader with only an MHHHHHH peptide at the N-terminus of EndoII (total molecular mass 16.8 kDa). *Escherichia coli* BL21(DE3)·pLysS (Novagen) was used for overexpression of EndoII. Plasmids are listed in Supplementary Table S1 of ref. (4). Plasmid DNA was purified by Qiaprep Spin Miniprep Kit (Qiagen) and DNA concentrations estimated by EtBr fluorescence or using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Oligonucleotides, radiolabelling and polymerase chain reaction

Oligonucleotides (Figure S1) were purchased from Sigma Genosys. Radiolabelling and polymerase chain reactions were carried out as previously described (4).

Preparation and activity assays of EndoII

Mutant EndoII was overexpressed from plasmids *in vivo* and purified by affinity chromatography, using HiTrap Chelating HP columns charged with $NiSO_4$ (Amersham Pharmacia Biotech), eluting and desalting as previously described (4). EndoII E118A and R57A concentrations were determined using a BioRad protein assay with bovine gamma globulin as standard; concentrations of other mutant enzymes were determined by comparing staining intensities of the EndoII bands in western blots relative to those of different amounts of EndoII R57A analyzed on the same blot, as previously described (4).

Endo II nicking activity was assayed as previously described (4).

Protein gels and western blots

Proteins were analyzed on discontinuous 5% (stacking) 14% (separating) sodium dodecyl sulfate polyacrylamide gels (37.5 : 1, BioRad) with 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3 as running buffer. Gels were run in a Mini protean II cell apparatus (BioRad) at 170 V for 65 min. After electrophoresis the gels were fixed and silver stained essentially as described by Oakley *et al.* (16) and finally dried between cellophane sheets (for qualitative analysis), or transferred to Immobilon-P (Millipore) transfer membranes and probed with monoclonal anti-His_6 antibody (Amersham) followed by secondary horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies (Amersham) and development with Enhanced Chemical Luminescence reagent (Amersham) and exposure to X-ray film (for quantification of EndoII, using EndoII purified R57A as standard).

EndoII binding assay

EndoII binding was analyzed by electrophoretic mobility shift assays (EMSA) as described (4), mixing varying amounts of EndoII with substrate on ice in 10 mM Tris–HCl (pH 8.3 at room temperature), 5 mM Na_2EDTA, 30 mM NaCl, 10% glycerol, 0.3 mg/ml BSA in a final volume of 10 μl and incubating at 30°C for 15 min before electrophoresis at +4°C on 5% (37.5 : 1) non-denaturing polyacrylamide gels in 1× TEB pH 8.3. Long substrates (148 bp) were prepared by polymerase chain reaction as described (4); shorter substrates were prepared by annealing oligonucleotides, 30 or 44 bp long, with the 807C cleavage site located in the middle (Supplementary Figure S1). In experiments with two competing forms of EndoII, these were mixed together before being added to the substrate; in experiments with two competing substrates these were mixed together before addition of the enzyme.

In-gel cleavage

For in-gel cleavage, gel slices from EMSA gels were cut out and soaked in cleavage buffer (4), which contains 10 mM $MgCl_2$, for 5–15 min and then crushed with a pipette tip and eluted overnight in 1 mM Na_2EDTA pH 8, 10 mM NaCl. The samples were then analyzed by electrophoresis in 15% polyacrylamide (37.5 : 1) gels containing 7 M urea in 1× TEB (4).

Gel filtration and crosslinking

Gel filtration assays were run on SMART System from Pharmacia at 4°C. Ten to forty micrograms of enzyme in 20 μl of 50 mM sodium phosphate pH 7.9, 20% glycerol and 10 mM or 1 M NaCl, with or without 1 μg of 30 bp substrate, was loaded on a Superdex 200 PC 3.2/30 column (Pharmacia Biotech) equilibrated in 50 mM sodium phosphate pH 7.9, 40 or 155 mM NaCl (these differences did not affect the results) and 10% glycerol. Samples with enzyme and substrate were incubated at

30°C for 15 min to allow binding before being subjected to gel filtration. The eluate was collected in 25 μl fractions. For size determination the column was calibrated with ribonuclease A, ovalbumin, aldolase and ferritin from the Gel Filtration Calibration Kits (LMW or HMW) (GE Healthcare). Crosslinking was performed with bis[sulfosuccinimidyl]suberate (BS^3) (Pierce Biotechnology) as described by the manufacturer except for using 10 mM BS^3 for 15 min.

