Plasmid pAL5000 represents a family of relatively newly discovered cryptic plasmids in gram-positive *Actinomycetes* bacteria. The replication regions of these plasmids comprise a bicistronic operon, *repA-repB*, encoding two replication proteins. Located upstream is a cis-acting element that functions as the origin of replication. It comprises an ~200-bp segment spanning two binding sites for the replication protein RepB, a low-affinity (L) site and a high-affinity (H) site separated by an ~40-bp spacer sequence. The trajectory of the DNA in the RepB-origin complex has been investigated, and it has been found that the origin undergoes significant bending movements upon RepB binding. RepB binding not only led to local bending effects but also caused a long-range polar curvature which affected the DNA sequences 3′ to the H site. These movements appear to be essential for the in-phase alignment of the L and H sites that leads to the formation of a looped structure. A novel property of RepB unearthed in this study is its ability to form multimers. This property may be an important factor that determines the overall trajectory of the DNA in the RepB-origin complex. The results presented in this study suggest that the origins of replication of pAL5000 and related plasmids are highly flexible and that multimeric, RepB-like initiator proteins bind the origin and induce local deformations and long-range curvatures which are probably necessary for the proper functioning of the origin.

The cryptic *Mycobacterium fortuitum* plasmid pAL5000 has gained a lot of importance due to its “tuberculosis connection.” A large number of vectors have been designed for *Mycobacterium* using the replication region of this plasmid (8, 19). Plasmids similar to pAL5000 have been reported in several other bacteria (19): *Corynebacterium* (pXZ10142), *Brevibacterium* (pRBL1 and pBAL8), *Rhodococcus* (pFAJ2600 and pNC903), *Bifidobacterium longum* (pMB1), and *Neisseria* (pJD1). Except for *Neisseria*, these bacteria belong to the gram-positive *Actinomycetes* family. Most of these plasmids feature a *repA-repB* bicistron which encodes the two replication proteins RepA and RepB (Fig. 1A). There is some similarity in the replication region of pAL5000-related plasmids with those of the ColE2 plasmids of *Escherichia coli* (27). The similarity is mostly discernible in the case of RepA, which shows homology with the CoE2 Rep (replication) proteins.

RepB (14 kDa) is a small protein possessing a high positive charge (pI, 9.5). It is expressed from the translationally coupled operon *repA-repB* (2, 3). It has been shown that RepB plays a critical role in the replication process by binding to two sites, a high-affinity (H) site and a low-affinity (L) site (2, 3, 23, 24, 25). Unlike RepA, RepB is poorly conserved. There is no significant similarity of RepB with DNA binding proteins in the databases outside the pAL5000 family. Even within the family, the conservation is poor. Nevertheless, RepB seems to belong to the larger family of helix-turn-helix DNA binding proteins possessing a three-helix bundle core (1). Based on sequence comparison, it has been suggested that, whereas in CoIE2, the primase and DNA binding activities are present in the same protein (19), in the case of pAL5000 and its related plasmids, these functions are distributed between RepA and RepB, respectively. Therefore, in all probability, RepB binding is a prerequisite for RepA primase to act.

Although members of the “pAL5000 group” replicate in taxonomically unrelated hosts, they still share a conserved 15-bp sequence (19). This sequence (Fig. 1A), located within an ~40-bp spacer that connects the two RepB binding sites, may therefore represent an evolutionarily conserved module having a specific function in the context of replication. The organization of the origin elements, two RepB binding sites separated by about four turns of the DNA helix, suggests that, as in the case of several other replication origins (12, 14, 16, 18), a bending movement may occur following the binding of RepB to the origin. Given this possible scenario, an investigation into the ability of RepB to bend the DNA was considered necessary to explain its functions. The results presented here show that the binding of RepB induces significant bending movements within the origin, which appears to culminate in the “melting” of the DNA strands.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *E. coli* strain XL1 Blue was used for routine manipulation of plasmid DNA. The vector pBend2 (11) used for DNA-bending experiments was a gift from S. Adhya, NIH. Chemicals. Ni²⁺-nitrilotriacetic acid agarose for chromatography was purchased from QIAGEN (Valencia, CA). Other chemicals for protein expression, purification, and analysis, of the highest grade of purity, were obtained from SRL Laboratories, India. Radiochemicals were purchased from BRIT (Mumbai, India). Restriction enzymes and DNA-modifying enzymes, such as polynucleotide

