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A leucine zipper motif determines different functions in a DNA replication protein

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RepA is the replication initiator protein of the *Pseudomonas* plasmid pPS10 and is also able to autoregulate its own synthesis. Here we report a genetic and functional analysis of a leucine zipper-like (LZ) motif located at the N-terminus of RepA. It is shown that the LZ motif modulates the equilibrium between monomeric and dimeric forms of the protein and that monomers of RepA interact with sequences at the origin of replication, *oriV*, while dimers are required for interactions of RepA at the *repA* promoter. Further, different residues of the LZ motif are seen to have different functional roles. Leucines at the d positions of the putative α-helix are relevant in the formation of RepA dimers required for transcriptional autoregulation. They also modulate other RepA–RepA interactions that result in cooperative binding of protein monomers to the origin of replication. The residues at the b/f positions of the putative helix play no relevant role in RepA–RepA interactions. These residues do not affect RepA autoregulation but do influence replication, as demonstrated by mutants that, without affecting binding to *oriV*, either increase the host range of the plasmid or are inactive in replication. It is proposed that residues in b/f positions play a relevant role in interactions between RepA and host replication factors.

Keywords: DNA replication/leucine zipper/pPS10/RepA/transcriptional autoregulation

Introduction

The leucine zipper (LZ) motif was first described as a dimerization domain in the yeast transcriptional factor GCN4 and in the oncogenic proteins Fos and Jun (Landschulz *et al.*, 1988; Hurst, 1994). The LZ motif consists of a repetition of 4–5 leucines spaced seven residues apart. The residues of the motif adopt an α-helical secondary structure in which leucines are arranged on the same face of the helix (position d; according to the pattern: abcdefgabcdefg...; Figure 1) every two helical turns, organizing a hydrophobic spine. Dimerization implies the facing of the hydrophobic spines of two α-helices in parallel orientation, leading to a coiled coil (O'Shea *et al.*, 1989, 1991; Saudek *et al.*, 1991; Ellenberger *et al.*, 1992; König and Richmond, 1993; Glover and Harrison, 1995). The leucines therefore play an essential role in the formation of the coiled-coil (Hodges *et al.*, 1988; Kouzarides and Ziff, 1988; Landshulz *et al.*, 1989; Hu *et al.*, 1990; Zhou *et al.*, 1992).

The role of residues of the motif other than leucines has also been analysed. It has been shown that hydrophobic residues placed in position a belong to an additional hydrophobic interface that contributes towards increasing dimer stability (O'Shea *et al.*, 1991; Saudek *et al.*, 1991; Trophsa *et al.*, 1991; Ellenberger *et al.*, 1992; König and Richmond, 1993; John *et al.*, 1994; Glover and Harrison, 1995). The roles of residues in positions a, d, e and g have been examined: (i) a, e and g residues determine the specificity of heterodimerization in Fos and Jun (Schuermann *et al.*, 1991; O'Shea *et al.*, 1992; John *et al.*, 1994)—in GCN4 these residues contribute moderately towards dimer stability (Hu *et al.*, 1993); (ii) residues at positions a, d, e and g determine the degree of oligomerization between LZ coiled-coils (Harbury *et al.*, 1993, 1994). The contributions of residues at positions b and f have not been investigated so fully. Eukaryotic LZs are generally associated with N-terminal basic regions involved in DNA interactions which form a continuous helix with the LZ motif (Pu and Struhl, 1991; Ellenberger *et al.*, 1992; König and Richmond, 1993; Glover and Harrison, 1995).

RepA is the replication initiator protein of the *Pseudomonas* plasmid pPS10 (Nieto *et al.*, 1992). RepA is a basic protein of 230 amino acid residues (M_r 26.6 kDa) whose N-terminal region shares homology with a LZ motif (Figure 1). This LZ motif is homologous to other LZ motifs found in the replication proteins of pSC101, F and R6K plasmids (Giraldo *et al.*, 1989). In RepA of pPS10, the LZ motif extends over four leucines (Leu13, 20, 27 and 34; Figure 1) within a region of potential α-helical structure (Giraldo *et al.*, 1989). There are no additional hydrophobic residues in position a, indicating that potential hydrophobic interactions should be restricted to leucine residues in position d. In the LZ of RepA (pPS10) there are no basic regions at the N-terminus. Recent information indicates that interactions of RepA with specific sequences in DNA are mediated by a putative helix–turn–helix (HTH) motif at the C-terminus of the protein (Figure 1a): therefore the LZ and DNA binding motifs of RepA are clearly different. (Giraldo *et al.*, 1989; García de Viedma, 1996).

Previous results have shown that RepA, in addition to promoting replication of pPS10 from the origin region, is able to repress its own transcription (Nieto *et al.*,1992), via interactions with two inverted repeats that overlap with the *repA* promoter (García de Viedma *et al.*, 1995; Figure 1a). RepA binding to the operator occurs first by RepA–DNA interactions and subsequently is reinforced by protein–protein interactions. These last interactions

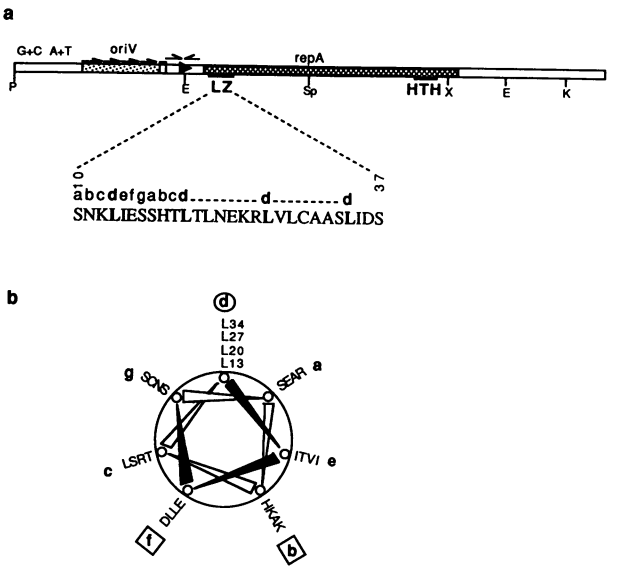

Fig. 1. (**a**) Relevant features of the basic replicon of pPS10. The origin region (oriV) includes: a dnaA box (□); four identical iterons of 22 bp (→); an AT-rich region (A+T) and a GC-rich region (G+C). (⌒) Two inverted repeats of 9 bp flank the promoter for *repA* (▶). (□) Open reading frame corresponding to the plasmid replication protein (repA). LZ and HTH indicate respectively the location of the LZ and the HTH motifs of RepA. The amino acid sequence corresponding to the LZ motif and coordinates for residues are indicated. On top of the sequence is shown the a–g letter code for the different residues in the LZ. P, *Pvu*II; E, *Eco*RI; Sp, *Sph*I; X, *Xho*I; K, *Kpn*I. (**b**) Helical wheel representation of the LZ motif in RepA. The positions analysed in this work, d and b/f, are marked with a circle and squares respectively.

