5048–5054 Nucleic Acids Research, 1995, Vol. 23, No. 24

Specific binding of the replication protein of plasmid pPS10 to direct and inverted repeats is mediated by an HTH motif

Darío García de Viedma*, Ana Serrano-López and Ramón Díaz-Orejas

Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain

Received August 4, 1995; Revised and Accepted November 15, 1995

ABSTRACT

The initiator protein of the plasmid pPS10, RepA, has a putative helix–turn–helix (HTH) motif at its C-terminal end. RepA dimers bind to an inverted repeat at the *repA* promoter (*repA*P) to autoregulate RepA synthesis. [D. García de Viedma, *et al.* (1996) *EMBO J.* in press]. RepA monomers bind to four direct repeats at the origin of replication (*oriV*) to initiate pPS10 replication This report shows that randomly generated mutations in RepA, associated with defficiencies in autoregulation, map either at the putative HTH motif or in its vicinity. These mutant proteins do not promote pPS10 replication and are severely affected in binding to both the *repA*P and *oriV* regions *in vitro*. Revertants of a mutant that map in the vicinity of the HTH motif have been obtained and correspond to a second amino acid substitution far upstream of the motif. However, reversion of mutants that map in the helices of the motif occurs less frequently, at least by an order of magnitude. All these data indicate that the helices of the HTH motif play an essential role in specific RepA–DNA interactions, although additional regions also seem to be involved in DNA binding activity. Some mutations have slightly different effects in replication and autoregulation, suggesting that the role of the HTH motif in the interaction of RepA dimers or monomers with their respective DNA targets (IR or DR) is not the same.

INTRODUCTION

pPS10 is a plasmid isolated from *Pseudomonas savastanoi* that is also able to establish in *P.aeruginosa* and *P.putida* (1). Its basic replicon consists of an origin of replication and an open reading frame (ORF) that codes for a replication protein, RepA, that is also a transcriptional regulator of its own synthesis (1). RepA is a basic protein of 231 amino acids (26.7 kDa) subjected to a monomer–dimer equilibrium in solution (1,2). Dimerization is modulated by a leucine zipper (LZ) motif located at the N-terminal end of RepA (1–3). RepA monomers promote pPS10 replication by binding to four direct repeats (DR) of 22 bp (iterons) present at the origin of replication (*oriV*) (1,2; Fig. 1). Dimers of RepA repress *repA* expression by interacting with an inverted repeat (IR) formed by two arms of 8 bp that overlap the promoter (*repA*P) and that share homology with the central region of the iterons (Fig. 1; 2,4). At the C-terminus of RepA a homologous region with a putative helix–turn–helix (HTH) motif is present (1,3; Fig. 1). The HTH motif is a DNA binding motif frequently found in transcriptional regulators. It is formed by a region of 20–22 residues organised in two α-helices. The helices are separated by a turn that introduces a 120° angle between them (5,6). Usually HTH motifs cannot fold independently and are included in a broader domain with additional structural elements (5). The N-terminal helix lies along the major groove of the DNA and the C-terminal helix, called the recognition helix, is involved in sequence-specific recognition of DNA (5).

When searching for HTH motifs using the matrix developed by Brennan (7) the putative HTH in RepA (Fig. 1) shows a good fit (1). This is indicated by the following: (i) the potential of the amino acids in the region to form two α-helical stretches; (ii) the presence of Gly9 and Val15, two highly conserved residues of the motif; (iii) the residue in position 5 (Met), which participates with Val15 in formation of the hydrophobic core of the motif, contains no β-branched groups; (iv) the presence of Gln in position 1, which is involved in the initial nucleation of the protein–DNA complex (8); (v) a non-charged residue (Val15) is present in the central position of the putative C-terminal helix.

The only feature of the HTH motif found in RepA that differs from the consensus is the presence of a charged residue in the middle of the N-terminal helix (Asp4; Fig. 1).

The initiator protein of replication in plasmid F, RepE, includes two putative HTH motifs (9). However, it has recently been reported that the C-terminal end of the RepE protein, but not the putative HTHs, is relevant in DNA binding (10). The C-terminal regions of the initiator proteins π of plasmid R6K and RepA of plasmid pSC101 are also involved in DNA binding, but no homologies with any known DNA binding domain have so far been found (11–13). Genetic analyses performed with plasmid RK2 have failed to define a DNA binding domain in the TrfA replication protein (14).