**REFERENCES**


kinase, T4 DNA ligase, and exonuclease III (ExoIII), were obtained from New England Biolabs. Recombinant DNA. Earlier it was demonstrated that RepB attains an active structure when its expression is translationally coupled to that of RepA (2, 3). In order to purify RepB in its native conformation, a construct (pTAB3) was made in a pT7-7 vector (26) (Table 1). Briefly, the repA-repB coding region was amplified by using primers corresponding to the 5′/H11032 end of repA and the 3′/H11032 end of repB. The 3′ end of the downstream primer contained additional sequences coding for six histidine residues. The amplified fragment was cloned in pT7-7 (26), and expression was performed in E. coli strain BL21(DE3). The BL21-

![Diagram of RepA and RepB](image)

**FIG. 1.** The replication region of pAL5000. (A) The minimal replication region of pAL5000 (1.8 kb) comprises a 200-bp origin and two translationally coupled open reading frames that express RepA and RepB, respectively. The core DNA sequence (90 bp; nucleotides [nt] 4531 to 4620) spanning the origin (L and H sites) is shown below. Approximately at the middle is located a conserved element (hatched box), which is present in the origins of the pAL5000 and ColE2 families of plasmids. (B to E) Concentration-dependent (0, 50, 100, 200, 400, and 800 nM; lanes 1 to 6, respectively) binding of RepB to the indicated probes. L-H (nt 4459 to 4663), H (nt 4589 to 4623), and L (nt 4531 to 4552) represent either the complete origin or the individual H and L RepB binding sites, respectively. The complexes were numbered C1 to C3. Complexes formed with the individual H and L sites were named by adding the letter H or L in parentheses. (F and G) Top-strand (F) and bottom-strand (G) DNase I footprinting using the L-H probe and RepB at a concentration of 800 nM. The A+G lane is indicated. Black bars indicate the H region, and white bars indicate the L region. + and − represent footprinting in the absence or presence of RepB, respectively.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT7-7</td>
<td>T7 promoter-based expression vector; expressed proteins carry no tags</td>
<td>26</td>
</tr>
<tr>
<td>pTAB3</td>
<td>Expresses C-terminally His-tagged RepB</td>
<td>This study</td>
</tr>
<tr>
<td>pBend2</td>
<td>DNA-bending vector which can be used to generate probes having the binding site in circular permutations</td>
<td>11</td>
</tr>
<tr>
<td>pBendH2</td>
<td>A double-stranded oligonucleotide derived from the pAL5000 origin (nt 4590-4623) with XbaI overhangs cloned in pBend2 at the XbaI site</td>
<td></td>
</tr>
<tr>
<td>pBendLH2</td>
<td>A PCR-amplified 90-bp fragment from the origin of pAL5000 (nt 4531-4620) of pAL5000) with XbaI cleavage sites at its ends was digested with the same restriction enzyme and cloned at the XbaI site of pBend2</td>
<td></td>
</tr>
<tr>
<td>pMC2</td>
<td>A pAL5000-derived fragment (nt 3895-1257) was cloned at the Accl/HincII site of pBCl, a pUC19-derived plasmid</td>
<td>5</td>
</tr>
<tr>
<td>pMC2-1</td>
<td>5 bp (ATCCCC) was inserted at nt 4557 of pMC2</td>
<td></td>
</tr>
<tr>
<td>pMC2-1</td>
<td>10 bp (ATCCTGAG) was inserted at nt 4557 of pMC2</td>
<td></td>
</tr>
</tbody>
</table>

*nt, nucleotides.*
Mutagenic primer derived from 4F which harbors a 5-bp insertion (underlined); this primer was used with 4R for PCR.