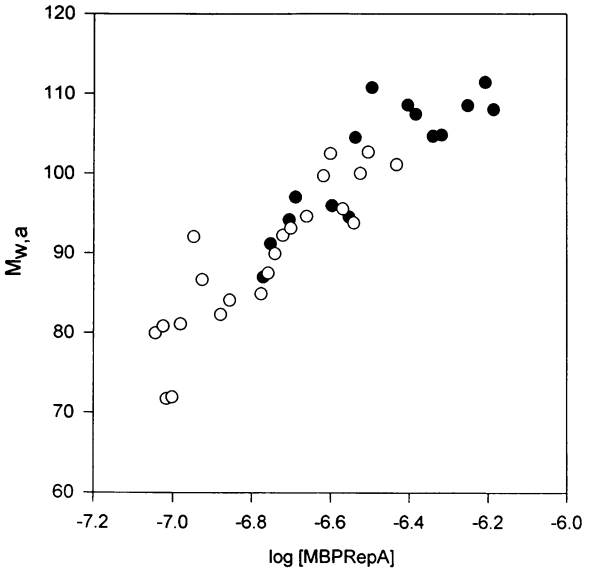

Fig. 2. Apparent weight-average molecular weight of MBPRepA as a function of protein concentration. Molecular weight values were calculated from the local slopes at defined radial intervals using a linear transformation of the data (ln*A* versus r^2, see Materials and methods). The conditions of the sedimentation equilibrium experiment were 20 000 r.p.m. and 4°C. The black and white dots represent two different ranges of loading protein concentrations.

result in the formation of high order RepA–DNA complexes and are important in ensuring efficient repression of the promoter (García de Viedma *et al.*, 1995).

Some mutants have been isolated mapping in, or close to, the homologous LZs of the replication proteins of pSC101 and R6K plasmids. It has been proposed that these mutations could affect protein–protein interactions (Ingmer and Cohen, 1993; York and Filutowicz, 1993; Miron *et al.*, 1992). At present, none of these LZs has been analysed in detail. This paper reports the first detailed characterization of the LZ motif in a DNA replication initiator protein.

Results

RepA is subjected to a monomer–dimer association equilibrium

RepA was overexpressed and purified as MBPRepA (73.6 kDa), an amino-translational fusion with the maltose binding protein MBP (47 kDa) (see Materials and methods; Figure 3b). MBPRepA is soluble and is able to repress a *repA*P–*lacZ* transcriptional fusion, indicating that it retains RepA autoregulatory activity (García de Viedma *et al.*, 1995). MBPRepA also retains the ability of RepA to interact *in vitro* with DNA fragments containing the *repA* promoter–operator region (*repA*P); interactions that lead to the formation of different RepA–DNA complexes (García de Viedma *et al.*, 1995).

With the aim of characterizing the association state of RepA in solution, the apparent average molecular weight of MBPRepA was determined—as a function of protein concentration—by sedimentation equilibrium analysis. The results indicated that this parameter increased with protein concentration from values close to those expected for a monomer, to values close to those expected for a dimer (Figure 2). This is an indication of a dimerization equilibrium (McRorie and Voelker, 1993). The global fitting of the experimental data to a monomer–dimer equilibrium model (see Materials and methods), indicates that MBPRepA forms dimers with an affinity constant of 2.1×10^6 (1.3×10^6; 3.3×10^6)/M (bracketed figures indicate the estimation of uncertainty in K_2^M with a confidence limit of 95%). As MBP is a monomer in solution (Duplay *et al.*, 1984), the sedimentation equilibrium behaviour of MBPRepA must be determined by RepA.

RepA dimers interact with the operator and are also responsible for the formation of sequential regulatory complexes

The *repA* operator region is organized as two inverted repeats, overlapping the *repA* promoter, to which RepA binds (García de Viedma *et al.*, 1995). Inverted repeats are usually recognized by dimeric factors (Freemont *et al.*, 1991; Pabo and Sauer, 1992). To analyse the RepA form that interacts with the operator, the heterodimerization assay developed by Hope and Struhl (1987) was used. This assay requires the obtention of two forms of RepA, differing in size, that form protein–DNA complexes with different mobility in electrophoretic mobility shift assay (EMSA). If dimers of the protein bind to the DNA target, EMSA, using mixtures of both protein forms, should show complexes corresponding to (i) homodimers of the large form, (ii) homodimers of the small form and (iii) heterodimers between both protein forms. The mobility of these heterodimeric complexes should be intermediate with respect to those which are homodimeric.