The dual binding of different forms of RepA of pPS10 to two differently arranged (DR or IR) DNA targets calls for analysis of its DNA binding domain. This report identifies the HTH motif

* To whom correspondence should be addressed

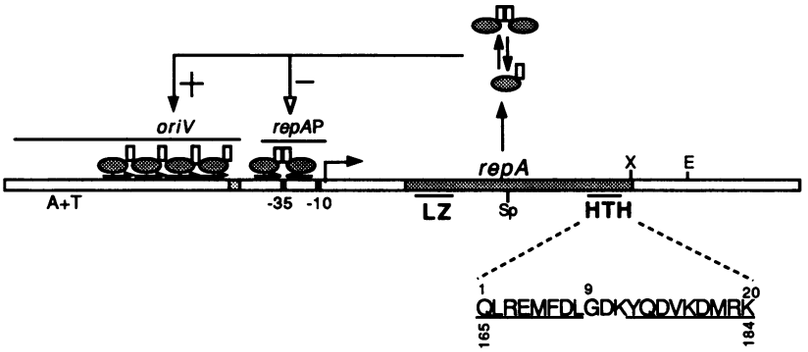

Figure 1. Relevant features of the basic replicon of pPS10. The origin region (*oriV*) includes a dnaA box (dotted square), four identical iterons of 22 bp (→) and an AT-rich region (A+T). (Dotted rectange) Open reading frame corresponding to plasmid replication protein *repA*. (Head-to-head arrows) An inverted repeat of 8 bp flanking the *rep*A promoter (*rep*AP). Putative leucine zipper (LZ) and helix–turn–helix (HTH) motifs in the RepA protein are indicated. The HTH amino acid sequence is expanded and the putative helices of the motif are underlined. Numbers above and below the sequence correspond to the coordinates of residues in the motif and in the whole protein respectively. (Top) RepA monomers bind to the iterons at *oriV* to initiate (+) pPS10 replication and RepA dimers bind to the inverted repeats at the promoter repressing (–) *repA* expression. Dimers are assembled by an LZ motif. E, *Eco*RI; Sp, *Sph*I; X, *Xho*I.

present in the replication protein of plasmid pPS10 as the essential part of the DNA binding domain of the protein. RepA mutants mapping in the HTH motif and adjacent regions were isolated by selecting mutants deficient in autoregulation. Analysis of these mutants indicated that the HTH motif is the DNA binding motif required for interactions of RepA with the inverted repeat of the *repA*P region and with homologous directed repeat sequences at the *oriV* region (iterons). It is also shown that specific RepA binding to these sequences is influenced by amino acids outside the HTH motif.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *Escherichia coli* K-12 strains used in this work were CC118 (15) and CSH50 (16). The *P.aeruginosa* strain used was PAO1024 (r^-, m^+; obtained from K. Nördstrom). The plasmids used were: pUC18*Not*, a general cloning vector (17); pMMBRepA, an RSF1010-based *tac*P–*repA* recombinant (1); pMal-c2 (BioLabs), an expression vector to fuse proteins with maltose binding protein (MBP); pCN51, a pPS10-pBR322 shuttle vector (1); pFusCE (fusion *Cla*I–*Eco*RI), a *repA*P–*lacZ* transcriptional fusion (4). pUCProm (*repA* promoter) and pUCIt (*oriV* iterons) are pUC18*Not* recombinants that include the *repA*P (coordinates 4–445) and *oriV* (coordinates 445–536) sequences respectively (4).

Media and growth conditions

Cultures were grown at 30°C (*Pseudomonas* and strains containing *lacZ* fusions) or 37°C, as indicated, in LB medium (18). Rich medium supplemented with glucose (BioLabs) was used to prepare the MBP–RepA wild-type and mutants. McConkey agar (DIFCO) was used to grow cells containing *lacZ* fusions. Media were supplemented with 50 μg/ml kanamycin or ampicillin (Sigma) to select for the presence of 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 carried out as described elsewhere (19). Electroporation of *Pseudomonas* cells was performed using the BioRad system (2.5 kV). DNA elution from agarose gels was performed using the Gene-clean kit (BIO 101 Inc.). β-Galactosidase assays were performed as described elsewhere (16). To map the mutations, the *Sph*I–*Eco*RI fragments corresponding to each mutant (coordinates 853–1500, which includes the 3′-end of the *repA* gene) were subcloned into the pUC18*Not* vector. This fragment was sequenced using a T7 sequencing kit (Pharmacia). The restriction sites corresponding to this mutant are indicated in Figure 1.

Relevant plasmid constructs

Plasmids of the series pMMBRepAH (H2, H4, H6, H11, H18, H36 and H38; mutations within or close to the HTH motif) and pMMBRepAHt (Ht1, Ht2, Ht5 and Ht8; thermosensitive mutants) contain independent *repA* mutations obtained by hydroxylamine treatment or PCR mutagenesis and were constructed by substitution of the *Sph*I–*Eco*RI *repA* fragment of pMMBRepAwt by the corresponding mutant fragments. pMMBRepAHR plasmids (HR7 and HR8: revertants of the H38 mutant) were obtained by a second round of mutagenesis of recombinant pMMBRepAH38. The pCN51H series of plasmids was obtained by substitution of the *Eco*RI–*Eco*RI fragment (coordinates 503–1500) of pCN51 by the corresponding fragments of the pMMBRepAH series. The MBP–RepA expression vectors pMalRepA, pMalRepAHt5, pMalRepAH36, pMalRepAH38 and pMalRepAHR8 are pMal-c2 recombinants containing a fragment that includes the *repA* gene (from the ATG codon to the *Eco*RI site, coordinates 577–1500) obtained respectively from pCN51, pCN51Ht5, pCN51H36, pCN51H38 and pCN51HR8 by PCR amplification. Sequence analysis indicated that no new mutations were introduced during the amplification reaction.

