In vitro DNA-binding properties of VirB, the *Shigella flexneri* virulence regulatory protein

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Abstract The DNA-binding activity of the *Shigella flexneri* VirB transcription factor was studied in vitro. The protein was found to bind non-specifically to DNA, but showed preferential binding to VirB-dependent promoter sequences. DNA binding was contingent on the presence of an intact helix-turn-helix motif. While high molecular mass protein–DNA complexes were formed in both specific and non-specific interactions with DNA, mutant derivatives of VirB lacking a leucine zipper domain or a carboxyl-terminal-located oligomerisation domain formed discrete complexes, indicating that an ability to oligomerise on DNA was responsible for the formation of high molecular mass complexes by the wild-type protein.

Key words: VirB protein; Transcription factor; Gene regulation; Leucine zipper; Helix-turn-helix motif; *Shigella flexneri*

1. Introduction

Shigella flexneri is a Gram-negative facultative enteroinvasive pathogen which infects the lower intestinal epithelia of humans and primates to cause bacillary dysentery. Shigellosis is a serious global health problem, causing around 600 000 deaths per annum, predominantly in the developing world [1]. Invasion of host cells by *S. flexneri* requires expression of virulence genes located on a 230 kb plasmid. The products of these genes include the Ipa invasins, which mediate the invasion of gut epithelia and macrophage apoptosis [2,3], the Mxi and Spa proteins which form a type III secretion system [4], and the IcsA, IcsB and VirA proteins which are responsible for intercellular spreading of bacteria in the lower gut [5–7]. The virulence genes are expressed only under conditions approximating those found at the site of infection, i.e. at 37°C, a pH of 7.4 and moderate osmolarity [8–10]. A transcriptional regulatory cascade, involving both chromosomally encoded proteins including IHF (integration host factor) and H-NS (histone-like nucleoid structuring protein) and plasmid-borne regulatory proteins, VirF and VirB, integrates environmental signals and restricts the expression of structural virulence genes to the appropriate conditions [7,11–14].

The transcriptional cascade regulating the activation of virulence gene expression is initiated with the expression of the VirF protein, an AraC-like transcriptional regulator [10,14]. VirF is responsible for the activation of the *virB* regulatory and *icsA* structural genes [9,15]. The VirB protein then activates the other structural genes required for virulence [16–18]. The expression of *virB* is also regulated by H-NS, which acts as an antagonist to VirF at the promoter, and together with levels of negative DNA supercoiling, this appears to be responsible for the thermo-sensitivity of *virB* expression [19–21].

The role of the VirB protein in the regulation of virulence gene expression in *S. flexneri* was identified through transposon mutagenesis of the large virulence plasmid, when it was shown to be essential for the expression of almost all of the structural virulence genes [18]. VirB is an unusual candidate for a transcriptional activator, sharing homology with a family of plasmid partition proteins, including the ParB and SopB proteins, rather than previously described conventional transcriptional regulators [18,22,23]. Small and basic, VirB has been shown to form dimers, trimers and higher oligomers both in vivo and in vitro [17]. Oligomerisation is independent of DNA binding [17] and occurs through two domains, a leucine zipper located between residues 193 and 228 and a region at the C-terminus predicted to form a triple coil structure (Fig. 1A) [17]. Also, VirB binds specifically to target structural gene promoters in vivo [17]. The ability of VirB to interact with DNA in vivo is dependent on the presence of the N-terminus and the integrity of a helix-turn-helix (HTH) DNA-binding motif, located between residues 148 and 171, and essential for structural gene activation (Fig. 2A) [17]. Oligomerisation of the protein contributes to DNA binding since oligomerisation mutants bind to DNA with a lower preference in vivo [17]. The interaction with virulence gene promoters implies that activation by VirB is direct, however the mechanism is unclear (Fig. 1B). It has recently been shown that H-NS also binds directly to these promoters where it acts antagonistically to VirB [24].