In experimental assays, purified preparations of RepA

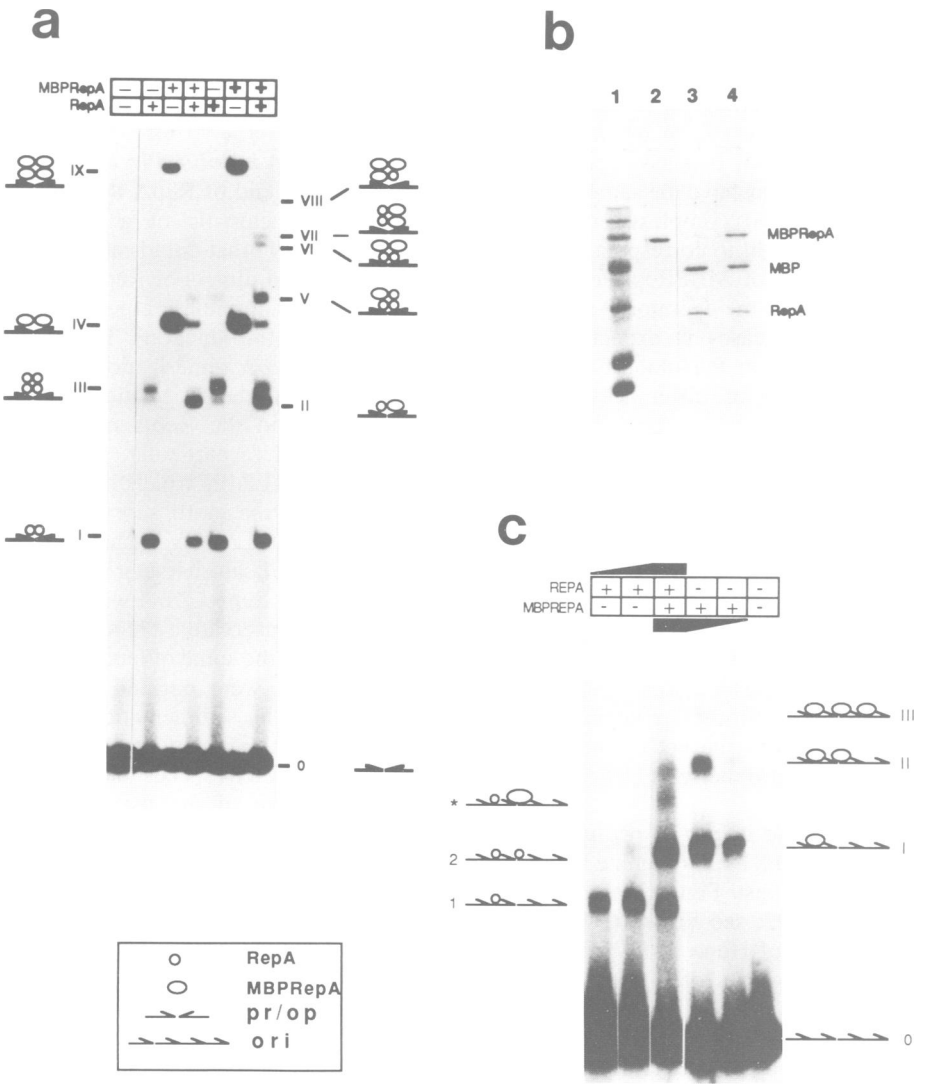

Fig. 3. (**a**) Interactions of MBPRepA and/or RepA at the promoter–operator region. EMSA analysis was performed incubating 15 ng of labelled promoter–operator probe with 10 ng (lanes 2–4) or 50 ng (lanes 5–7) of MBPRepA, RepA or a mixture of both proteins (see panel at the top). An interpretation of the different compositions of complexes (I–IX) is provided. On the left side of the picture are complexes that include homodimers and on the right those which include heterodimers. Although one cannot discriminate fully between the composition of the complexes VI and VII, they should correspond to two homodimers or two heterodimers (see text). (**b**) SDS–PAGE including the purified protein samples used in the heterodimerization assays in operator and origin. Lane 1: molecular weight markers (Bio-Rad); lane 2: purified MBPRepA; lane 3: MBP and RepA obtained by digestion of MBPRepA with factor Xa; lane 4: mixture between MBPRepA and excised RepA. Bands corresponding to the fusion and the processed proteins (MBP and RepA) are indicated. (**c**) Interactions of MBPRepA and/or RepA at *oriV*. EMSA analyses were performed using 15 ng of labelled origin probe and two different quantities of RepA or MBPRepA proteins (100 and 150 ng). In the central lane, 150 ng of each protein were incubated together prior to the addition of DNA. An interpretation of the composition of the different complexes is indicated on both sides of the figure. In the central lane, complexes 2 and I overlap. MBPRepA complexes are indicated in Roman numerals, RepA complexes in arabic numbers. The intermediate mobility complex corresponding to the entrance of one MBPRepA monomer and one RepA monomer, is indicated with an asterisk. The symbols used to represent the monomers of RepA, MBPRepA and the DNA probes are indicated within the squared box.

(26.7 kDa) and MBPRepA (73.6 kDa) were incubated with *repA*P DNA probes under conditions that lead to the formation of two sequential retardation complexes with each protein preparation (Figure 3a, complexes I and III for RepA and complexes IV and IX for MBPRepA). The band with the highest mobility results from RepA–DNA interactions. The more retarded band is due to additional molecules of RepA entering into the RepA–DNA complex by protein–protein interactions (García de Viedma *et al.*, 1995). When RepA and MBPRepA are incubated together prior to DNA binding, and at low protein concentration (Figure 3a, lane 4), a new complex appears (II). This complex displays intermediate mobility with respect to complexes I and IV, obtained with RepA and MBPRepA respectively. This intermediate complex (II) should correspond to heterodimers between RepA and MBPRepA, indicating that the RepA form that binds to the operator region is a dimer. Heterodimers were also detected in EMSA using *in vitro* translation extracts co-expressing MBPRepA and RepA together (data not shown). At higher protein concentrations (Figure 3a, last three lanes), the mixture of RepA and MBPRepA yielded four new retardation complexes (complexes V–VIII) with intermediate mobilities compared with the slower mobility complexes detected with RepA and MBPRepA alone (III and IX, respectively). Each of these four bands could correspond

to each one of the possible heterotetrameric combinations that can be formed between MBPRepA and RepA (Figure 3a). Thus, dimeric forms are also involved in the sequential RepA–RepA entries to the operator. The mobility of the different heterotetramers depends on the number of monomers of RepA or MBPRepA. It is assumed that the two possible isomers of a complex have the same mobility (Andera *et al.*, 1994). The two complexes in central positions (VI and VII) are thought to correspond either to two homodimers (one of each of MBPRepA or RepA), or to two heterodimers bound to the operator. As the total protein mass present in both cases is expected to be the same, the slight difference in the mobility of these complexes could be the result of differences in their compaction.

Bands of weak intensity can also be seen (with mobilities that correspond to heterocomplexes) in lanes containing only RepA. As RepA was obtained by digesting MBPRepA with factor Xa (Figure 3b), these minor bands reflect the presence of traces of undigested MBPRepA in the RepA preparations.

The joint incubations of MBPRepA and RepA were performed at protein concentrations of 1 mg/ml in which, according to the association constant, RepA in solution must be mainly in the dimeric form. The appearance of intermediate complexes indicates the active interchange of monomers between different dimers.

RepA monomers bind to the origin of replication

The origin of replication of pPS10 is organized as a set of four directed repeats (iterons; Figure 1a) to which RepA binds (Nieto *et al.*, 1992; see Figure 6, RepAwt). The heterodimerization assay of Hope and Struhl (1987) was also used to test if monomers or dimers of RepA interact with the iterons of the origin (Figure 3c). Assays were performed at RepA or MBPRepA protein concentrations giving the two first retardation complexes with the *oriV* probe—corresponding to one-iteron- or two-iteron-bound complexes (a faint complex due to three iterons bound by MBPRepA can also be appreciated). When MBPRepA and RepA were incubated together prior to binding to DNA, no new complexes of intermediate mobility were detected between those complexes corresponding to protein bound to just one iteron (complexes 1 and I, Figure 3c). As indicated in the previous section, heterodimers are readily formed when RepA and MBPRepA are incubated together. Therefore, in this experiment, the absence of intermediate bands between the first two retardation complexes indicates that monomers of RepA, rather than dimers, bind to the *oriV* region. An alternative explanation for the absence of intermediate complexes could be that the presence of MBP prevents binding of heterodimers to *oriV*. This is not probable, however, since MBPRepA homodimers conserve protein–protein and protein–DNA binding functions. Independent data indicate that a mutant (LZ12), that shifts the association equilibrium to the monomeric fraction, is impaired for binding to the promoter where dimers are required. However, binding to *oriV* is not impeded (see below). All these data favour the interpretation that monomers of RepA contact the *oriV* region. It can be predicted that when two iterons are occupied (complexes 2 and II, Figure 3c), one monomer of each MBPRepA and RepA protein could be part of the same complex. Therefore, a band of intermediate mobility should appear when using mixtures of both protein forms. This band can indeed be detected (indicated with an asterisk in Figure 3c).