Random mutagenesis methods

DNA was mutagenized *in vitro* with hydroxylamine (Sigma) under conditions that introduce, on average, single nucleotide changes in the DNA molecule (20). The *Sph*I–*Eco*RI mutated fragments were used to substitute for the corresponding wild-type

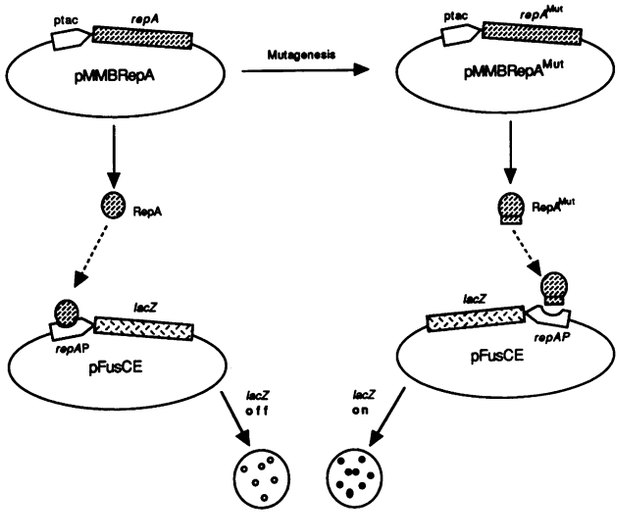

Figure 2. Scheme of the two-plasmid method used to screen RepA mutants that fail to bind to the *repA*P region. (Left) White colonies are obtained when RepA (expressed from a *tac*P–*repA* recombinant, pMMBRepA) binds *in trans* to the *repA* promoter of a transcriptional *lacZ* fusion (pFusCE), inhibiting *lacZ* expression. (Right) Red colonies are obtained when a mutation in RepA affects the binding of this protein to the *repA* promoter. Colonies were grown in McConkey agar supplemented with IPTG (1 mM) to induce RepA synthesis.

fragment of pMMBRepAwt. Mutagenesis of DNA by PCR techniques was performed taking advantage of the low fidelity of *Taq* DNA polymerase. A pCN51 *repA* fragment (coordinates 577–1500) was amplified using standard reaction conditions and 15, 25 or 50 amplification cycles. *Sph*I–*Eco*RI fragments obtained from the amplified products were also used to substitute for the corresponding wild-type fragment of pMMBRepAwt. As autoregulation-defective mutants were obtained in all cases, the clones obtained with the lower number of amplification cycles (15 cycles) were selected for the analysis, since these have a higher probability of containing single mutations.

Purification of wild-type and mutated RepA proteins

The purification of MBPRepA, MBPRepAHt5, MBPRepAH36, MBPRepAH38 and MBPRepAHR8 was performed as follows. Strain CC118 containing pMalRepA, pMalRepAHt5, pMalRepAH36, pMalRepAH38 or pMalRepAHR8 was inoculated into 1 l rich glucose medium and grown to A_{600} 0.6. At this point MBP–RepA expression was induced by addition of 10 μM IPTG (Boehringer) dissolved in the same medium (one third of the initial volume). The culture was then 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 lb/in^2. The lysate was spun down at 9000 *g* for 20 min at 4°C. MBP–RepA was found mainly in the soluble fraction. The supernatant was diluted (1:5) in buffer A and loaded onto an amylose column (Biolabs). After loading the sample the column was washed with 8 vol buffer A. MBP–RepA was eluted in buffer A supplemented with 20 mM maltose. Column fractions were analysed on 15% (w/v) polyacrylamide gels by SDS–PAGE (22). MBP–RepA activity of the different fractions was evaluated by their efficiencies at binding specifically to a *repA*P fragment using an electrophoretic mobility shift assay (EMSA). Quantification of protein was carried out using the BioRad protein assay kit based on Bradford (21) and by densitometry of protein bands in SDS–PAGE (22).

Electrophoretic mobility shift assays (EMSA)

To obtain the *repA*P and *oriV* probes 20 μg pUCProm and pUCIt were digested with *Not*I and labelled with 40 μCi α-^{32}P and 1 U Klenow fragment. Labelled DNA fragments were fractionated in native PAGE and eluted from the gel. Interactions between purified MBP–RepA proteins (wild-type and mutants) and labelled DNA fragments were evaluated by EMSA assays as previously described (4).

RESULTS

Direct screening of RepA mutants affected in binding to DNA

RepA mutants were obtained by random *in vitro* mutagenesis of a *tac*P–*repA* recombinant (pMMBRepA) using either hydroxylamine treatment or PCR mutagenesis. The mutants affected in DNA binding were identified using a two-plasmid system that takes advantage of the ability of RepA to repress *in trans* transcription from *repA*P, its own promoter (1,4; Fig. 2). In this system RepA is provided by a *tac*P–*repA* recombinant (pMMBRepA). The activity of *repA*P is monitored by the level of *lacZ* expression of a *repA*P–*lacZ* transcriptional fusion placed *in trans* on a plasmid, pFusCE, compatible with the *tac*P–*repA* recombinant. RepA mutants that fail to bind to *repA*P should give red colonies (*lacZ*$^+$) on McConkey agar. RepA mutants unaffected in binding to DNA should repress *repA*P, leading to white colonies (*lacZ*$^-$). Mutants clearly affected in autoregulation were obtained with a frequency of 1%.