The HTH DNA-binding motif in VirB is 80% identical to the HTH in the homologous ParB protein of plasmid P1, which mediates binding of ParB to the *parS* site leading to plasmid partitioning [17,25,26]. Interestingly there appears to be no consensus sequence analogous to *parS* for VirB DNA binding, and no homology exists between the four promoters to which VirB binds.

Although chromatin immunoprecipitation experiments indicate that VirB interacts specifically with target promoters in vivo [17], binding has never been demonstrated directly. This

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study investigates the binding of the VirB protein to target promoter DNA in vitro and assesses the importance of the HTH motif and the role of oligomerisation in VirB binding to DNA. Evidence is presented that the VirB protein polymerises on DNA in vitro echoing a phenomenon observed in vivo with the ParB and SopB proteins [27,28]. This raises interesting questions about the nature of DNA binding in vivo and the mechanisms by which VirB may activate structural gene expression.

2. Materials and methods

2.1. Bacterial strains plasmids and growth conditions

Plasmids were derivatives of the pET19b Novagen vector containing VirB, or VirB mutants K152E, K164E, L224R, ΔLZ, or Δ244–309 [17]. Proteins were expressed in *Escherichia coli* K-12 strain BL21DE3 containing the LacI-expressing plasmid pDIA17. Cells were grown in Luria–Bertani broth containing chloramphenicol (20 μg/ml) and carbenicillin (50 μg/ml) at 30°C.

2.2. Purification of VirB

Expression of N-terminally His-tagged VirB was induced with 0.1 mM IPTG in exponentially growing 500 ml cultures. After 3 h the cells were harvested and lysed in a French pressure cell. Lysates (~15 ml) were applied to a His-Bind® Quick column (Novagen) pre-equilibrated with binding buffer [17]. The column was then washed with binding buffer (50 ml) and wash buffer (25 ml) [17] and the protein eluted in 1 ml fractions of 2×15 ml of elution buffer (10% glycerol, 50 mM Tris–HCl pH 7.9, 0.5 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) containing 100 mM imidazole and 500 mM imidazole. Fractions were analysed by SDS–PAGE and those containing VirB were pooled and dialysed three times against 1 l 50 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8, 300 mM NaCl, 5% glycerol, 0.1 mM PMSF, 1 mM dithiothreitol (DTT). VirB was estimated to be approximately 95% pure. Preparation and use of anti-VirB rabbit polyclonal antibodies was described previously [17]. The His-tagged wild-type protein was found to be fully functional in vivo (data not shown).

2.3. Small scale protein purification

VirB and VirB mutants for use in electrophoretic mobility shift assays were purified from crude extracts by metal affinity chromatography using the His-Bind Magnetic Beads (Novagen). Induction of protein expression and cell lysis were performed as described above. 1.5 ml of the soluble extract was applied to 500 μl of pre-equilibrated beads in a 2 ml microfuge tube and incubated for 5 min. The magnetic beads were pelleted using a magnetic rack (Novagen) and the supernatant removed. The remaining soluble extract was applied to the beads as before and the beads were then washed three times with 2 ml wash buffer (as before) to remove contaminating proteins. Bound protein was eluted with 500 mM imidazole and analysed by SDS–PAGE. Proteins purified in this manner were deemed to be 85–90% pure by SDS–PAGE followed by Coomassie staining, and were dialysed in storage buffer (as above) and stored at −20°C.