A LZ motif is involved in RepA dimerization

At the amino end of RepA there is a region with leucine heptads characteristic of a LZ motif (Giraldo *et al.*, 1989; Figure 1) that could modulate the balance between monomers and dimers of RepA. RepA LZ has a peculiar feature; its hydrophobic core is restricted to the leucine residues (see Introduction). To determine if this LZ was relevant in RepA dimerization, leucines were substituted for valines in the d position and the effect of these substitutions in the RepA monomer–dimer equilibrium was analysed. In particular, analysis was made of a LZ mutant, RepALZ12, which has the two first leucines in position d of the motif substituted by valines. It should be noted that Leu→Val changes should conserve the potential α-helical character of the motif. As a control, the LZ mutant RepALZ141 was also analysed. This mutant includes a conservative change, Ala32→Val, in position b, opposite to the spine of leucines on the putative α-helix. Both mutants were purified as MBPRepA fusions and were assayed by sedimentation equilibrium analysis.

Global fitting of analytical ultracentrifugation data (performed at different speeds and concentrations) shows that the substitution of the two first leucines (RepALZ12) reduced by 13-fold the dimerization constant of the protein [1.8×10^5 (1.1×10^5; 3.1×10^5)/M; K_2^M is 2.1×10^6 for the wild-type protein, as previously shown]. The change of a residue in the motif other than leucine (RepALZ141) does not alter this constant significantly [5.7×10^6 (2.0×10^6; 2.6×10^6)/M]. Figure 4 indicates that at the same concentration interval at which the equilibrium sedimentation profile of RepAwt is close to that expected for a pure dimer, the profile of RepALZ12 overlaps with that expected for a pure monomer. This indicates that the leucine for valine substitutions severely affect RepA dimerization behaviour in solution. Note that the equilibrium sedimentation profile corresponding to RepALZ141 is equivalent to that obtained for RepAwt (Figure 4). These results indicate that the leucine residues in position d are involved in the dimerization of RepA, and support the functional relevance of the LZ motif of RepA.

The LZ motif is relevant for RepA transcriptional autoregulation and pPS10 replication

The results above suggest that dimers and monomers are two forms of RepA which bind to the operator and origin of replication respectively, and that the LZ motif modulates the balance between these forms. Therefore, mutations in the LZ motif would be expected to have effects upon autoregulation and replication.

To test this proposal, the number of LZ mutants was increased, using site-directed mutagenesis, by the introduction of single or double Leu→Val conservative substitutions in all four leucine residues of the LZ motif, single and double substitutions in positions b and f and an in-phase deletion of the whole LZ motif (Materials and methods; Figure 5).

To evaluate the effect of these mutations on replication of pPS10, pPS10–pBR322 recombinants carrying the LZ

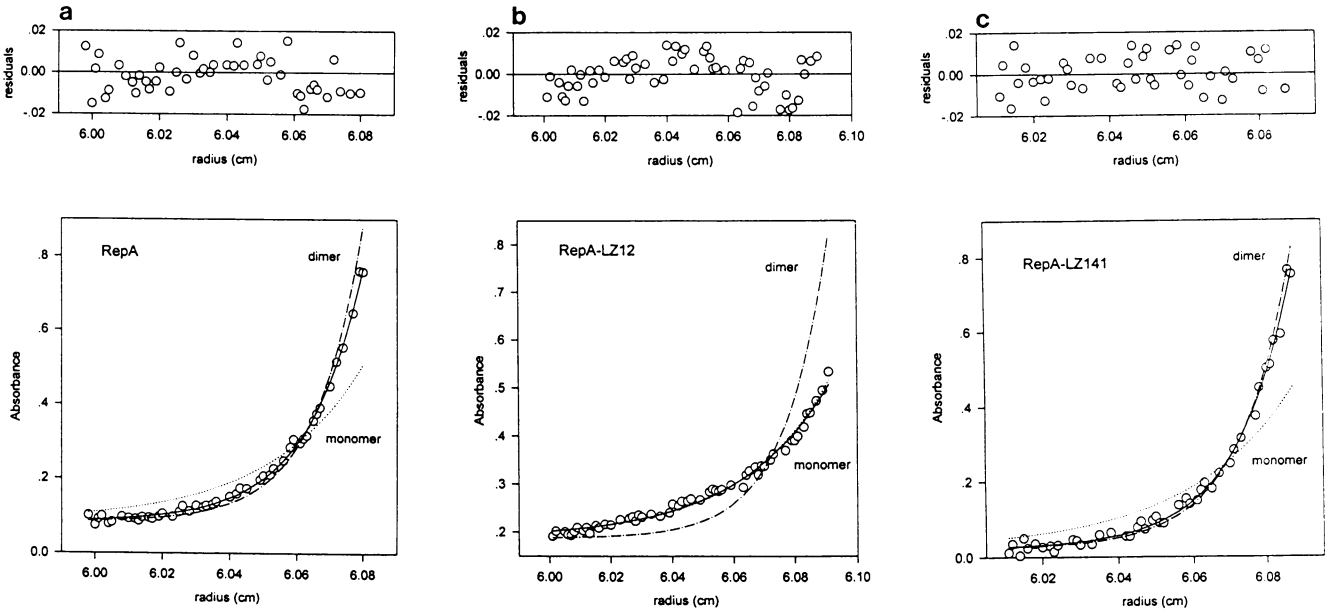

Fig. 4. Sedimentation equilibrium profiles of (**a**) MBPRepA, (**b**) MBPRepALZ12 and (**c**) MBPRepALZ141, taken at 25 000 r.p.m. and 4°C. For illustrative purposes, the theoretical radial distribution of absorbances corresponding to pure monomers (·····) and pure dimers (–·–·–) are indicated.