RepA binds to its operator as a dimer (2) that is assembled by the LZ motif located at the N-terminal end of the protein (2,3). Therefore, mutations in the LZ motif could affect autoregulation without necessarily altering the DNA binding motif. In addition, mutants in the *tac* promoter of pMMBRepA could decrease *repA* expression, giving red colonies that would not correspond to deficiencies in RepA DNA binding activity. To discard possible mutants in both *tac*P and the LZ motif, *Sph*I–*Eco*RI fragments (which include the 3′ half of the *repA* gene; Fig. 1) from the mutagenized pMMBRepA sample were used to substitute for the corresponding fragment of a pMMBRepA recombinant that had not been subjected to mutagenesis. Recombinants were introduced by transformation in the *lac*$^-$ strain CSH50. A pool of these transformants containing the pMMBRepA derivatives was transformed with pFusCE and plated onto McConkey agar to select for red colonies. Thermosensitive and non-conditional mutants were selected. Thermosensitive mutants give white colonies at 30°C and red colonies at 42°C and were chosen in order to counterselect deletion mutants.

The β-galactosidase activity of coloured colonies containing pFusCE and pMMBRepA mutants was measured and the percentage of repression of the *repA* promoter for each mutant was determined. Clear reductions in the autoregulatory activity of RepA were obtained for all mutants assayed (Fig. 3), indicating defects in RepA binding to the *repA* promoter.

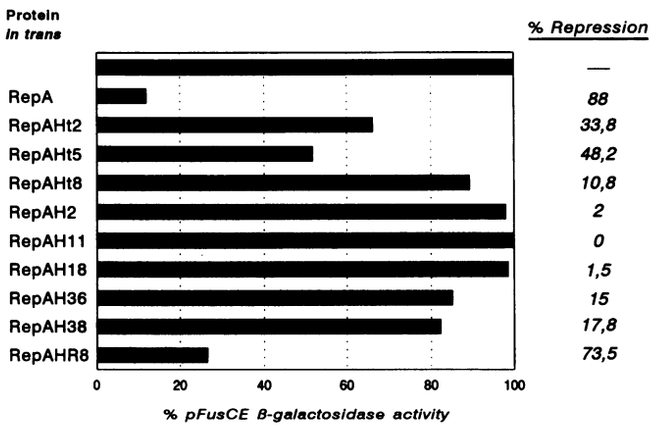

Figure 3. Determination of autoregulation activity in RepA mutants. The histogram and figures represent the percentage of β-galactosidase activity of pFusCE in the presence of *in trans* RepAwt, the RepAH mutants and the revertant RepAHR8. RepA was expressed in all cases from corresponding *tac*P recombinants (pMMBRepAs) in the presence of IPTG. The 100% β-gal activity (no repression, ~4400 Miller U) corresponds to the level of β-gal expressed by cells containing the pFusCE recombinant and the *tacP* vector (pMMB67EH). Thermosensitive mutants Ht2, Ht5 and Ht8 were analysed at 42°C.

Mutations in RepA affecting autoregulation map in the HTH motif or adjacent regions

The *Sph*I–*Eco*RI region of all clones affected in transcriptional regulation mediated by RepA was sequenced. Deletions and substitutions, both affecting single nucleotides, were found. Hydroxylamine treatment and PCR mutagenesis lead, with high frequency, to non-conditional mutants containing single base deletions. For the thermosensitive mutants (Ht1, Ht2, Ht5 and Ht8) all the changes analysed corresponded to substitutions (as would be expected for a protein active at 30°C). All the mutants mapped in a region between residues 144 and 219, which includes a putative HTH motif (1). Three substitutions mapped within the helices, Ht1 and Ht5 (carrying the same mutation $G_{1076}\rightarrow A$) in the first helix and H36 ($G_{1115}\rightarrow A$) in the second helix. Substitutions were also obtained in mutants H37 and H38 (carrying the same mutation $T_{1005}\rightarrow C$), Ht2 ($C_{1040}\rightarrow T$), H18 ($G_{1130}\rightarrow A$) and Ht8 ($A_{1232}\rightarrow G$). The amino acid changes corresponding to these missense mutations and their positions with respect to the HTH motif are indicated in Figure 4. Mutants H2, H4 (in the second helix) and H6 contain single base deletions at positions 1168, 1106 and 1219 that introduce frame-shifts and which lead to premature protein termination. H11 carries a deletion at position 1154 that introduces a stop codon.