RESULTS

EndoII forms dimers and tetramers in solution

Gel filtration analysis showed that EndoII G49A with PelB as well as R57A and E118A lacking PelB are present as dimers in solution (Figure 1) under the low-salt conditions used for our activity assay and EMSA (4). Monomers formed a small fraction of the total enzyme population (data not shown). At higher salt concentrations also tetramers were formed (see Figure 7); both EndoII dimers and the size standards eluted at the same times at both salt concentrations. Preferential formation of tetrameric EndoII at high-salt concentrations is consistent with the crystal structure of EndoII showing hydrophobic interactions in the tetramerization interface (Andersson *et al.*, manuscript in preparation). The apparent sizes of the proteins were ~90–95% of what was expected for dimers and tetramers, suggesting an enzyme slightly more compact than those used for calibration. Peak fractions were subsequently subjected to SDS–PAGE followed by Coomassie or silver staining, with or without prior crosslinking with BS^3. Crosslinked material from the fractions that were expected to contain tetramers (gel filtration at 1 M NaCl initial concentration) revealed monomers, dimers, trimers
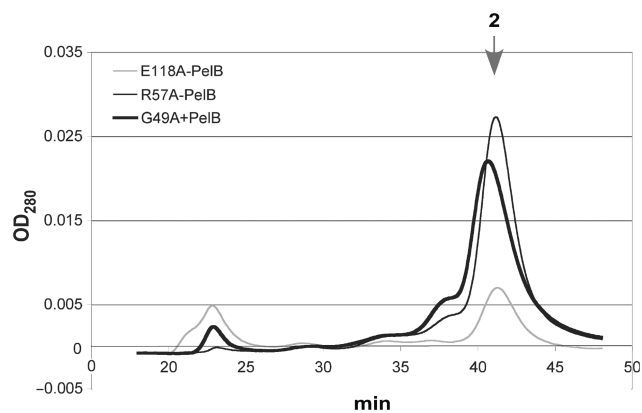
and tetramers (Figure 2) confirming the multimerization of the enzyme. Material stuck at the top of the separating gel may be aggregates. The occurrence of trimers, dimers and monomers in the crosslinked tetramer material could reflect instability of the tetramer (not all remaining tetrameric upon concluded gel filtration) or incomplete penetration of the crosslinking agent. The total intracellular concentrations of cations in *E. coli in vivo* are quite high, ~220 mM K$^+$ and ~80 mM Na$^+$ in exponentially growing cells in media with approximately physiological osmolarity (17), though some of these ions likely are bound to various intracellular constituents. Thus, higher order multimers of EndoII may be common *in vivo*.

EndoII needs >30 bp for stable binding

Sites recognized by EndoII *in vivo* or *in vitro* are characterized by a 16 bp ambiguous sequence (15,18). Although EndoII does nick a substrate where the recognition site is embedded in a 24-bp oligonucleotide duplex (5), this nicking is erratic and no stable gel shifts were found with this substrate (data not shown), suggesting that stable binding of EndoII requires more DNA than the 16 bp that are conserved around the scissile bond. We prepared blunt-ended 30 and 44 bp substrates containing the same 23 bp 807C core as the previously used 148 bp substrate (4) flanked by random GC-poor DNA (sequences shown in Supplementary Figure S1), and tested them with different EndoII mutants.

The 44 bp substrate was nicked reproducibly at the same positions as previously found for the 807C site (4,5) (data not shown), showing it is long enough to function as substrate for EndoII. Also with this short substrate, the E118A enzyme formed fast-moving
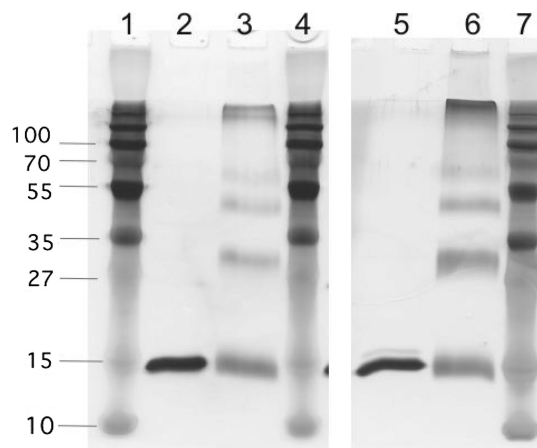

Figure 1. Gel filtration of EndoII G49A + PelB (black, thick line), EndoII R57A–PelB (black, thin line) and E118A–PelB (gray) at 10 mM initial NaCl concentration in the sample. The concentration of NaCl in the column was 155 mM. Separately filtered size standards were used to calibrate the column (elution times: ferritin, 440 kDa, 29 min; aldolase, 158 kDa, 34 min; ovalbumin, 43 kDa, 40 min; ribonuclease A, 13.7 kDa, 45 min). The arrow points to the eluted dimer with apparent sizes 37 kDa for G49A + PelB (expected 39.6 kDa); 33 kDa for EndoII R57A–PelB and E118A–PelB (expected 33.6 kDa). The peak at 23 min is the void.