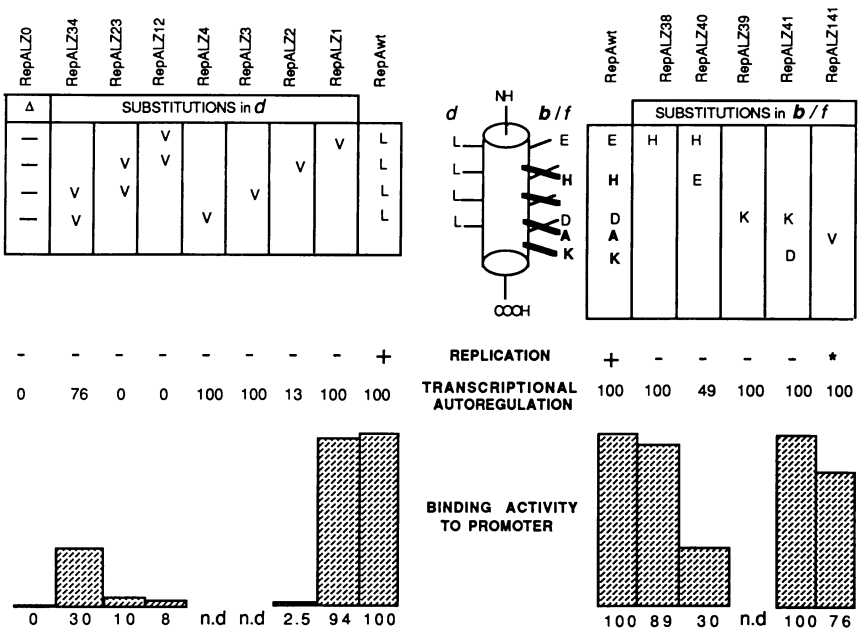

Fig. 5. Summary of replication and autoregulation efficiency of the different LZ mutants. At the centre, the putative α-helix of the RepA LZ is represented with the hydrophobic leucine spine d on the left side and the residues corresponding to b/f positions on the right side. The substitutions introduced at these positions, with names of the mutants, are indicated on the left and right hand panels respectively. Δ indicates an in-phase deletion of the motif (RepALZ0 mutant protein). The phenotypes of all the mutants in replication and autoregulation are indicated in the lower part of the figure. +/– indicate positive or negative establishment in *Pseudomonas* and *E.coli*. The asterisk indicates that the mutation increases the plasmid host range. The autoregulation values indicate the efficiency of the protein in repressing *in trans* synthesis of β-Gal promoted by a compatible *repA*P–*lacZ* transcriptional fusion. Values were standardized with respect to the repression modulated by the RepA wt protein (88%). The histogram at the lower part of the figure represents the efficiency of the different mutant proteins to bind to the promoter–operator region *in vitro* as analysed by EMSA (15 ng of labelled DNA probe and 10–40 ng of protein). The autoradiographic signals corresponding to the retarded complexes and the free DNA were quantified by densitometry. The values are standardized with respect to the proportion of retarded DNA obtained with RepA wt (equivalent to 46% of the total DNA assayed at the protein concentration range selected). n.d indicates that the analysis was not performed with those mutants.

mutations were constructed and isolated in *Escherichia coli*. Mutants were tested by their ability to transform *Pseudomonas aeruginosa*. An equivalent assay was performed using an *E.coli polA*$^-$ strain (BT1000) which allows the establishment of pPS10 at 30°C (Fernández-Tresguerres *et al.*, 1995). In these hosts, the pBR322 replicon is silent and therefore failure in transformation indicates deficient pPS10 replication.

To evaluate the ability of RepA mutants to autoregulate *repA*P, *tac*P–*repA* derivatives containing the different LZ substitutions were constructed (Materials and methods) and introduced into *E.coli* cells containing a *repA*P–*lacZ* transcriptional fusion (pFusCE; García de Viedma *et al.*, 1995). The effects on *lacZ* expression were determined in the presence of IPTG. In all cases, the same *tac*P–*rep*A derivatives carrying *repA* in the opposite orientation were assayed as controls.

Replication and regulation results are summarized in Figure 5. In-phase deletion of the LZ motif prevented both replication and regulation. All Leu→Val substitutions failed to promote the establishment of the pPS10 replicon. Four out of five substitutions in b/f positions, obtained by site-directed mutagenesis, fully impaired pPS10 replication. The same results were obtained when replication was tested in either *E.coli* or in *Pseudomonas*.

The autoregulatory activity of the LZ mutants was tested in *E.coli*. It was found that mutations in different leucines of the motif affected autoregulation properties (Figure 5). The second leucine seems especially important as it is the only residue in which a single mutation shows an effect. All double mutations at the leucines affect autoregulatory properties. Double mutations in the two first or two middle leucines fully impair regulation mediated by RepA. The evaluation of replication and autoregulation in the same host, *E.coli*, rules out that results could reflect differential effects of the host on the stability of the proteins.

In autoregulation-affected mutants, it is unlikely that RepA overexpression is responsible for failure to replicate. Expression of RepA from a ptac–*repA* derivative has no effect on the copy number of a pPS10 replicon placed *in trans* (data not shown). The possibility that the deficiency of mutants to establish the pPS10 replicon in *Pseudomonas* could reflect killing by a run-away type of replication is highly improbable, since it implies a hyperactive replication protein. In addition, LZ1, LZ3, LZ4, LZ34, LZ38, LZ39 and LZ41 mutants fail to replicate without being substantially affected in autoregulation, suggesting that, at least in these cases, impaired replication is not associated with deficiencies in autoregulation.

All these data indicate that the LZ motif is important in both the autoregulation and replication activities of RepA. Replication is more sensitive than is regulation to substitutions in the motif. Thus, it is possible to discriminate between residues of the LZ involved in replication but not in autoregulation. Leucine residues (position d) seem to be more relevant in autoregulation than residues at b/f positions.

RepA LZ mediates the assembly of a functional dimer into the operator

To analyse further the role of the LZ motif in autoregulation, EMSA analysis was performed using purified preparations of the different RepA mutants and promoter–operator probes. Complexes were quantified by densitometry. Data are shown in Figure 5. RepALZ2, RepALZ12 and RepALZ23, which showed the highest reductions in autoregulation activity, are also severely affected in their binding to the operator *in vitro*. RepALZ34 and RepALZ40, both moderately affected in autoregulation, retained a higher degree of interaction with the operator.

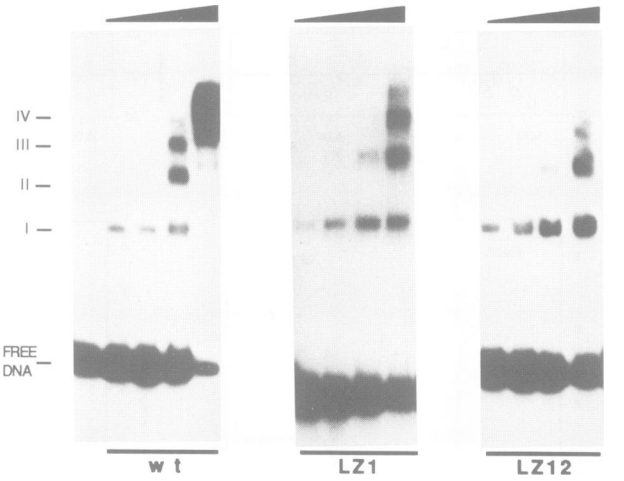

Fig. 6. Binding to the *oriV* region of the RepA wt protein and the mutants in the first (LZ1) or two first leucines (LZ12). In all cases, RepA protein was assayed by EMSA as a purified fusion with MBP. RepAwt is included as a reference. Increasing quantities of protein (50, 75, 150 and 300 ng) were incubated with 15 ng of labelled origin probes. Positions of retarded complexes (I, II, III and IV) and free DNA are indicated.

As in the autoregulation assays, changes in the leucines have, in comparison with changes in b/f positions, the greatest effects on binding to the operator. The deletion of the motif prevents binding to this region. This deletion is associated with an increased sensitivity to proteolysis (data not shown), suggesting that the mutation affects the structure of the protein. Other mutations that do not affect autoregulation mediated by RepA do not interfere substantially with the binding of the protein to the *repA*P region.

Thus, there is a clear correlation between autoregulation and binding to the operator. It seems that an important role of the LZ motif in autoregulation is to allow the formation of the RepA dimer, the protein form active in binding to the operator.