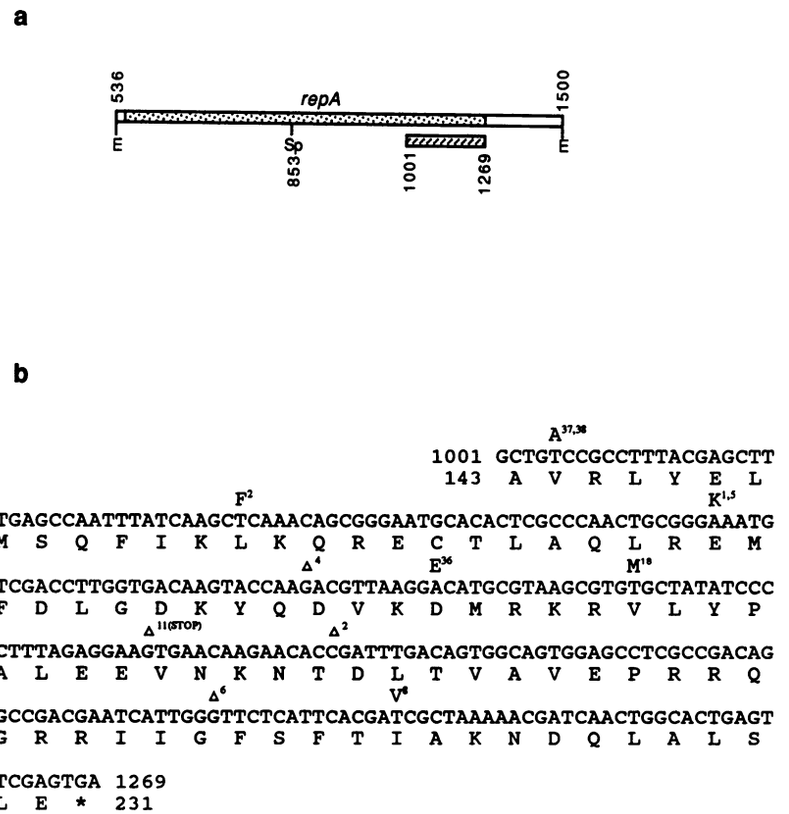

Figure 4. Localization of the mutations in RepA that affect binding to the *repA*P region. (**a**) Scheme of the *Eco*RI–*Eco*RI pPS10 fragment including *repA* (dotted box). The region that includes the HTH motif, in which the mutations were localized, is indicated (striped box). Nucleotide coordinates are indicated. E, *Eco*RI; Sp, *Sph*I. (**b**) Nucleotide and amino acid sequence of the region (nucleotide coordinates 1001–1269, amino acid coordinates 143–231) that includes the HTH motif and the different mutations found. The α-helices (boxed) and the turn (dashed underlined) of the HTH motif are indicated. The amino acid substitution corresponding to each missense mutation is indicated above the sequence at the corresponding residue. Δ, the mutation is a deletion of the base located below; superscripts, the numbers assigned to each mutant; STOP, the deletion introduces a stop codon.

All the mutations are located within or are adjacent to the putative HTH motif (Fig. 4). This indicates that this motif is relevant in the binding of RepA to *repA*P.

Revertants are not obtained for mutations in the helices of the HTH motif

To evaluate further the relevance of the putative HTH motif in binding to the *repA* promoter an attempt was made to obtain and characterize suppressor mutations for the mutants mapping in each of the putative helices of the motif (Ht5 and H36) and for a mutation mapping in its proximity (H38). It was reasoned that mutations affecting residues that are a direct part of the DNA binding motif would require true reversions to restore activity. In contrast, mutants in residues that play a role in, for example, packing the motif could probably be compensated for by intragenic suppressions as well. This would predict that the reversion frequency would be higher for mutants of the second type.

Plasmid DNA was prepared from plasmids containing mutations Ht5, H36 and H38. The preparations were again mutagenized *in vitro* with hydroxylamine. Using the two-plasmid screening assay (Fig. 2) reversions that restore RepA repressor activity would give white colonies. The H38 mutant, which maps upstream of the first helix, gave revertants with the so-called 'fish-eye' phenotype (white colonies coloured at the centre) with high frequency (1.7%, 35 revertants in 2500 colonies tested). However, revertants of mutations that mapped in either of the two putative helices were not isolated. This indicated that the reversion frequency was at least an order of magnitude lower for the mutations within the helices. The 'fish-eye' phenotype in the revertants obtained for the H38 mutant indicates some degree of β-galactosidase expression by the transcriptional fusion pFusCE (Fig. 2) and therefore an incomplete restoration of repression mediated by RepA. Two of these revertants (HR7 and HR8) were sequenced. They were found to contain, in addition to the mutation of their parental H38, the same change, $G_{812}\rightarrow A$, leading to the substitution $D_{79}\rightarrow N$. Quantification of the β-galactosidase activity promoted by pFusCE in the presence of RepAHR8 protein indicated a clear restoration of the regulatory activity in this revertant when compared with the levels obtained with H38 (Fig. 3). The amino acid change introduced by the second mutation is far upstream of the HTH motif, indicating some contribution to DNA binding function of residues distant in the primary structure of the protein (Fig. 1).