2.4. Electrophoretic mobility shift analysis of protein–DNA interactions

A 340 bp DNA fragment encompassing the *icsB-ipgD* promoter region was amplified by the polymerase chain reaction using oligonucleotide primers IcsB5′ 5′-TATAGGTCCTGTATTGCTTGCCTGAAT-3′ and IcsB3′ 5′-AAATGAGGATCCATGCAATCCCAAATTAGT-3′. A 350 bp DNA fragment internal to the *icsP* gene was amplified with primers IcsP5′ 5′-ACTCTCGACTTTAAAAGGATGGGA-3′ and 5′-CTACTCACCTTCTTGTTCTCTCGC-3′. Radiolabelled probe DNA was incubated with increasing concentrations of His-tag-purified wild-type VirB or mutant VirB for 20 min at room temperature in a 20 μl reaction cocktail containing 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20 mM Tris–HCl pH 8, 50 mM KNa and 2 mM spermidine. Similar results were obtained when these mixtures were incubated at 30°C or 37°C. Each reaction mixture contained 1.45 μM DNA (5 ng) and VirB protein concentrations in the range 0–500 μM. Protein–DNA complexes were resolved by electrophoresis through an 8% polyacrylamide gel, for 4 h at 4°C. The gel was dried under vacuum onto Whatmann 3 MM paper and examined by autoradiography.

3. Results and discussion

3.1. Ability of purified VirB protein to bind to target promoter DNA

The *icsB-ipgD* intergenic region was selected as the target DNA in electrophoretic mobility shift assays, as this region contains the promoters for two divergently transcribed VirB-dependent operons (Fig. 1). A DNA fragment internal to the open reading frame of the *icsP* gene was used as a negative control. Binding reactions with increasing concentrations of $(His)_{10}$-VirB protein were carried out in an optimised buffer at room temperature.

Purified VirB showed a higher preference for binding to *icsB-ipgD* DNA than to *icsP* DNA (Fig. 2). At a protein:DNA molar ratio of 4:1, the *icsB-ipgD* probe begins to be shifted to the wells in the presence of 6.25 μM VirB (Fig. 2A). In contrast, the *icsP* probe requires a protein:DNA molar ratio of 80:1 (125 μM VirB in the reaction mixture) to achieve a comparable shift (Fig. 2B). VirB was observed to display a similar preference for binding to the *spa* and *virA* structural gene promoters in vitro as it does for *icsB-ipgD* (data not shown). These results indicate that VirB probably binds directly to the target promoters in vivo to effect the activation of structural gene expression, which is consistent with previous findings from in vivo analyses [17].

Interestingly, no discrete protein–DNA complexes, which normally indicate the attachment of an active protein to its binding site on DNA via an HTH motif [29], were observed in the binding of VirB to either probe. One possible explanation for this pattern is that VirB binds to DNA as a large complex. Another possibility is that on binding, VirB polymerises rap-

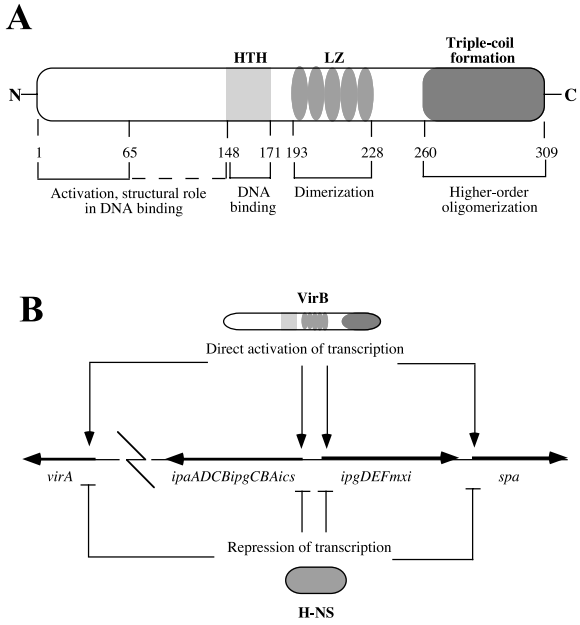

Fig. 1. Model of structural domains and activity of the VirB protein. The organisation of structural domains within the VirB protein is shown in schematic form in A. Structural features of the protein, their positions and their functions are highlighted on the diagram. Mutagenesis data have shown that a domain with a role in gene activation and a structural role in DNA binding is located between residues 1 and 65 and may extend to residue 148 [17]. A model for the activation of structural gene expression is shown in B. VirB binds directly at these promoters to activate gene expression, probably acting as an antagonist to the H-NS repressor [24].