Figure 2. Analysis of crosslinked EndoII R57A and E118A lacking PelB. Tetramer peaks from gel filtered samples were collected and crosslinked with BS^3. Equal amounts of crosslinked and non-crosslinked samples (from the same peak fraction) were loaded on SDS–PAGE and subsequently silver stained. Expected sizes: EndoII monomer 16.8 kDa, dimer 33.6 kDa, trimer 50.4 kDa and tetramer 67.2 kDa. Lanes 1, 4 and 7, molecular weight standards, sizes in kDa shown to the left; lane 2 R57A not crosslinked, lane 3 R57A crosslinked with BS^3, lane 5 E118A not crosslinked, lane 6 E118A crosslinked with BS^3.
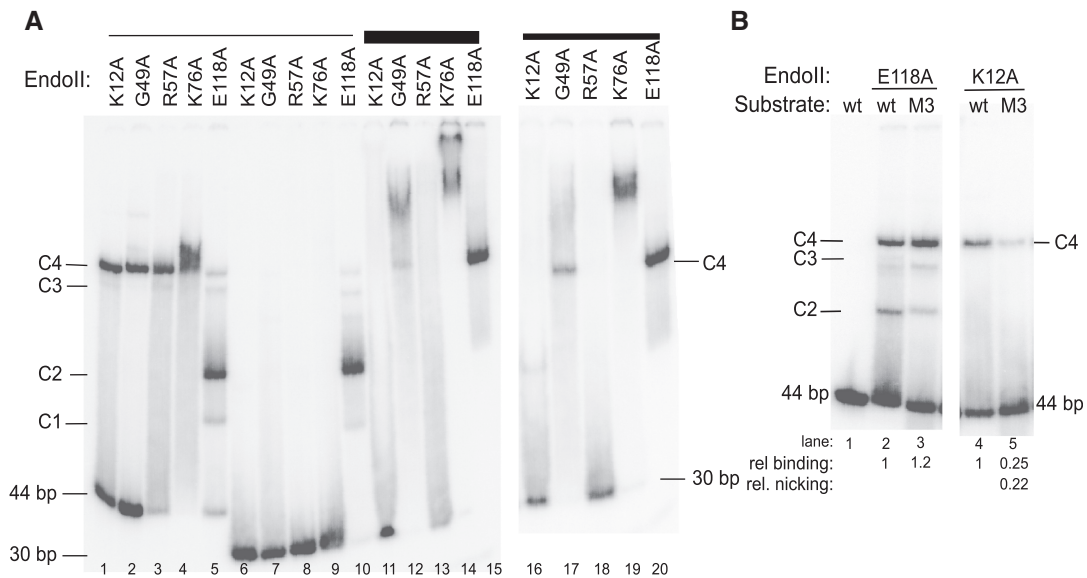
Figure 3. (**A**) EMSA with five representative mutant enzymes (K12A, G49A, R57A, K76A, E118A, all with the PelB leader) shifting the wt. 44 bp (lanes 1–5) or 30 bp (lanes 6–20) substrates. Lanes 1–15 and 16–20 are from separate gels. Enzyme quantities in lanes 1–5 were chosen to produce complexes with the 44 bp substrate (3.7 nM): K12A, 46 nM; G49A, 45 nM; R57A, 420 nM; K76A, 272 nM; E118A, 122 nM. The same amounts of the different mutant enzymes that were used to shift the 44 bp substrate in lanes 1–5 were also used with the 30 bp substrate (3.7 nM) in lanes 6–10. For lanes 11–15, 50 times as much enzyme was used, and for lanes 16–20, 20 times as much enzyme was used. C1–C4 indicate positions of different enzyme–DNA complexes. (**B**) EMSA with EndoII E118A and K12A on 44 bp substrates having (44 wt.) or lacking (44M3, see Supplementary Figure S1) preferred nick sites. The same molar ratios of the respective enzymes to substrate were used as for panel A, lanes 1–10. Numbers below the lane numbers show relative efficiency of complex formation (at standard binding conditions, 30 mM NaCl) and relative nicking efficiency (at standard nicking conditions, 1 mM NaCl). Lanes 1–3 and 4–5 are from different portions of the same gel.

complexes [C1 and C2, Figure 3, cf Figure 5 in (4)], which were absent with other mutants. The more slowly moving complex C4 was formed by all mutants; some also formed a slow-moving complex C3 in low amounts. Fewer complex species were seen with these short substrates than with the 148 bp substrate previously used (4), as expected since the 148 bp substrate contains several more binding sites. Binding of the two catalytically least active mutants, E118A and R57A, was tested also in the presence of 10 mM MgCl$_2$ without EDTA, yielding essentially the same EMSA patterns as in the absence of divalent cations (data not shown).

Also the 30 bp substrate was nicked at the same positions (data not shown), but except for EndoII E118A, enzyme quantities yielding stable complexes with the 44 bp substrate failed to do so for the 30 bp substrate (Figure 3A), showing that binding to this short substrate was less stable. EndoII E118A, which we previously identified as one of the best binding mutants (4), formed complexes to the same extent with this substrate as with the 44 bp substrate. The mutant enzymes G49A and K76A shifted the 30 bp substrate when 20-fold more enzyme was used (Figure 3A, lanes 17 and 19), while others, e.g. K12A and R57A, did not produce distinct shifted bands even with 50-fold higher enzyme concentration, (Figure 3A, lanes 11 and 13).

In Figure 3B, formation of complexes with a less efficiently nicked substrate was tested. Substrate 44M3 lacks the most prominent nick sites of the wt. 44 bp substrate (sites 2–4, Supplementary Figure S1), and was

nicked with about 5-fold lower efficiency by catalytically proficient EndoII K12A. The same complexes C1–C4 were formed by EndoII E118A and complex C4 by G49A, R57A and K12A with 44M3 as with wt. 44 (Figure 3B and data not shown). Using standard conditions for binding and nicking assays, complex formation by the cleavage-proficient EndoII K12A to this variant substrate was reduced to approximately the same extent as nicking, while the catalytically inactive E118A mutant bound equally well or better to this mutant substrate than to the wt. substrate. When both assays were performed at the same salt concentration (10 mM NaCl), EndoII K12A binding to the 44M3 substrate was less reduced than its nicking of this substrate, in comparison to activities on the wt. 44 bp substrate. Thus, EndoII appears to be able to bind to varying extent also to substrates that are not nicked. This is supported by our observation that EndoII can be chased out of its DNA complexes by poly(dAdT).