The LZ motif mediates cooperative interactions between RepA monomers at the origin of replication

To define the binding pattern of RepA wt at the origin, and to analyse comparatively the effect of different mutations, EMSA was also performed with *oriV* probes. RepA wt binds to this region, leading to the formation of four complexes (Figure 6). As there are four iterons at the origin, each complex probably corresponds to RepA binding to one, two, three or all four repetitions. Initial binding to the origin is linear, but successive addition of protein induces the complete occupation of all four iterons in a cooperative way.

Comparative EMSA assays performed with the RepA wt protein, and with a LZ mutant in a single leucine (RepALZ1), indicate that this mutation affects the cooperative RepA–RepA interactions at the origin (Figure 6). At a protein concentration in which *oriV* molecules have all four iterons bound by RepA wt, RepALZ1 still binds to one or two iterons. Only a reduced proportion of DNA molecules have the four iterons occupied. This defect increases in the double Leu→Val mutant, RepALZ12. EMSA analysis using this mutant showed an

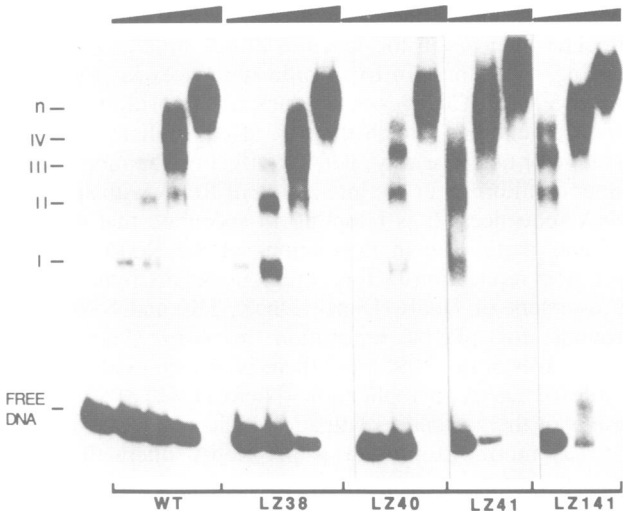

Fig. 7. Binding to the *oriV* region of the RepA wt protein and RepA mutants in b/f residues of the LZ. In all cases, RepA protein was assayed by EMSA as a purified fusion with MBP. RepAwt is included as a reference. Increasing quantities of protein were incubated with 15 ng of labelled origin probes (wt: 50, 100, 200, 400 ng; LZ38: 50, 100, 200, 400 ng; LZ40: 50, 200, 400 ng; LZ41: 100, 200, 400 ng; LZ141: 100, 200, 400 ng). Positions of retarded complexes (I, II, III, IV and n) and free DNA are indicated. n indicates high order complexes (observed at the higher protein concentrations used). These could be the result of additional RepA molecules entering the complex after occupation of the four iterons at the origin.

accumulation of complex I, formed by the binding of RepA to a single iteron (Figure 6). The cooperative behaviour of RepA in the origin seems to be essential for replication, since, as shown above, neither RepALZ1 nor RepALZ12 can initiate replication. Note that at low protein levels the binding of both mutants at the origin are comparable with that observed with the wt protein. This indicates that the mutations do not interfere with the initial binding of the protein to the *oriV* sequences. Note also that there is a clear reduction in the free DNA fraction when, at high RepA wt concentrations, all the retarded complexes correspond to molecules with all four iterons bound. This could reflect a stabilization of the protein–DNA complex mediated by RepA–RepA interactions when all four iterons are occupied. This stabilization would reduce the population of DNA that dissociates from the RepA–DNA complex when it enters the gel matrix. At high protein concentrations the free DNA fraction is much larger in the mutants. This may be explained by the reduced stabilization of the RepA–*oriV* complexes expected for these mutants, due to defective RepA–RepA interactions at the origin.

Mutations in the LZ motif affecting replication but not affecting binding to oriV

EMSA analysis was performed with *oriV* probes and purified LZ mutants in motif positions b/f (Figure 7). The lowest mobility complex (n), which appears at the highest protein concentrations, could be due to additional entries of RepA in *oriV* when all the iterons have been occupied. This has been described for DnaA in *oriC* (Bramhill and Kornberg, 1988). Apart from variations in the binding patterns, probably due to slight differences in the protein concentration and/or the contribution of a partially denatured protein fraction, all the mutants assayed showed, as did RepA wt, cooperative binding to the origin. The efficient formation of the high order RepA–*oriV* complexes in these mutants suggests that changes in the b/f region have no major effect in RepA–*oriV* interactions. Nevertheless, as shown above, *in vivo* analysis indicates that RepALZ38, RepALZ39, RepALZ40 and RepALZ41 are not able to promote replication. This could indicate that the b/f residues are involved in some kind of additional interactions relevant to replication. This possibility is underlined by the fact that another mutant in position b, RepALZ141, increases the host range of the plasmid (Fernández-Tresguerres *et al.*, 1995; Figure 5) without altering binding to *oriV* regions (Figure 7). This suggests that mutations in this region of the helix could also optimize these additional interactions. Hence, positive or negative effects in replication are not necessarily due to differences in RepA binding to *oriV*. As discussed below, this indicates a dual role of the LZ motif in replication, one consisting of promoting RepA–RepA cooperative interactions, and another consisting of promoting interactions between RepA and other host replication factors.

Discussion

The LZ in RepA dynamically regulates a monomer–dimer bifunctional system

Previous results had indicated that RepA–DNA interactions involved in transcriptional regulation and replication are different (García de Viedma *et al.*, 1995). Now it is shown that RepA is subjected to a monomer–dimer association equilibrium and that monomers interact with the origin of replication whereas dimers of the protein interact with the *repA*P region. Dimers of RepA are also involved in the formation of high order complexes in the *repA*P region by RepA–RepA interactions (see Introduction). The involvement of dimers of a replication protein in autoregulation, and of monomers in activation of the origin, has been proposed previously for the replication proteins of plasmids F (Ishiai *et al.*, 1994) and pSC101 (Manen *et al.*, 1992). The relevance of monomers of the replication protein in initiation of plasmid replication has been reported in plasmid P1 (DasGupta *et al.*, 1993; Wickner *et al.*, 1994). The successive entries of dimers at the operator has also been found in TF1, a *Bacillus subtilis* type II DNA binding protein (Andera *et al.*, 1994).

The LZ motif found in RepA is important in the regulation of the monomer–dimer distribution. Double mutation at the leucines severely diminishes dimerization and this prevents the interaction of RepA in the *repA*P region, though not in *oriV*. This is consistent with the involvement of dimers of this protein in autoregulation, and monomers in replication. Mutations in LZ residues other than the leucines, on the opposite side of the potential α-helix, do not affect dimerization or binding to the *repA*P region. This underlines the essential and specific role of the leucines of the motif in dimerization.