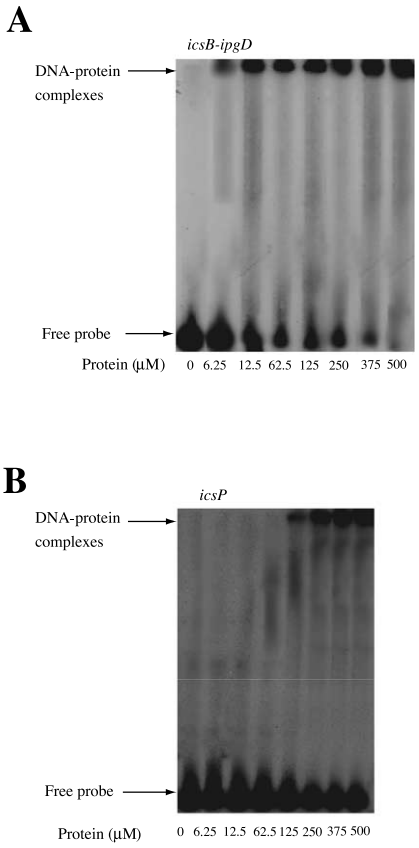

Fig. 2. Binding of purified recombinant VirB to DNA in vitro. Gel mobility shift analysis of VirB interactions with the target promoter *icsB-ipgD* DNA and control *icsP* DNA. Increasing amounts of purified recombinant $(His)_{10}$-VirB (indicated below each gel) were incubated with 1.45 μM ^{32}P-labelled *icsB-ipgD* (A) and *icsP* (B) DNA fragments for 20 min at room temperature. Bound and free probes were separated by polyacrylamide gel electrophoresis and visualised by autoradiography. The migration of free and bound probes is indicated with arrows.

idly along the DNA, forming a large complex that traps the probes in the gel wells. With the exception of differing binding preferences, the pattern of binding appears similar for both the target promoter DNA and the negative control DNA. Indeed, in further bandshift experiments VirB was capable of shifting many unrelated DNA probes in a similar manner (data not shown), but it always showed a strong preference for binding to target DNAs that contained VirB-dependent promoters. We are confident that VirB is not aggregating in these in vitro assays, and that it does not aggregate in vivo. Previously we have performed cross-linking experiments in vitro (without DNA) and in vivo (with DNA) ([17] and unpublished data). Complexes of VirB up to 10-mer could be detected in the presence of cross-linker but the protein was not found to form complexes that became trapped in the wells of the gel. In the absence of cross-linker dimers and trimers were dominant, something that would not be expected if the protein was merely prone to aggregation.

3.2. Purification of VirB mutants

To test the effect of mutations in the HTH putative DNA-binding motif of VirB on the ability of the protein to bind DNA, the K152E and K164E mutants were purified for use in electrophoretic mobility shift assays. Both of these proteins contain mutations at key residues in the motif, proposed to disrupt protein–DNA interactions [17]. These changes were previously shown to abolish the ability of VirB to activate structural gene transcription, without affecting the oligomeric state of the protein [17]. To test the requirement for oligomerisation in the DNA-binding activity of VirB, several representative mutants that had been shown to be defective in oligomerisation, L224R, ΔLZ, and Δ244–309 [17], were also purified. An attempt was made to purify a truncate lacking the N-terminus of the protein, to examine the role of that region in DNA binding, but this protein proved unstable when overexpressed. The proteins were purified as described in Section 2 and were judged to be >90% pure by SDS–PAGE and densitometric analysis (Fig. 3).