Enzyme titrations (Figure 4) showed that complexes C1 and C2 formed by EndoII E118A with the 30 bp substrate were chased into complexes C3 and C4, but no complex moving more slowly than C4 was formed with this substrate (Figure 4A, see also Figure 6B). In contrast, EndoII E118A shifted the 44 bp substrate from complexes C1 and C2 into progressively more retarded complexes C3, C4 and C5. Complex C4 formed by EndoII R57A was similarly chased into a more slowly moving complex C5 with increasing enzyme concentration (Figure 4B). The most retarded complexes C5 did not form until all the DNA was bound by the enzyme.
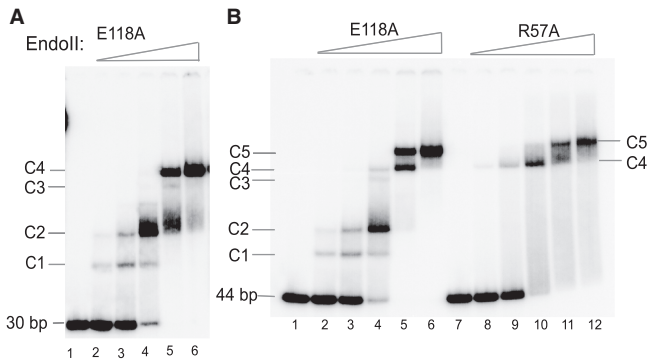
Figure 4. EMSA titration of EndoII mutants with PelB. (**A**) EndoII E118A and the wt. 30 bp substrate. Lane 1, no enzyme; lanes 2–6, 6.6, 26, 104, 415 nM; and 1.66 µM EndoII E118A per lane; all lanes, 3.7 nM of the wt. 30 bp substrate. C1–C4 indicate positions of different enzyme–DNA complexes. The smear above the C2 band at high enzyme concentrations is likely caused by degradation products from higher-order complexes. (**B**) EndoII E118A and R57A and the wt. 44 bp substrate. Lanes 1 and 7, no enzyme added. Lanes 2–6, the same amounts of EndoII E118A as for lanes 2–6 in (A); lanes 8–12, 23, 91, 367 nM, 1.46 and 5.86 µM EndoII R57A per lane; all lanes, 3.7 nM of the wt. 44 bp substrate. C1–C5 indicate positions of different enzyme–DNA complexes. Panels A and B are from different gels.

Fast-moving complexes contain one DNA molecule and an EndoII monomer or dimer

EndoII of two different sizes [with or without the PelB leader, which does not affect recognition or catalysis by EndoII (4,15), 19.8 and 16.8 kDa, respectively] were used to determine the nature of the fast-moving complexes seen with the E118A mutant. Gel shifts were performed with the two forms of EndoII separately and mixed (Figure 5), in amounts that would shift similar fractions of the substrate. As expected, the larger enzyme (carrying the leader peptide) produced more slowly moving complexes than the smaller enzyme (lacking the leader peptide) (compare lanes 1 and 4 with lanes 3 and 6, respectively). If the same bands are formed in the mixed reaction as in the separate reactions, this would indicate that only one molecule of EndoII was bound to the substrate in these bands. An additional intermediate band, not corresponding to any of the bands in the separate reactions, would indicate a mixture of two enzymes bound to the substrate, one of each size. In the region of complex C1, a mixture of the two E118A enzymes (lane 2) with the 44 bp substrate produced the same two bands as seen separately in lanes 1 and 3. Thus, this complex contains only one EndoII monomer per DNA. In the region of complex C2, an intermediate band could be seen, suggesting that this complex contains one dimer (or two monomers) per DNA. The 30 bp substrate gave the same results (lanes 4–6).

We also used the 30 and 44 bp substrates together to verify the number of DNA molecules in the E118A complexes. In the region of complex C1 two bands formed from the mixed substrates (Figure 5, lane 7), corresponding to the bands seen separately with only one substrate (lanes 6 and 8). Thus, only one DNA molecule is present in complex C1. Complexes C2 (as well as
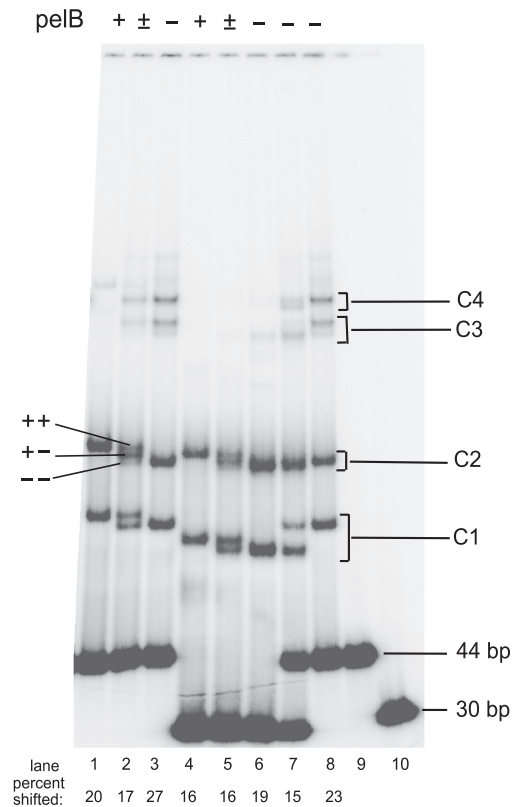

Figure 5. EMSA analysis of wt. 30 bp and wt. 44 bp substrates with E118A ± the PelB leader. The substrates were 44 bp (lanes 1–3, 8–9; 3.7 nM) or 30 bp (lanes 4–6, 10; 5.4 nM), or both together at these concentrations (lane 7). Enzyme preparations were E118A with or without the PelB leader, in quantities shifting approximately the same fraction of the added substrate (actual fractions shifted are noted below the lane numbers). For lanes 2 and 5 mixtures of the preparations with and without PelB were used. No enzyme was added for lanes 9 and 10. Arrows C1–C4 indicate the different enzyme–DNA complexes formed. Bands formed by combinations of enzyme with or without PelB in the C2 area of lane 2 are shown with arrows (+, with PelB; –, without PelB). In the areas denoted C3 and C4 several different complexes can be seen, but are not well resolved in these gels.

C3 and C4) with 30 or 44 bp DNA migrated largely at the same rate regardless of substrate length (lanes 6–8), preventing determination of their DNA content by this method. Instead a cold, 148 bp substrate was used to compete the radiolabelled 44 bp substrate (44*). If only one DNA molecule was included in each complex, the complexes containing 44* bp DNA would still migrate at the same rate while the complexes containing unlabelled 148 bp DNA would not be visible on the autoradiogram. With increasing amounts of unlabelled competitor, the 44* bp substrate would be competed out of its complexes. On the other hand, if the complexes contain more than one DNA molecule, those containing both substrates (44* and 148 bp DNA) should be visible on the gel and be significantly more retarded than those containing only 44* bp DNA molecules. Since there is more than one EndoII binding site in the 148 bp substrate (4), larger complexes could form with, for example two or three 44* bp substrates bound to the same 148 bp molecule.