TABLE 2. List of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Description of amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F CTCTGGTTGATCGAGTGGTTTGGG R GCTGCCTAAATTTGCGGCG</td>
<td>This ~200-bp amplicon (nt 4460–4663) which spans both the L and H regions of the pAL5000 origin was used as a probe in EMSA and footprinting experiments</td>
</tr>
<tr>
<td>2</td>
<td>F GCCGCTCGGACGACATAACACCG R GCTGCCTAAATTTGCGGCG</td>
<td>74-bp H-site probe (nt 4584–4663)</td>
</tr>
<tr>
<td>3</td>
<td>F CTCTGGTTGATCGAGTGGTTTGGG R GGGGCCCACCCCAACACCTG</td>
<td>110-bp L-site probe (nt 4460–4560)</td>
</tr>
<tr>
<td>4</td>
<td>F GCCGCGGAAGTGTGCACG R ACCACGATATACCCGGC</td>
<td>90-bp origin core (nt 4531–4620) spanning L and H sites used in phasing-related EMSA experiments</td>
</tr>
<tr>
<td>5</td>
<td>F GCCGCGGAAGTGTGCAGTTGTGAATTCGGATCCGGGCCC</td>
<td>Mutagenic primer derived from 4F which harbors a 5-bp insertion (underlined); this primer was used with 4R for PCR</td>
</tr>
<tr>
<td>6</td>
<td>F GCCGCCGAAGTGTGCAGTTGTGAATTCGGATCCGGGCC</td>
<td>10-bp insertion; other features same as 5F</td>
</tr>
<tr>
<td>7</td>
<td>F GCTCTAGAGCGGCGGAGTGTCGCA R GCTCTAGAATATACCCGGC</td>
<td>Primer pairs same as 4, but harboring XbaI restriction sequence (underlined) at both ends</td>
</tr>
</tbody>
</table>

* nt, nucleotides.

Based expression system was preferred as this strain of E. coli lacks OmpT, an outer membrane protease of E. coli, and RepB is highly sensitive to OmpT (3).

To study the DNA-bending activity of RepB, a construct (pBendH2) was made by cloning a double-stranded oligonucleotide representing 34 bp of the H site (Table 1) with XbaI-compatible overhangs into the XbaI site of the binding probe vector pBend2 (11). A similar construct (pBendLH2) was made by cloning a PCR amplicon with XbaI ends representing the minimal L-H region (Tables 1 and 2) in the same vector and at the same cloning site. Insertional mutagenesis was performed by using a GeneI Insite PCR-based mutagenesis kit (Bangalore Genei, India). An EcoRI fragment was excised from pMC2 (5), an E. coli-mycobacterium shuttle vector (Table 1), and cloned into the mutagenesis vector pRK22. Oligonucleotide-directed insertion mutagenesis was then performed using the protocols given by the manufacturer of the kit. The mutagenized fragments were then recloned into pMC2 in such a way that the original sequence was replaced by its mutagenized counterparts. Finally, the mutagenized region was sequenced as a confirmatory measure.

**Protein purifications.** C-terminally six-histidine-tagged RepB was isolated from isopropyl-β-D-thiogalactopyranoside-induced E. coli BL21(DE3) cells harboring pTAB3 using procedures described earlier (3). The RepB protein obtained by this method was >98% pure, as judged by 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Comassie blue. The concentration of RepB was determined using a standard method (13).

**Electrophoresis techniques.** Electrophoretic mobility shift assay (EMSA) was performed as described earlier (2, 3). Desired DNA fragments (Table 2) were derived from the pAL5000 origin of replication by radiolabeled using PCR-based techniques (2), and the labeled PCR products were purified using a PCR purification kit (QIAGEN). The binding reaction mixture contained (30–100 fmol final volume) 3 μl 1× binding buffer (100 mM Tris [pH 8], 600 mM NaCl, 50 mM MgCl2), 1 mM EDTA, 1 mM dithiothreitol, 0.1 μg salmon sperm DNA, and 10,000 cpm of labeled DNA, which corresponds to about 3 ng of the DNA and the required amount of purified proteins. The reaction mixtures were preincubated for 10 min and then incubated for an additional 20 min on ice after the probes were added. The DNA-protein complexes were separated on a 4% native PAGE gel by electrophoresis in 0.5× Tris-borate buffer (50 mM Tris-borate, 1 mM EDTA) at 200 V for 3 h at 4°C after a prerun at 100 V for 1 h. Following the electrophoresis, the gel was dried, and the bands were visualized by autoradiography.