3.3. Ability of VirB HTH mutants to bind DNA in vitro

The observation that mutagenesis of key charged residues in the HTH DNA-binding motif of VirB abolished the ability of the protein to activate structural gene expression and reduced in vivo binding of VirB to promoters of the virulence structural genes [17], indicated that this motif was functional and contributed to DNA binding by VirB in *S. flexneri*. To

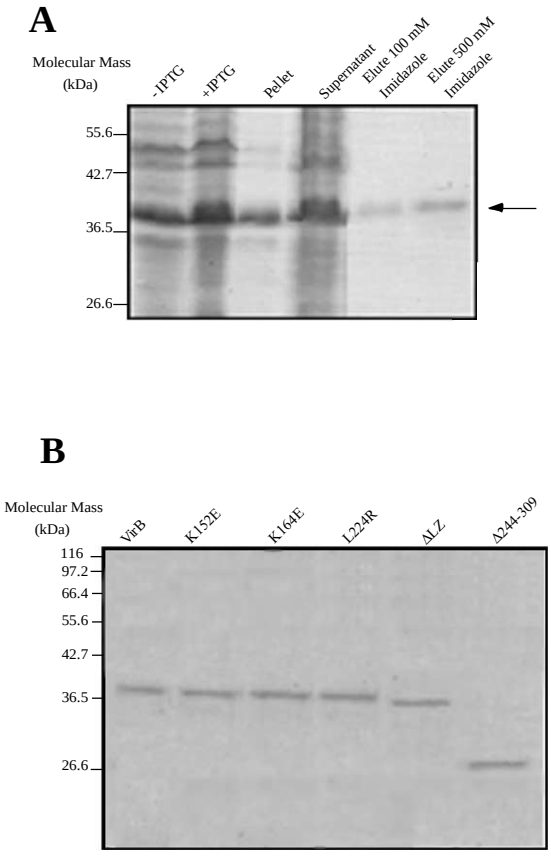

Fig. 3. Purification of recombinant VirB mutants. SDS–PAGE analysis of overexpression and purification of the VirB mutant K164E is shown in A. Strain BL21 (DE3)/pDIA17 harbouring the overexpression plasmid pET19K164E was grown to the mid-exponential phase, and *virB* expression induced by the addition of 0.1 mM IPTG. Protein samples of induced (+) and uninduced (−) culture, the insoluble and soluble fraction prior to passage over a Ni^{2+}-charged column, and the elution fractions are indicated on the gel. The band corresponding to K164E is indicated with a horizontal arrow. B shows SDS–PAGE analysis of 1 μg of each of the VirB mutants purified to show concentration, purity and integrity of the proteins.

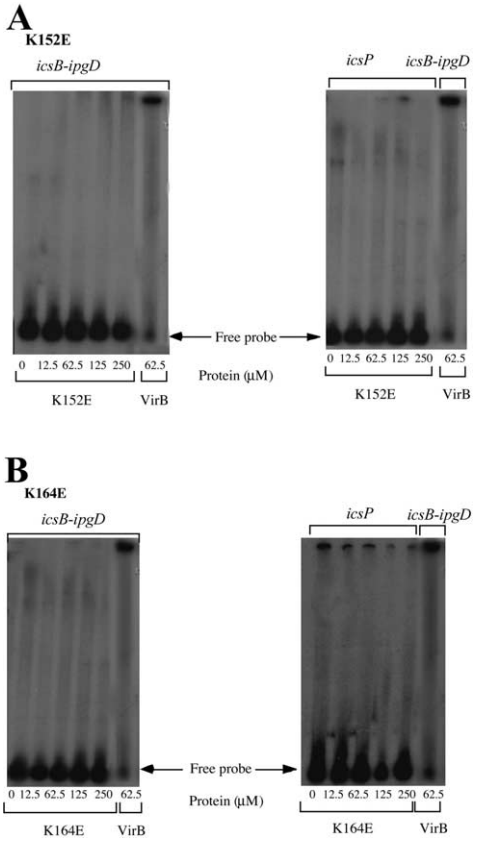

Fig. 4. Ability of VirB HTH mutants to bind DNA in vitro. Gel mobility shift analysis of interactions of the K152E and K164E HTH mutants of VirB with the target promoter *icsB-ipgD* DNA and control *icsP* DNA. Increasing amounts (indicated below each gel) of purified recombinant $(His)_{10}$-K152E (A) and $(His)_{10}$-K164E (B) were incubated with 1.45 μM ^{32}P-labelled *icsB-ipgD* or *icsP* DNA fragments for 20 min at room temperature. Bound and free probes were separated by polyacrylamide gel electrophoresis and visualised by autoradiography. The migration of free probes is indicated with arrows. In each case, the mobility shift achieved with 62.5 μM wild-type VirB protein binding to the *icsB-ipgD* probe in a 40:1 protein:DNA molar ratio is shown for comparison in the lane on the extreme right.