In EMSA, bands corresponding to DNA-bound RepA–MBPRepA heterodimers are observed when mixtures of the two RepA forms and *repA*P probes are used. The dimerization constant of RepA, as evaluated by equilibrium centrifugation analysis, indicates that at the protein concentration used in heterodimerization assays, RepA should

be present mainly as a dimer. This indicates that the formation of heterodimers reflects a dynamic interchange of subunits between pre-formed RepA dimers. The situation is different in other proteins such as the replication protein of pSC101 or in GCN4. Here, heterodimers are observed when there is co-expression of two protein forms of different size *in vitro*, but not in mixtures of both proteins (Hope and Struhl, 1987; Manen *et al.*, 1992). This indicates the non-existence of subunit rearrangements in pre-formed dimers.

In addition to the hydrophobic leucine spine found in the d position, LZ motifs frequently show a hydrophobic spine in position a which contributes towards the stability of the dimer (Tropsha *et al.*, 1991; John *et al.*, 1994). In RepA, the great sensitivity of dimerization to changes in leucines, and the dynamic interchanging of subunits in the dimer, could both result from the absence of the additional hydrophobic spine in position a. The flexible association–dissociation rate acquired by this 'single spine attachment' could confer advantages in a system in which monomers and dimers are both functionally relevant.

The LZ of RepA has different roles in transcriptional regulation and DNA replication

The LZ in RepA is relevant in replication and autoregulation. Changes in the different residues have a greater effect on replication that on regulation. This could indicate that the interaction of the partially active LZ mutants with inverted repeats at the operator somehow compensates for their dimerization defects. However, a mutant severely affected in dimerization (RepALZ12) is not able to regulate and binds very poorly to the operator. This effect cannot be a result of a major alteration in the protein, as RepALZ12 retains its ability to bind to the origin of replication. It should be noted that binding to DNA is not mediated directly by the LZ motif but by the HTH motif at the carboxy end of RepA (García de Viedma, 1996).

The heterodimerization assays indicate that RepA binds as a monomer to *oriV* sequences. It was therefore unexpected that conservative mutations in the leucines, that reduce the dimerization constant of the protein, should have a negative effect in replication. A detailed analysis of RepA interactions at the *oriV* region indicated that the leucine residues of the motif affect cooperative interactions of the RepA protein at the *oriV* region. This reveals that leucine residues modulate RepA–RepA interactions needed to nucleate an active replication initiation complex. As RepA monomers bind to directed repeats at *oriV*, steric restrictions could be limiting potential interactions between leucines of two adjacently bound RepA monomers. Contacts between leucine residues in a RepA monomer and other hydrophobic surfaces of the adjacently bound RepA monomer could elude those steric restrictions. Nevertheless, as DNA binding and dimerization domains are independent in RepA (see Introduction), one cannot discard the possibility that rearrangements of RepA–*oriV* complexes could allow additional contacts between leucines of two *oriV*-bound RepA monomers. In any case, the absence in RepA LZ of an additional hydrophobic spine in position a could make the requirements for different RepA–RepA contacts at *oriV* more flexible.

The results also indicate the different contributions to replication and regulation made by residues at the d position (leucines) and at the b/f positions of the putative α-helix. Changes in the leucines affect dimerization and binding of the protein to specific sequences in *oriV* and *repA*P regions. Changes in residues at b/f positions do not affect regulation, though they do affect replication (in a positive or negative way) without affecting the monomer–dimer equilibrium or the interaction of RepA with specific DNA sequences. It is tempting to speculate that the b/f residues participate in interactions of the RepA protein with host replication factors. Previous results indicate the requirement of DnaB, DnaG, DnaK, HU and SSB host proteins for pPS10 replication *in vitro* (Fernández-Tresguerres *et al.*, 1995) and there is *in vivo* evidence for a role of DnaA in replication (Nieto *et al.*, 1992). The results of the present investigation indicate a dual role for the LZ motif: promoting RepA–RepA interactions in dimerization and cooperativity at *oriV* (via leucines at position d), and promoting (via residues at b/f positions) interactions between RepA and other host replication factors involved in replication.

This is the first detailed analysis of an LZ motif present in a replication protein that reveals a multifunctional role for the motif. It shows that residues in particular positions of the putative α-helix are essential for replication and/or autoregulation. For the first time, a functional role for the residues in positions b/f of a LZ motif is described.

Materials and methods

Bacterial strains

The *E.coli* K-12 strains used were: C600 (Bachmann, 1972), CC118 (Manoil and Beckwith, 1985), CSH50 (Miller, 1972) and BT1000 (Wechsler *et al.*, 1973). PAO1024 (r^-, m^+; from K.Nördstrom) was the *P.aeruginosa* strain used.

Media and growth conditions

Cultures were grown at 30°C (*lacZ* fusions) or 37°C in LB medium (Lennox, 1955). Rich glucose medium (BioLabs) was used to prepare MBPRepA. 2× TY (Amersham) was used in M13 derivative infections. Media were supplemented with kanamycin or ampicillin (50 μg/ml) to select for plasmids conferring resistance to these antibiotics.

General methods

DNA cloning, gel electrophoresis of DNA and proteins, basic manipulations with enzymes, mini and maxi preparations of DNA and transformation of *E.coli* cells were performed as described by Sambrook *et al.* (1989). DNA elution from agarose gels was performed using a Geneclean kit (BIO 101). β-Galactosidase assays were performed as described by Miller (1972). For DNA mutagenesis, the 'oligonucleotide-directed *in vitro* mutagenesis system' (Amersham) was used.

Plasmid constructions

Substitutions in the residues of the LZ motif of RepA were introduced by oligonucleotide-directed mutagenesis of the M13mp19 *repA* derivative carrying the *Eco*RI *rep*A fragment of plasmid pCN51 (Nieto *et al.*, 1992). Plasmids pME38–pME41 include single and double substitutions in residues b/f of the LZ motif. In single mutants, pME38 and pME39, Glu15 and Asp36 were changed to His and Lys, respectively. Double mutants pME40 and pME41, in addition to the substitutions of pME38 and pME39, included the changes His18→Glu and Lys39→Asp, respectively. Single and double Leu→Val substitutions in the four leucines (position d) of the LZ motif were introduced to obtain the M13 derivatives pRG3111–pRG3114 (single substitutions in Leu13, Leu20, Leu27 and Leu34, respectively) and pRG3112, pRG3123 and pRG3134 (double substitutions in the two first, two middle and two last leucines of the LZ motif, respectively). An in-phase deletion of all the motif, pRG310, was also obtained (affecting residues 13–34). pME141 is a broad host range pPS10 derivative obtained by *in vitro* mutagenesis with hydroxylamine (Fernández-Tresguerres *et al.*, 1995).

The series pSE38–pSE41 and pRG321–pRG324 and the recombinants

pRG3212, pRG3223, pRG3234, pRG320 and pSB141 were obtained by substitution of the wt *Eco*RI *repA* fragment of pCN51 (Nieto *et al.*, 1992) by the corresponding fragments of pME38–pME41, pRG3111–pRG3114, pRG3112, pRG3123, pRG3134, pRG310 and pME141, respectively.