**DNase I footprinting.** DNase I footprinting analysis was performed essentially as described earlier (3) with the 200-bp origin probe (Table 2). Approximately 0.3 pmol labeled DNA was incubated with various amounts of RepB for 20 min at 4°C. Then, 50 ng DNase I (Sigma) was added and the mixture incubated for 3 min at room temperature. The digestion was stopped by adding DNase I stop solution (50 mM Tris HCl [pH 8], 30 mM EDTA, 2% [wt/vol] SDS, 0.4 mg/ml proteinase K). Digested DNA fragments were resuspended in loading buffer (50% [vol/vol] deionized formamide, 10 mM EDTA, 0.025% [wt/vol] xylene cyanol, and 0.025% [wt/vol] bromophenol blue), boiled for 5 min, chilled rapidly, and separated by gel electrophoresis on 8% (wt/vol) Polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie blue. The concentration of RepB was determined using a standard method (13).

**Circular permutation analysis.** DNA fragments were isolated from two plasmids, namely, pBendH2 and pBendLH2 (Table 1), by digestion with MluI, NheI, XhoI, EcoRV, and BamHI. These fragments were dephosphorylated by using calf intestine alkaline phosphatase and end labeled using T4 polynucleotide kinase in the presence of [γ-32P]ATP using standard procedures. EMSAs were performed mainly as described above, except that the protein-DNA complexes were separated on 8% (wt/vol) polyacrylamide gels run at 200 V for 5 h at 4°C. The magnitudes of the apparent bending were calculated from the variations in the mobilities of the protein-DNA complexes in the circular permutation analysis using the formula RL = cos(α/2). RL (relative mobility) is defined as the ratio between the mobilities of the maximally (μmax) and minimally (μmin) reared bands. Mobility (μ) is in turn defined as the distance migrated from the origin. α is the angle by which the DNA is bent from linearity (11).

**Ligase-mediated circularization assay.** The ligase-mediated circularization assay was performed essentially as described earlier (10). The labeled, 210-bp probe (~0.04 μM) was generated by excising an XhoI fragment from pBendLH2 (Table 1) and kinasing its ends with [γ-32P]ATP. In this probe, the L-H fragment is centrally located and flanked by symmetrical, vector-derived sequences. The probe was preincubated with various amounts of RepB on ice for 20 min in T4 DNA ligase buffer in a final volume of 50 μl. The ligation reaction was initiated by the addition of 0.025 U of T4 DNA ligase (New England Biolabs), followed by incubation at 16°C for 30 min. The reaction was stopped by the addition of five volumes of stop solution (0.5% SDS, 10 mM EDTA, 1 mg/ml proteinase K). Following incubation at 90°C for 2 h, the ligated DNA was extracted with phenol-chloroform-isooamyl alcohol and then with chloroform-isoamyl alcohol. Following incubation at 50°C for 2 h, the ligated DNA was extracted with phenol-chloroform-isooamyl alcohol and then with chloroform-isooamyl alcohol. For detecting circular DNA, the purified DNA was digested with 100 U of XhoI (New England Biolabs) at 37°C for 30 min. The extracted samples were run on a 5% nondenaturing polyacrylamide gel. The gels were dried and autoradiographed at ~80°C.