confirm directly the disruption of protein–DNA interactions by these mutations, purified K152E and K164E were used in electrophoretic mobility shift assays. Fig. 4 shows the results of binding reactions of the HTH mutants with an *icsB-ipgD* promoter probe and an *icsP* control probe. Clearly both of the HTH mutants were unable to bind to either promoter DNA or to the negative control *icsP* DNA, in contrast to the wild-type VirB protein binding to the *icsB-ipgD* probe (Fig. 4). In some cases, small quantities of probe were trapped in the wells spontaneously, even in the absence of any protein (for example, Fig. 4B; *icsP* probe). The reasons for this are not known. These results are consistent with the HTH motif in VirB being functional and contributing to the DNA-binding ability of the protein. By implication, HTH mutations would block gene activation by VirB in *S. flexneri* because they render the protein unable to bind to DNA. In previous work we have shown that these HTH mutants remain fully functional for oligomerisation in vitro [17] so the effects reported here are unlikely to involve deficiencies in protein–protein interaction. VirB differs from other DNA-binding proteins having an HTH motif in apparently not binding to

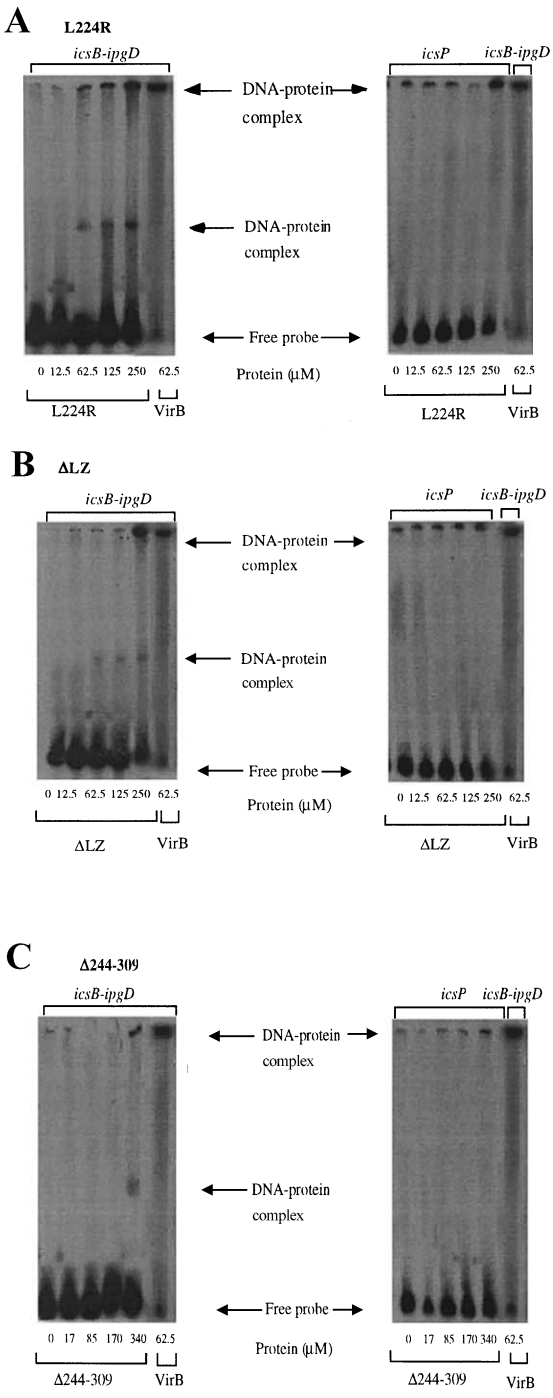

Fig. 5. Ability of oligomerisation deficient VirB mutants to bind DNA in vitro. Gel mobility shift analysis of interactions of the L224R, ΔLZ and Δ244–309 oligomerisation deficient mutants of VirB with the target promoter *icsB-ipgD* DNA and control *icsP* DNA. Increasing amounts (indicated below each gel) of purified recombinant $(His)_{10}$-L224R (A), $(His)_{10}$-ΔLZ (B) and $(His)_{10}$-Δ244–309 (C) were incubated with 1.45 μM ^{32}P-labelled *icsB-ipgD* and *icsP* DNA fragments for 20 min at room temperature. Bound and free probes were separated by polyacrylamide gel electrophoresis and visualised by autoradiography. The migration of free and bound probes is indicated with arrows. In each case, the mobility shift achieved with 62.5 μM wild-type VirB protein binding to the *icsB-ipgD* probe in a 40:1 protein:DNA molar ratio is shown for comparison in the lane on the extreme right.

a consensus binding site [29,30] and may, like its antagonist H-NS, bind to a consensus structure in DNA [31]. If so, other aspects of VirB may contribute to the specificity of its binding activity.

3.4. Ability of oligomerisation deficient mutants of VirB to bind DNA in vitro