The series pMMBLZ38–pMMBLZ41, pMMBLZ1–pMMBLZ4 and the recombinants pMMBLZ12, pMMBLZ23, pMMBLZ34, pMMBLZ0 and pMMBLZ141 were constructed by cloning the *Eco*RI *repA* fragments of pSE38–pSE41, pRG321–pRG324, pRG3212, pRG3223, pRG3234, pRG320 and pSB141, respectively, in the *Eco*RI site of the RSF1010-type *tac*P expression vector pMMB67EH (Furste *et al.*, 1986). The orientation that leads to *repA* expression was selected. The opposite orientations were also selected as negative controls.

To construct pMalRepA, a pCN51 fragment that included the *repA* gene [coordinates 577–1500, according to Nieto *et al.* (1992)] was obtained by PCR using *Pfu*I and the oligonucleotides 5′-ATGGTCGAGAACAAAGTCACG-3′ (start codon underlined) and 3′-CGATACCGTGTCTCTTAAGGCC-5′ (*Eco*RI site underlined) as primers. The ATG oligonucleotide was phosphorylated before amplification. PCR products were digested with *Eco*RI and cloned between the *Xmn*I and *Eco*RI sites of the pMal-c2 expression vector (BioLabs), generating a translational fusion between MBP and RepA. The *repA* gene was sequenced to ensure the absence of nucleotide changes after the amplification reaction. To construct the LZ mutant variants pMalRepLZ0, LZ1, LZ2, LZ12, LZ23, LZ34, LZ141, LZ38, LZ40 and LZ41, an equivalent process was followed but by amplifying *repA* from pRG320–pRG322, pRG3212, pRG3223, pRG3234, pMM141, pSE38, pSE40 and pSE41.

pFusCE is a transcriptional fusion between the *rep*A promoter and the *lacZ* reporter gene (García de Viedma *et al.*, 1995). In pMMBRepA, *repA* is under the control of *tac*P (Nieto *et al.*, 1992).

pUCProm and pUCIt were obtained by cloning the pPS10 *repA* promoter–operator region (coordinates 445–536) and origin of replication sequences (coordinates 4–445) respectively, in pUC18*Not*.

Purification of MBPRepA

Strain CC118, containing pMalRepA, was inoculated into 1 l of rich glucose medium and grown to A_{600} = 0.6. At this point, MBPRepA expression was induced by addition of 10 μM IPTG dissolved in the same medium (1/3 of the initial volume). The culture was incubated overnight at 37°C. Cells were collected by centrifugation at 8000 *g* for 10 min. The sediment was resuspended in buffer A (10 mM Tris–HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and lysed in a French cell press at 20 000 p.s.i. The lysate was spun down at 9000 *g* for 20 min at 4°C. MBPRepA was found mainly in the soluble fraction. The supernatant was diluted (1:5) in buffer A and loaded in an amylose column (BioLabs). After loading, the column was washed with eight volumes of buffer A and MBPRepA was eluted with the same buffer supplemented with 20 mM maltose. Column fractions were analysed on 15% (w/v) polyacrylamide gels by SDS–PAGE (Laemmli, 1979). MBPRepA activity was measured by its ability to shift a promoter–operator fragment in EMSA.

To excise RepA from MBP, 40 μg of purified MBPRepA were digested with 0.5 μg of factor Xa at 4°C for 3 h. The reaction was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. The efficiency of the reaction was checked by SDS–PAGE.

EMSA analysis

pUCProm and pUCIt (20 μg) were digested with *Not*I and labelled with 40 μCi of [α-^{32}P] and 1 U of Klenow fragment. Fragments were fractionated in native PAGE and eluted from the gel as previously described (García de Viedma *et al.*, 1995); the conditions followed for EMSA have been described elsewhere (García de Viedma *et al.*, 1995). For heterodimer assays, RepA was incubated with MBPRepA for 5 min at 4°C (a 1 mg/ml solution of each protein was used) prior to addition of the labelled DNA probe. Quantification of RepA and MBPRepA in these assays was performed by the Bio-Rad protein assay kit, based on Bradford (1976) and by densitometry of protein bands in SDS–PAGE.

Analytical ultracentrifugation assays

Short column sedimentation equilibrium experiments were performed as follows. Samples of 50 μl of MBPRepA (equilibrated in 10 mM Tris, 10 mM maltose, 200 mM NaCl, 1 mM EDTA, pH 7.4) ranging in protein concentration from 0.01 to 1.2 mg/ml were centrifuged at 4°C and at different speeds (20 000 and 25 000 r.p.m.) in a Beckman XL-A analytical ultracentrifuge using centrifuge cells with 12 mm six channel centrepieces of epon charcoal. When the sedimentation equilibrium was reached, radial scans with 10–20 averages and 0.001 cm step size were taken at different absorbances (230 and 280 nm). Baseline offsets were determined at 42 000 r.p.m.

In order to obtain the apparent weight-average molecular weight (Mw_a) of MBPRepA samples, a preliminary analysis of individual data sets was performed using the programs EQASSOC and XLAEQ (supplied by Beckman Instruments Inc., see Minton, 1994), with 0.735 ml/g as the partial specific volume of protein, calculated from its amino acid composition and corrected for temperature according to Durshlag (1986). The dependency of Mw_a on protein concentration was used as a diagnostic test of association behaviour. Average molecular weights were obtained either from the whole cell (as described above) or from measurements of local slopes at defined radial intervals, using $\mathrm{Ln}A/r^2$ transformed data (Laue, 1992; Cole *et al.*, 1993).

Experimental data at different speeds and protein concentrations were globally fitted to a monomer–dimer association equilibrium model (Equation I) using the MicroCal Origin version of the NONLIN algorithm (Johnson *et al.*, 1981).

$$A(r)=A(r_0)\exp[\mathrm{H}\,M_1\,(r^2-r_0^2)]+A(r_0)^2\exp[\ln K_2^{\mathrm{A}}+2\,\mathrm{H}\,M_1(r^2-r_0^2)]+\zeta \quad (\mathrm{I})$$

In equation (I), $A(r)$ is the total protein concentration (in absorbance) at a given radius r, r_0 is an arbitrary radial distance of reference, $H = (1-v\rho)\,\omega^2/2RT$, where v is the partial specific volume of the protein, ρ is the solution density, ω is the angular velocity of the rotor, R is the gas constant, T is the absolute temperature, M_1 is the monomer molecular weight of MBPRepA (73 600 g/mol), and ζ is a baseline offset. The equilibrium constant of dimerization, K_2^{A}, was transformed in the equivalent constant in molar units, K_2^{M}, using the following equation: $K_2^{\mathrm{M}} = K_2^{\mathrm{A}}(\varepsilon l/2)$ (Lewis, 1991), where ε is the molar absorption coefficient of the protein at 280 nm (62 500 M/cm) and l is the optical pathlength of the centrifuge cell.

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