**KmO₃ footprinting.** The determination of the sensitivities of RepB-bound binding sites to potassium permanganate (KmO₃) oxidation was performed as described earlier (4). Briefly, end-labeled substrates (prepared as described above) were incubated with various amounts of RepB for 15 min at 4°C in EMSA.
binding buffer lacking reducing agents and containing 400 ng salmon sperm DNA in a final volume of 50 μL. Subsequently, 2.5 μL of 50 μM K3[O4]3 was added and the mixture incubated for 2 min at room temperature, after which the reaction mixtures were quenched by the addition of β-mercaptoethanol and EDTA. The DNA was ethanol precipitated in the presence of glycerol, washed with 70% ethanol, and cleaved by the addition of 100 μL of 1 M piperidine for 20 min at 90°C. Cleaved DNA was precipitated with ethanol and resolved through 8% (wt/vol) urea-Tris-borate-EDTA sequencing gel containing 30% formamide. The gels were dried and autoradiographed at −80°C.

Cross-linking experiments. Glutaraldehyde cross-linking of RepB was performed for 5 min at room temperature using glutaraldehyde at a final concentration of 0.05%. The reaction was stopped by adding SDS-PAGE loading buffer, followed by boiling. The cross-linked proteins were separated on a 13.5% SDS gel, and RepB-specific bands were detected by immunoblotting using anti-RepB sera raised in an earlier study (2).

Size exclusion chromatography (SEC). RepB oligomers were separated on the basis of size exclusion using a Protein Pak 3000 SW column (Waters). The protein was loaded at a concentration of 15 μM and eluted with buffer (20 mM phosphate [pH 7.0], 150 mM KCl, and 5% glycerol) at a flow rate of 0.5 ml/min. Separation was monitored using UV absorbance at 220 nm. The peak fractions were concentrated and analyzed by Western blotting using anti-RepB sera.

Transformation efficiency. Transformation efficiency experiments were performed using the E. coli-mycobacterium shuttle vector pMC2 (5), which contains the 2.5-kb pAL5000-derived origin cloned in a modified pUC19 vector. Transformants were selected by using kanamycin (25 μg/ml). The plasmid pMC2 and its isolation mutants pMC2-1 (5 bp) and pMC2-2 (10 bp) were transformed into mycobacteria using electroporation as described earlier (5, 22). Plasmid DNA for transformation was isolated using commercial miniprep kits (Qiagen). The estimation of DNA concentration was done by measurement of optical density at 260 nm. The plasmid DNAs were also analyzed using gel electrophoresis. Only those preparations having high quality (>95% supercoiled DNA) and comparable concentrations (approximately 0.5 μg/ml) were used. Each set of transformations were performed in triplicate, and each plating was done in duplicate. The average colony counts from the duplicate plates were then averaged and expressed as mean relative transformation efficiency.

RESULTS

Specificity of RepB binding. EMSA was performed using the 200-bp origin probe (Fig. 1A) which spans the L and H sites and flanking sequences. A dose-dependent increase in the binding of RepB to the L-H probe was observed (Fig. 1B). At the highest concentration (800 nM), three complexes (C1, C2, and C3) were formed (Fig. 1B). The complexes were specific, as an unrelated probe did not show any binding (Fig. 1C). Moreover, a significantly higher level of binding to the H site than to the L site was observed (Fig. 1D and E), which is consistent with the high and low affinities of these sites, respectively, for RepB (24, 25). In the case of the H site, two complexes were formed. The intensity of the larger complex, C2(H), increased abruptly, indicating cooperativity. In the case of the L site, a single complex, C1(L), was formed. A faint band in between the free and C1(L) bands is a nonspecific one which is present in all the lanes. The binding phenomena thus obtained with the H and L sites are in agreement with the proposition made earlier (25) that RepB binds cooperatively as a dimer to the H site and as a monomer to the L site. Footprinting experiments were also done to study the extent of occupancy at the two sites under saturating conditions (Fig. 1F and G). The results show that the protection of the H region from DNase I cleavage was relatively stronger than that of the L region, particularly in the case of the bottom strand (Fig. 1G). In the case of the top strand, the respective footprints appear to be equally strong (Fig. 1F). There may be some difference in the manner in which RepB interacts with the two strands, as proposed earlier (24).