Results from these competition experiments with EndoII E118A are shown in Figure 6A. At a molar
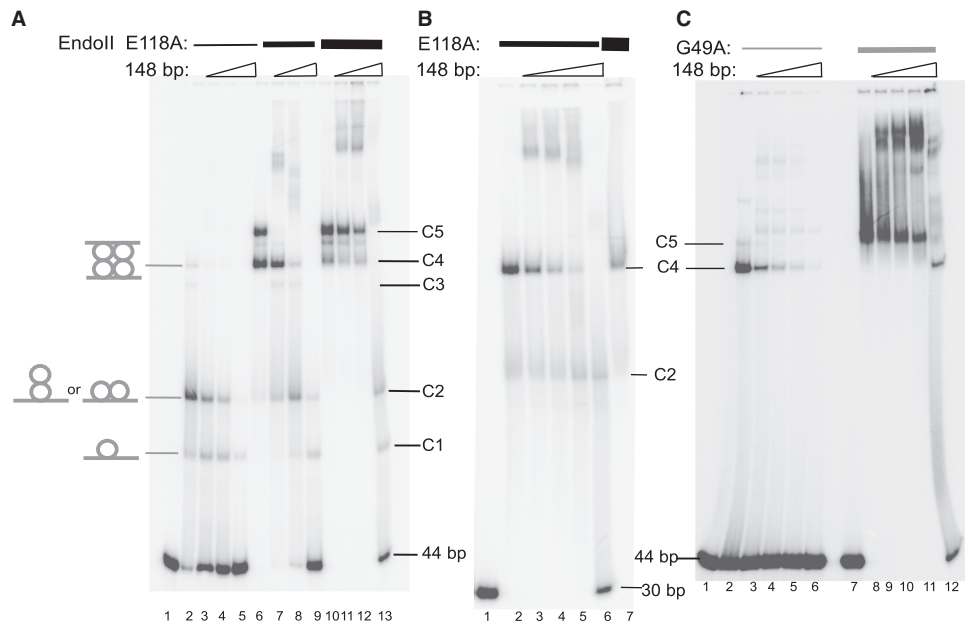
Figure 6. EMSA analysis of EndoII E118A (with PelB) binding to competing substrates. A, B and C are from different gels. (**A**) Radiolabelled wt. 44 bp substrate (1 ng; 3.7 nM) was competed by increasing amounts of cold 148 bp substrate for binding to EndoII E118A. Lane 1, no enzyme added; lanes 2, 6 and 10, no 148 bp substrate added. Lanes 2–5, 100 nM EndoII E118A; lanes 6–9 410 nM EndoII E118A; lanes 10–13, 1.66 μM EndoII E118A. Of the competitor 148 bp substrate, 1.7 ng (1.9 nM) was added for lanes 3, 7 and 11; 3.4 ng (3.8 nM) for lanes 4, 8 and 12; and 33.6 ng (37.4 nM) for lanes 5, 9 and 13. C1–C5 indicate positions of different enzyme–DNA complexes; drawings to the left indicate probable compositions of the complexes. (**B**) Radiolabelled wt. 30 bp substrate (5.4 nM per assay) was competed by increasing amounts of cold 148 bp substrate for binding to EndoII E118A. Lane 1, no enzyme added; lanes 2 and 7, no 148 bp substrate added. Lanes 2–6, 1.95 μM EndoII E118A per lane; lane 7, 31.2 μM EndoII E118A. Of the competitor 148 bp substrate, 1.7 ng (1.9 nM) was added for lane 3; 3.4 ng (3.8 nM) for lane 4; 8.4 ng, 9.3 nM for lane 5; and 33.6 ng (37.4 nM) for lane 6. C2 and C4 indicate positions of different enzyme–DNA complexes. (**C**) EMSA analysis of EndoII G49A (with PelB) binding to competing substrates of different sizes. Radiolabelled wt. 44 bp substrate (1 ng per assay; 3.7 nM) was competed by increasing amounts of cold 148 bp substrate for binding to EndoII G49A. Lanes 1 and 7, no enzyme added; lanes 1, 2, 7 and 8, no 148 bp substrate added. Lanes 2–6, 43 nM EndoII G49A per lane; lanes 8–12, 2.26 μM EndoII G49A per lane. Of the competitor 148 bp substrate, 1.7 ng (1.9 nM) was added for lanes 3 and 9; 3.4 ng (3.8 nM) for lanes 4 and 10; 8.4 ng (9.3 nM) for lanes 5 and 11; and 33.6 ng (37.4 nM) for lanes 6 and 12. C4 and C5 indicate positions of different enzyme–DNA complexes.

ratio EndoII E118A/44* bp substrate of 27:1 primarily the fast-moving complexes C1 and C2 were formed (lanes 2–5). Addition of the competitor did not result in altered migration rates for these complexes, confirming that they only contain one DNA molecule each.

Slow-moving complexes contain more than one DNA molecule and more EndoII than one dimer

Since complexes C1 and C2 could be chased into C3 and C4 with progressively more enzyme added (Figure 4B), these slow-moving complexes must contain more EndoII than one dimer. Using higher molar ratios EndoII E118A/ 44* bp substrate of 110:1 or 450:1 virtually all of the substrate was found in the slow-moving complexes C4 and C5 (Figure 6A, lanes 6 and 10). When the 148 bp substrate was added to these complexes (lanes 7–9, 11–13) additional complexes with increased migration rate were formed. Complex C5 disappeared completely even with the lowest amount of competitor, and since the amount of faster-moving material did not increase in proportion, at least some of the label in this complex must be present in the newly-formed slow-moving complexes in lane 7. Thus, complex C5 must contain more than one DNA molecule. The nature of complex C4 was more difficult to elucidate from this experiment.