RepA mutants deficient in autoregulation are affected in binding to the *repA*P region

It was thought that *repA* mutants defective in autoregulation would be deficient in RepA binding to the *repA*P region. To test this assumption an analysis was made of *in vitro* binding to *repA*P of RepA containing mutations Ht5 or H36 (which affect each of the putative helices of the motif), H38 (located in the proximity of the HTH motif) and its revertant, HR8. This was performed by EMSA using promoter probes and purified wild-type/mutant RepA proteins. Proteins were purified and assayed as MBP–RepA fusions. Figure 5 shows that, at the protein concentrations assayed, the wild-type RepA fusion binds specifically to the *repA*P probe, whilst the three mutant proteins have lost this binding ability. Note that the RepAHt5 preparation does not bind to *repA*P, although *in vivo* the mutant is able to autoregulate expression of the *repA* promoter at the permissive temperature

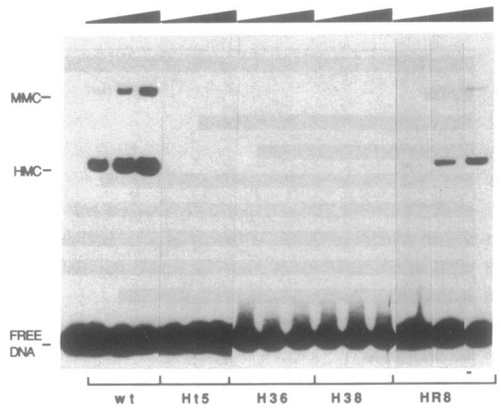

Figure 5. Binding of RepA mutants to the *repA*P region analysed by EMSA. Analyses were performed with RepAwt and the mutants RepAHt5, RepAH36, RepAH38 and RepAHR8. Increasing quantities of protein (10–40 ng) were incubated with 15 ng labelled operator probe. The proteins used in these assays were purified as MBP fusions. Free DNA and high and medium mobility complexes (HMC and MMC) are indicated.

(30°C). This could indicate that the activity of this protein is more labile *in vitro* than *in vivo*. RepAHR8, the RepAH38 revertant, regained the ability to interact with the *repA*P region. However, binding of RepAHR8 to this region was slightly reduced when compared with RepAwt. This is consistent with the incomplete restoration of the autoregulatory activity observed *in vivo* for this mutant (Fig. 3). These data establish a clear correlation between autoregulation detected *in vivo* and RepA ability to bind to the *repA*P region detected *in vitro*.

RepA mutants affected in binding to the *repA*P region are also affected in interaction with *oriV* sequences

If the HTH is the RepA DNA binding motif involved in making specific contacts with both the *repA*P and *oriV* regions, mutants in the motif that fail to bind to the *repA*P region should also be affected in binding to the *oriV* region. To evaluate this, EMSA analyses were performed using *oriV* probes and purified wild-type/mutant MBP–RepA proteins. It was found that all the mutants tested (Ht5, H36 and H38) were clearly affected in binding to the origin of replication (Fig. 6). However, there are differences among these mutants. Binding to the origin is lost in RepAHt5, is clearly affected in RepAH36 and greatly affected in RepAH38. The RepAHR8 'revertant' protein, although recovering binding activity to the origin, shows some deficiency in the formation of stable protein–*oriV* complexes, as indicated by the smearing of the different retardation complexes (23).

RepA mutants affected in binding to *oriV* have reduced ability to promote plasmid replication

If mutant RepA proteins are defective in binding to *oriV*, their efficiency in promoting plasmid replication should also be affected. To evaluate replication activity of the mutants, the *Eco*RI–*Eco*RI fragment of pCN51 (pBR322-pPS10wt recombinant), which contains the wild-type *repA* gene of pPS10, was substituted by the corresponding fragment of each of the mutants deficient in autoregulation or by that of the revertant. pCN51 and all the pCN51H mutant recombinants were rescued by transformation into an *E.coli* CSH50 background, where they replicate

Table 1. Number of colonies obtained when transforming *P.aeruginosa* with 0.5 mg plasmid DNAof the pCN51wt and pCN51H series of mutants

pCN51 derivatives	wt	Ht1	Ht2	Ht5	Ht8	H2	H4	H6	H11	H18	H36	H38	HR8
number of colonies	247	3	0	0	n.d.	4	5	3	3	5	4	1	0

n.d., not determined.
Colonies obtained with some of the mutants grew more slowly than the wild type colonies and were not viable after subculturing.

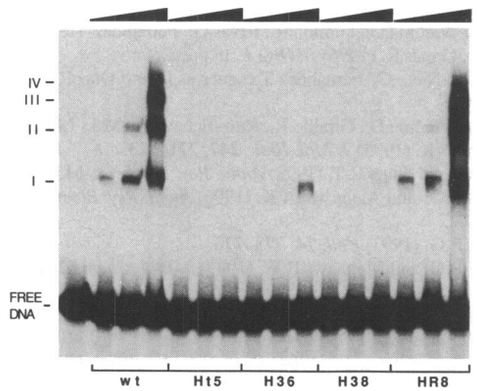

Figure 6. EMSA performed with different RepA mutants and *oriV* DNA probes. Analyses were carried out with RepAwt and the mutants RepAHt5, RepAH36, RepAH38 and RepAHR8. Increasing quantities of protein (50–300 ng) were incubated with 15 ng labelled origin probes. The proteins used in these assays were purified as MBP fusions. Relative positions of the different protein–DNA complexes and free DNA are indicated. I, II, III and IV, RepA binding to one, two, three or all four iterons at *oriV* respectively.