To test the role of oligomerisation in the ability of VirB to bind DNA, three mutants, L224R, ΔLZ, and Δ244–309, were purified for use in bandshift assays (Fig. 3B). These mutants had previously been shown to have a reduced capacity to oligomerise in vivo [17]. All of the oligomerisation deficient mutants showed a reduced preference for promoter *icsB-ipgD* DNA and the *icsP* internal DNA compared with wild-type VirB in vitro (Fig. 5). Unlike the native protein, none of the mutants was capable of fully shifting the DNA probes at any protein:DNA molar ratio tested, indicating that VirB probably interacts with DNA most efficiently in its oligomeric form. This is in agreement with in vivo experiments showing a reduced DNA-binding capacity for VirB mutants impaired in oligomerisation [17].

Interestingly the VirB mutants with an inability to oligomerise showed a different pattern of binding to the *icsB-ipgD* promoter DNA to the wild-type protein (Fig. 5). Wild-type VirB shifted the promoter DNA probe directly into the wells, whereas each of the oligomerisation deficient mutants produced a single intermediate complex of the protein with promoter DNA before a shift into the wells (Fig. 5). Thus it appears that the L224R, ΔLZ, and Δ244–309 mutants form an initial complex with DNA, followed by polymerisation of the protein on the DNA forming high molecular weight complexes that remain in the wells of the gel. The reduced ability of these proteins to interact with each other appears to slow this reaction sufficiently to separate initial complex formation from oligomerisation. The L224R and the ΔLZ mutants bound to and shifted promoter DNA at a protein:DNA molar ratio of 40:1, a ratio at which the wild-type shifted the probe completely to the wells. The Δ244–309 mutant formed the intermediate complex only at a protein:DNA molar ratio of 218:1, possibly indicative of the severity of the carboxyl-terminal deletion that removed a major oligomerisation domain.

Thus it appears that oligomerisation of the VirB protein plays a key role in the binding of the protein to DNA in vitro. Proteins defective for oligomerisation showed a reduced preference for promoter DNA and a different binding pattern compared with native VirB. The results presented here demonstrate direct binding of the VirB protein to target promoters in vitro for the first time. In addition they are consistent with an essential role for the HTH motif in this DNA-binding activity. Finally it appears that oligomerisation of VirB is also required for effective DNA binding and there is an indication that VirB possesses the ability to polymerise along DNA in vitro, possibly echoing a similar phenomenon described in vivo for the homologous ParB and SopB plasmid partitioning proteins [26–28]. Perhaps VirB has evolved from a plasmid partition function that was diverted to a role as a regulator of virulence genes. The *S. flexneri* plasmids possess dedicated partition functions related in amino acid sequence to VirB [32,33] and this redundancy might have permitted such an event to occur.

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