Figure 6B, however, shows that also complex C4 contains more than one DNA molecule: EndoII E118A formed no more slowly moving complexes than C4 with the 30 bp substrate (Figure 4A and 6B, lane 7), and this was chased into larger complexes with increasing amounts of 148 bp competitor. [The lack of complex C1 in Figure 6B is a consequence of the enzyme concentration used (cf lanes 6 and 7 in Figure 4A)]. A competition experiment with EndoII G49A, shown in Figure 6C, showed that also with this mutant complexes C4 and C5 gave rise to more slowly migrating complexes in the presence of the competitor (lanes 3–6 and 9–12, respectively), suggesting they both contain more than one DNA molecule.

It is not likely that two DNA molecules in a complex are held together by two separate EndoII dimers; in that case both dimers must bind both substrate molecules which means that complexes containing two DNA molecules held together by one dimer should also be present. The presence of 50% more DNA shifts the C2 complex marginally (cf. lanes 6 and 8 in Figure 5). Thus, the presence of 100% more DNA (two substrate molecules per dimer) should result in a complex migrating at approximately the same rate as C2 for all mutants, for which we see no evidence. Thus cooperativity in binding (an enzyme that has already bound DNA recruiting an

additional enzyme molecule to the same complex) is unlikely. As a further test for cooperativity in binding, binding assays were carried out with all mutants at varying enzyme/substrate ratios, using the 148 bp substrate. The fraction of complexed DNA increased linearly with enzyme/substrate ratios in titrations yielding from 5 to 90% of the substrate complexed, suggesting there was no cooperative binding by EndoII. Thus, the EndoII species forming complex C4 must be present in solution and bind as one unit—a trimer, tetramer or higher order complex—to the substrate.

Using the 44 bp substrate and soaking gel slices in cleavage buffer, complexes formed with the nicking-proficient K12A mutant enzyme were found to be catalytically active (data not shown). About 80% of the EndoII K12A C4-complexed 44 bp substrate was nicked after 15 min in-gel incubation with MgCl₂ at room temperature. This suggests that both substrates bound in C4 are nicked. However, in-gel dissociation and association (19) could produce complete nicking even if each complex nicks only one of the bound substrates.

Complex C3 never accounts for more than a small fraction of the total DNA added, making it difficult to elucidate its composition. It is not likely to be a dimer with two bound substrate molecules, since this would be expected to move almost as fast as complex C2. Preliminary results comparing the DNA content with the enzyme content in the C3 and C4 bands suggest that there is less enzyme per DNA in the C3 band than in the C4 band, so possibly C3 consists of two substrate molecules with an EndoII trimer or a dimer plus a monomer (implying C4 is at least a tetramer). Also the precise composition of complex C5 is elusive. Since the migration rate of the fast-moving C2 complex was not measurably affected by a 50% difference in the amount of bound DNA (30 versus 44 bp, Figure 5), a difference between C4 and C5 just in the amount of DNA is unlikely. Complex C5 therefore must contain more EndoII than C4; it may also contain more DNA.

Complex C4 most likely consists of an EndoII tetramer binding two DNA molecules

To analyze the nature of the slow-moving EndoII-DNA complexes, gel filtration was performed. Since the 44 bp substrate migrated at the same rate as the enzyme tetramer, the R57A mutant did not bind well to the 30 bp substrate (Figure 3) and the E118A mutant likely would cause a very complex pattern, the G49A mutant and the 30 bp substrate were chosen. Results are shown in Figure 7. The 30 bp substrate migrated at a rate in between those for dimeric and tetrameric EndoII, corresponding to a protein molecular mass of 52 kDa. When the enzyme had been permitted to interact with the 30 bp substrate, a new peak appeared, migrating at a rate corresponding to a protein with molecular mass of 170 kDa. This is consistent with an EndoII tetramer bound to two DNA molecules (apparent size expected from gel filtration of tetramer and DNA separately 179 kDa). The molar enzyme/substrate ratio in this experiment was 42:1. From the analysis in Figure 3 (lanes 12
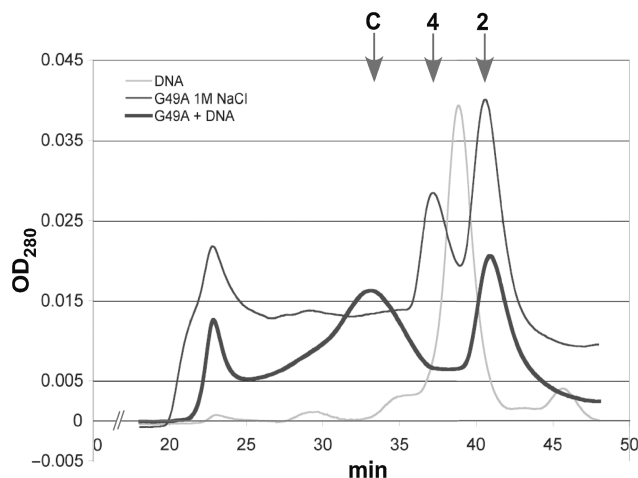