efficiently due to the pBR322 replicon. Subsequently it was determined whether these recombinants could be established by transformation into *P.aeruginosa*. Establishment of pCN51 in *P.aeruginosa* is due to the pPS10 replicon and therefore the efficiency of transformation indicates the efficiency of pPS10 replication. A total of 273 transformants were obtained in *Pseudomonas* with pCN51wt. Using the same amount of DNA no colonies or very few (not more than five colonies) were obtained with pCN51H mutants (Table 1). Where few colonies were obtained they grew much more slowly on the original selection plate than that obtained with pCN51wt and were not viable when subcultured. This suggests that they were not stable transformants. These data indicate that the mutations reduce the transformation frequency by at least two orders of magnitude.

The revertant HR8, though recovering most of the autoregulatory activity, gave no colonies in these assays. This could be due to instability of the RepA–*oriV* complexes detected *in vitro* (Fig. 6). The Ht (thermosensitive) mutants were deficient in establishment of the respective pCN51-Ht derivatives at 30°C, though at this temperature these mutants can autoregulate *repA* expression.

DISCUSSION

This work explores the region of the replication protein of plasmid pPS10, RepA, involved in specific interactions with DNA. Analysis of randomly generated RepA mutants affected in autoregulation indicated that they map either within or close to a putative HTH motif located at the C-terminal end of RepA. All these *repA* mutants were also impaired in replication. Purified proteins carrying mutations in each of the helices of the motif or in adjacent regions showed severe deficiencies in binding to both the *repA*P and *oriV* regions.

All *in vitro* data were obtained using MBP–RepA fusion proteins. It might therefore be argued that the results could be affected by the presence of the MBP tag. However, this interference is improbable, since: (i) *in vitro* binding to promoter probes is the same for MBP–RepAwt and RepAwt (2); (ii) analysis performed with mutants in the LZ motif in RepA (2) indicates a clear correlation between the activity of RepA, evaluated *in vivo* (autoregulation), and activity of MBP–RepA, evaluated *in vitro* (binding activity to the promoter). In addition, the results in this report show that the HTH mutants demonstrate similar correlations between repression activity and binding to promoter probes.

Usually HTH motifs are found as DNA binding domains in dimeric proteins that recognize IR sequences (5,6). The results suggest that the putative HTH motif present in RepA is the DNA binding motif required for binding of RepA to the IR in the *repA*P region and to the DRs present in *oriV*. RepA binds as a dimer to *repA*P and as a monomer to *oriV* (2).

The C-terminal helix of the HTH motif is generally considered to be the 'DNA recognition helix' (5,6). In the present analysis mutants mapping in the two helices were obtained, indicating that they are both relevant to DNA binding activity. This relevance is further supported by our failure to isolate intragenic suppressors of mutations affecting either of the two potential helices of the motif. This suggests that changes in either of these helices could severely disrupt the DNA binding domain, so that function is not easily restored by compensatory mutations. In other transcriptional regulators regions upstream of the second helix are also relevant to DNA binding (24). It might be speculated that the contribution of the first helix suggests a role in contacting the DNA phosphate backbone or a role in interactions that result in packing the second helix in such a way that it might enter the major groove.

There is a frequent location of mutations in regions adjacent to the HTH motif, which results in a DNA binding-deficient phenotype. As described for the replication proteins of pSC101 and RK2 (13,29), in RepA of pPS10 two DNA binding mutants map close to the C-terminal end of the protein. This suggests a contribution of this region to formation of an active DNA binding domain. The available structures of different DNA–protein complexes indicate that residues adjacent to HTH can make contacts with DNA (6,25) and that these are also relevant in the determination of DNA binding specificity (5,26–28). In RepA of pPS10, though these adjacent regions participate in DNA binding activity, they seem to be involved in more flexible interactions than the helices in the putative HTH motif. This is suggested by the fact that suppressors of mutants located close to the helices can be obtained more easily than mutations mapping within the helices. Obviously, a proper evaluation of the contribution of different regions to the DNA binding motif requires refined structural information.

Residues that are distant from the HTH motif can also contribute to formation of an active DNA binding domain, as indicated by the isolation, far upstream of the motif, of two suppressor mutations. The contribution of distal residues to an active HTH domain could be due to: (i) their direct participation in formation of this domain; or (ii) an indirect effect on the stability of the protein. No differences were detected between any of the mutants and the wild-type protein in *in vivo* expression levels. The purification of MBP–RepA proteins by affinity chromatography showed no evidence that the mutants were more sensitive to degradation. Evaluation of the contribution of mutations to stable folding of the protein could give additional information on the role of these regions located upstream of the HTH motif.

Although the results of this report show that the HTH motif is involved in binding both to the *repA*P and *oriV* regions, the role of this motif is not equivalent on both targets. This is indicated by small differences between the phenotypes of mutants in autoregulation and replication: (i) the mutant proteins assayed do not bind to the operator (IR) *in vitro*, though some (H36 and H38) retain a residual binding activity to the origin (DRs); (ii) thermosensitive mutants, deficient in autoregulation at 42°C but not at 30°C, are deficient in replication at both temperatures; (iii) a suppressor mutation (HR8) that restores autoregulation activity also recovers binding activity to *oriV in vitro*, though the stability of RepA–*oriV* complexes is reduced and promotion of plasmid replication is fully impaired. In the F replicon mutations in the putative DNA binding domain of the initiator, protein RepE, also affect binding to the operator in greater measure than to the origin of replication (10). It has been proposed that this reflects indirect effects of these mutations on RepE dimerization, as dimeric forms are responsible for binding to the operator (10). In RepA this is not probable, since dimerization is modulated via a LZ motif at the N-terminal end of the protein (2,3). There are alternatives that might explain the differences between the replication and autoregulation phenotypes of certain HTH mutants: (i) slight differences in the DNA residues involved in contacting RepA at the promoter and *oriV*; (ii) the same residues are involved in both contacts, but there are differences in these interactions due to different DNA structure in these regions (DRs at *oriV* share a core sequence with the IR at the promoter, but also include additional flanking nucleotides; 1); (iii) a combination of both possibilities. The evaluation of these alternatives requires refined structural information.

ACKNOWLEDGEMENTS

This work was supported by grants BIO88-0294, BIO91-1055 and BIO 92-1018-CO2O2 from the Spanish CICYT and by the European Net CHRX-CT92-0010. D. García de Viedma was supported by a fellowship from the Comunidad de Madrid and by contract reference BIO2CT920084 of the BIOTECH Program of the EC. The excellent technical assistance of C. Pardo is gratefully acknowledged. We are also very thankful to Drs R. Giraldo and M. Suzuki for discussion of the results.

REFERENCES

1 Nieto,C., Giraldo,R., Fernández Tresguerres,E. and Díaz,R. (1992) *J. Mol. Biol.*, **223**, 415–426.
2 García de Viedma,D., Giraldo,R., Rivas,G., Fernández-Tresguerres,M.E.R. and Díaz-Orejas,R. (1996) *EMBO J.*, in press.
3 Giraldo,R., Nieto,C., Fernández Tresguerres,E. and Díaz,R. (1989) *Nature* 342, 866.
4 García de Viedma,D., Giraldo,R., Ruíz-Echevarría,M.J., Lürz,R. and Díaz-Orejas,R. (1995) *J. Mol. Biol.*, **247**, 211–223.
5 Pabo,C.O. and Sauer,R.T. (1992) *Annu. Rev. Biochem.*, **61**, 1053–1095.
6 Harrison,S.C. and Aggarwal,A.K. (1990) *Annu. Rev. Biochem.*, **59**, 933–969.
7 Brennan,R.G. (1993) *Cell*, **74**, 773–776.
8 Brennan,R.G. and Matthews,B.W. (1989) *J. Biol. Chem.*, **264**, 1903–1906.
9 Masson,L. and Ray,S.D. (1988) *Nucleic Acids Res.*, **16**, 413–424.
10 Matsunaga,F., Kawasaki,Y., Ishiai,M., Nishikawa,K., Yura,T. and Wada,C. (1995) *J. Bacteriol.*, **177**, 1994–2001.
11 Germino,J. and Bastia,D. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4692–4696.
12 Greener,A., Filutowicz,M.S., McEachern,M.J. and Helinski,D.R. (1990) *Mol. Gen. Genet.*, **224**, 24–32.
13 Manen,D., Upegui-González,L.C. and Caro,L. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 8923–8927.
14 Cereghino,J.L., Helinski,D.R. and Toukdarian,A.E. (1994) *Plasmid*, **31**, 89–99.
15 Manoil,C. and Beckwith,J. (1985). *Proc. Natl. Acad. Sci. USA*, **82**, 8129–8133.
16 Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17 Herrero,H., De Lorenzo,V. and Timmis,K.N. (1990) *J. Bacteriol.*, **172**, 6557–6567.
18 Lennox,E.S. (1955) *Virology*, **1**, 190–206.
19 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn.Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
20 Hashimoto-Gotoh,T. and Sekeguchi,K. (1976) *J. Bacteriol.*, **127**, 1561–1563.
21 Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
22 Laemli,U.K. (1979) *Nature*, **227**, 680–685.
23 Carey,J. (1991) *Methods Enzymol.*, **208**, 103–117.
24 Jacob,J. and Drummond,M. (1993) *Mol. Microbiol.*, **10**, 813–821
25 Overdier,D.G., Porcella,A. and Costa,R.H. (1994) *Mol. Cell. Biol.*, **14**, 2755–2766.
26 Jordan,S.R. and Pabo,C.O. (1889) *Science*, **242**, 893–899.
27 Aggarwal,A.K., Rodgers,D.W., Drottar,M., Ptashne,M. and Harrison,S.C. (1988) *Science*, **242**, 899–907.
28 Freemont,P.S., Laue,A.N. and Sanderson,M.R. (1991) *Biochem. J.*, **278**, 1–23.
29 Cereghino,J.L. and Helinski,D.R. (1993) *J. Biol. Chem.*, **268**, 24926–24932.