Figure 7. Gel filtration of the 30 bp substrate (thin grey line), and of EndoII G49A carrying the PelB leader incubated with the wt. 30 bp substrate (molar ratio of monomeric enzyme to substrate 42:1) at 10 mM (thick black line) and without the substrate at 1 M (thin black line) initial NaCl concentration in the sample. The concentration of NaCl in the column was 40 mM in all cases. Arrows point to eluted EndoII complexes: 2, apparent size 35 kDa (expected for EndoII G49A + PelB dimer 39.6 kDa); 4, apparent size 74.7 kDa (expected for tetramer 79.2 kDa). C indicates the enzyme–DNA complex with apparent size 170 kDa. The apparent size of the 30 bp substrate corresponded to a protein of 52 kDa. The peak at 22 min is the void.

and 17), a molar ratio EndoII G49A/30 bp substrate higher than 240:1 is needed to produce significant quantities of complexes moving more slowly than C4. Thus, the complex seen in the gel filtration most likely is complex C4. Increasing the enzyme–substrate ratio in gel filtration experiments did not result in the formation of a peak of a faster-eluting complex corresponding to C5, but only increased the amount of enzyme in the void, suggesting that the C5 complex is too large to enter these beads. At lower enzyme–substrate ratios than the one shown, less and less of the shown complex was formed but no new peaks appeared between that of this complex and that of free enzyme.

DISCUSSION

Tetrameric EndoII binds to two substrates

For all mutants tested, except EndoII E118A, complex C4 was the only species formed at low enzyme/DNA ratios. Since these mutants carry very different substitutions affecting their catalytic activities in different ways, we consider it likely that this complex is representative also for the wildtype enzyme. EMSAs, gel filtration experiments and crosslinking studies suggest that complex C4 contains two DNA molecules to which an EndoII tetramer is bound.

In the gel filtration assays, free native EndoII was recovered primarily as dimers under the low ionic strength conditions used for activity and gel shift assays, and as dimers and tetramers at higher ionic strength (Figures 1 and 7 and data not shown). The EndoII E118A mutant also crystallizes as a tetramer formed by two primary

dimers that can bind one substrate molecule each (Andersson *et al.*, manuscript in preparation). It is unlikely that tetramerization is induced upon binding to the substrate, since this should have resulted in a cooperative binding pattern, of which we see no evidence. We suggest that only the tetramer can bind stably to DNA, and, as free tetramers are removed from the solution, new free tetramers will be formed from the free dimers to maintain the dimer–tetramer equilibrium.

The effect of Glu118 on binding

Different from all other mutants, EndoII E118A formed fast-moving complexes C1 (one copy of EndoII, one DNA molecule) and C2 (two copies of EndoII, one DNA molecule). EndoII E118A also was the only mutant binding equally well to substrates lacking efficiently utilized nick sites. The 16 bp EndoII site is located in the middle of the 30 bp substrate, and the active surface of EndoII (as modelled on UvrC (4) and seen in the EndoII E118A crystal) is 10 bp across. This leaves room for one enzyme molecule binding to the recognized sequence, either one EndoII monomer or half of one dimer. Binding of two monomers would force one or, more likely, both of them to bind outside of the recognition sequence, resulting in a less stable complex. Only a mutant binding more promiscuously would be expected to form stable complexes also outside the recognition sequence. At low enzyme/substrate ratios (Figure 4A and B, lane 3), complex C2 formed a sharp band indicating a unique composition; according to the reasoning above, we consider it most likely that this complex is formed by an EndoII dimer rather than two monomers.

Thus, EndoII E118A binds to single DNA molecules both in monomeric and in dimeric form, as well as to two substrate molecules simultaneously in the tetrameric form that was found with all other mutants. The E118A mutant enzyme did not seem to be compromised in its ability to form dimers or tetramers, since the free enzyme in solution was found in these forms in similar proportions as for other mutants, as shown by both gel filtration and crosslinking experiments. Thus, the difference between EndoII E118A and all other variants likely lies in its interaction with DNA, not in its multimerization capacity.

The E > A substitution removes a negative charge at the catalytic surface, which may affect binding directly. Alternatively, the glutamate may coordinate residues elsewhere in the same monomer or primary dimer in a way that actually reduces binding e.g. through steric hindrance or by affecting the structure of the dimer. Removing such interactions may facilitate binding to the substrate, though not necessarily in a manner permitting efficient catalysis. Such facilitated binding might explain why EndoII E118A can bind single substrates stably, both as monomer and dimer. Conversely, the need for a tetramer for stable binding may reflect a need for more interaction surface than provided by a monomer or dimer to generate sufficient binding energy for wildtype-like enzymes.

EndoII E118A also failed to produce complexes moving more slowly than C4 with the 30 bp substrate, though it produced complex C5 with the 44 bp substrate. The other mutants that formed complexes with the 30 bp substrate at reasonable enzyme concentrations (G49A and K76A) both produced complexes moving more slowly than C4 with this substrate. Both these mutants, however, show reduced base-specified contacts, especially at the distal parts of the recognized sequence (4), and therefore may bind more promiscuously to a shorter sequence, permitting them to load more than one tetramer on to the short 30 bp substrate.

Comparison to other endonucleases

A tetrameric complex was quite unexpected for EndoII, which recognizes a long and asymmetric DNA sequence (15,18,20) with relatively low sequence specificity. Proteins that bind to DNA as dimers or tetramers often recognize symmetric DNA sequences [e.g. (21–23)] or structures (24). Some restriction endonucleases that require two DNA recognition sites are related to recombinases and transposases, DNA binding proteins that bring distant DNA sites together [e.g. EcoRII (25); NaeI (26)], while others carry out recombination-like functions [type IIF enzymes catalyzing four-strand DNA breakage, e.g. SfiI (27)]. Among enzymes that cleave within their recognition sequence and bind to two DNA sites simultaneously, restriction endonucleases of type IIE (e.g. EcoRII, NaeI) engage their two targets using two separate and distinct domains (28,29), which distinguishes these enzymes from the homotetrameric EndoII. Type IIF restriction endonucleases (e.g. SfiI, Bse634I, SgrAI) cleave both targets coordinately (30). The majority of these endonucleases are homotetramers arranged as dimers of dimers, where each dimer binds and cleaves one copy of the target site (31,32). SgrAI may bind as a dimer to one DNA site, subsequently recruiting another dimer bound to another site forming a tetramer binding two DNA sequences (33); this is unlikely for EndoII as we see no complexes of dimeric EndoII to single DNA substrates (except in the special case of EndoII E118A, which is addressed above). Instead, the most likely scenario is that EndoII binds as a tetramer [dimer of dimers, as the type IIF enzyme Bse634I (34)]. Types I, IIB, IIS, III and IV restriction endonucleases usually interact with two DNA sites (35–39). However, all five types cleave DNA outside their recognition sequence, and types I, III and IV also differ significantly from EndoII in subunit organization and cofactor requirement.

The two investigated GIY-YIG restriction endonucleases Eco29kI and Cfr42I like EndoII bind their substrates without cation cofactor and require a metal ion cofactor for all catalytic events (14,40), but in contrast to EndoII recognize and cleave short, symmetric unambiguous sequences. Cfr42I presumably binds as a tetramer to two DNA molecules (14) while Eco29kI is a monomer in solution (13). The sequence similarity between EndoII and these two GIY-YIG restriction endonucleases is very low outside the conserved GIY-YIG motifs, preventing reliable alignments; manual alignment suggests some similarities in the NTR. The sequence recognized by EndoII is more similar to that of the homing

endonucleases of the GIY-YIG family I-TevI and its isoschizomer I-BmoI (8,41), which however bind as monomers and nick one of the strands in the absence of Mg^{2+} (9,42). Thus, EndoII is unique among sequence-specific GIY-YIG endonucleases in its interactions with its substrate.

Mechanism of EndoII activity

EndoII recognizes an asymmetric ambiguous sequence and shows varying catalytic activity depending on the exact DNA sequence at the site. Stable catalytic complexes are formed only after the tetrameric enzyme has bound two DNA substrates. Comparison of results obtained *in vivo* and *in vitro* suggests that catalysis involves either nicking on one strand only (mode I activity) or cleavage due to simultaneous nicking of both strands (mode II activity). Also mode I activity can result in double-stranded cleavage upon consecutive (more than one binding event) rather than concurrent (one binding event) nicks, but this activity is masked *in vivo* by the action of repair functions that seal single-stranded nicks. *In vitro*, both modes can be observed, but mode I activity is higher (5). *In vivo*, only mode II activity can be observed since nicks are rapidly sealed (5). A GC-rich sequence element centred four base pairs to one side of the incised position is shared in both catalytic modes; mode II activity in addition requires a second sequence element centred 2 bp to the other side (4,18). In mode I activity, both bound substrates may be nicked, since in-gel activity on the 44 bp substrate by EndoII K12A in complex C4 exceeded 50%. However, in-gel dissociation and association (19) could result in complete nicking even if only one substrate is nicked by each tetramer.

Mode II activity appears to be efficient only at one of the two bound sites (20). With long substrates, the two DNA sites bound by each tetramer likely are part of the same DNA molecule looped between the binding sites or searched via intersegment transfer, since the effective concentration of a site present in *cis* will be higher than that for a site present in *trans*. In support of a scanning mechanism, as little as 3–9% modified Cyt (glucosyl-hydroxymethylated) residues in the DNA, resulting in approximately one modified residue per 60 bp, completely prevents EndoII cleavage *in vivo* (43). We propose that upon binding of one site to one part (one dimer) of the EndoII tetramer, the DNA is translocated by the second dimer until it reaches a second site. If a modified Cyt is encountered during translocation the enzyme may be dissociated from its substrate, or a catalytically inactive complex may be formed. If no modified residue is encountered, the second dimer may bind the second site reached, resulting in formation of a catalytically proficient complex. When a preferred EndoII cleavage site was introduced into the substrate, *in vivo* cleavage (mode II activity) at neighbouring, originally less preferred, sites was reduced within ca. 1000 bp of the introduced preferred cleavage site (20), suggesting that only one of two neighbouring sites held by the same tetramer—the preferred one—is cleaved efficiently in mode II activity. The interference distance of around 1000 bp to each side

of a preferred site agrees with the 952 bp found by Reuter *et al.* (44) as the distance limit for two sites in *cis* to be bridged by an EcoRII dimer. Analysis of the EndoII structure (Andersson *et al.*, manuscript in preparation) shows that the enzyme needs to be distorted significantly to access both strands of a bound substrate, suggesting that this distortion could induce a conformation preventing the second dimer from incising its substrate and explaining how only one of two bound sites is cleaved. No such context effects on cleavage have been observed *in vitro* (15). However, *in vitro* double-strand breaks most frequently result from two consecutive single-strand nicks resulting from separate binding events (mode I activity), i.e. one strand nicked per binding, rather than mode II concerted cleavage of both strands in a single binding event (5). Mode II cleavage of only one of the bound substrates would be in stark contrast to homotetrameric restriction endonucleases binding two substrates, such as NgoMIV and Cfr101, that cleave both substrates concertedly and swiftly, likely concurrently rather than consecutively (45,46).

However, the limited double-stranded cleavage resulting from EndoII activity *in vivo* is quite adequate to cause degradation of host DNA by the very potent exonuclease encoded or controlled by genes *46* and *47* (47), while its predominant single-stranded nicking activity quite possibly may promote recombination (Carlson and Lagerbäck, unpublished). Thus, EndoII combines features of both restriction and homing endonucleases with its own unique mode of catalysis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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