L-H site phasing and RepB binding. The pAL5000 origin is comprised of two sites, L and H, which are separated by about 30 to 40 bp. To investigate the importance of phasing in the process of RepB binding, EMSA was performed using probes that amplify the core region (Table 2). To introduce phasing mutations, 5-bp (out of phase) and 10-bp (in phase) insertions were made in the forward primer (Fig. 2A; Table 2). The labeled, PCR-amplified fragments thus obtained were subjected to EMSA using RepB in the 400 to 800 nM concentration range. The binding of RepB to the origin resulted in a dose-dependent increase in the formation of the three complexes, C1, C2, and C3 (Fig. 2B, lanes 2 to 4). The 5-bp insertion inhibited the shift from C1 to C2 and then C3 (Fig. 2, lanes 6 to 8 in comparison to lanes 2 to 4), but in the case of the 10-bp insertion, the formation of C2 and C3 was nearly as efficient as their formation in the wild type (lanes 10 to 12 in comparison to lanes 2 to 4). The results, therefore, indicate that the formation of complexes C2 and C3 is phasing sensitive.

In order to test whether changes in the phasing affect the replication efficiency, the same insertion mutations were introduced by oligonucleotide-directed mutagenesis into pMC2, an E. coli-mycobacterium shuttle vector. The three plasmids, pMC2, pMC2-1, and pMC2-2, were then transformed separately into Mycobacterium smegmatis LR222. The results showed that in the case of pMC2-1 (5-bp insertion), the replication efficiency dropped sixfold relative to the efficiency in pMC2, whereas in the case of pMC2-2 (10-bp insertion), the efficiency increased by about twofold (Fig. 2C). The results suggest that proper phasing of the L and H sites and also, possibly, the distance separating them are important determinants of replication activity.

RepB-dependent looping of DNA. Ring closure assays (10) were performed using the L-H fragment as probe. The probe was generated by excising an XhoI fragment from the vector pBendLH2 sites (see below). This probe (210 bp) spans a centrally located L-H site and some additional sequences to about 60 bp on either side. The results (Fig. 3) showed that, in the absence of RepB, the DNA forms linear multimers (Fig. 3, lane 1). As RepB was added at increasing concentrations (200, 400, and 800 nM) the formation of multimers was inhibited (Fig. 3, lanes 2 to 4 in comparison to lane 1). ExoIII digestion experiments were then performed to investigate the extent to which RepB promoted circularization. The results showed that, in the absence of RepB, a prominent ExoIII-resistant species (lane 5) was found to be present in the ligated sample. With increasing amounts of RepB, the formation of the ExoIII-resistant species was suppressed, and instead, a range of intermediate ExoIII-resistant bands was formed (lane 7). At the highest concentration of RepB (800 nM), the intermediate bands were further suppressed and a single resistant band (lane 8) was visible. This band migrated just above the linear dimer, and hence, it apparently represents a monomeric circle.

The results were further corrected for differences in transformability arising out of minor differences in DNA concentrations by taking into account their relative transformation efficiencies in E. coli.
The results therefore indicate that RepB binding leads to looping in the pAL5000 origin.

Circular permutation assays for DNA bending. To study DNA bending by RepB, the core H site sequence was cloned into the central XbaI site of pBend2 (Table 1). DNA fragments (160 bp) were excised by using the permuted restriction sites available in pBend2. The excised DNA carries the H site in a circularly permuted order, as shown in the diagram in Fig. 4B. The fragments are presented in such a way that the orientation of the H site remains the same as is shown in Fig. 1A. The EMSA (Fig. 4A) was performed using two different concentrations of RepB, 250 and 1,000 nM, which resulted in the formation of either a subsaturation (250 nM) or a saturation (1,000 nM) complex. In the case of the H site, two complexes were seen, a minor one (C1H), which presumably is monomeric, and a major one (C2H), which is dimeric (Fig. 1B). The RL for the maximally retarded bend turned out to be 0.9, which translates to a bending angle of 50°. In the case of the H site, the maximal retardation (which is synonymous to maximal bending) occurred when the insert was centrally located (EcoRV fragment). This indicates that the bend center is located approximately at the midpoint of the H site (Fig. 4B). The bending associated with the minor band follows the same pattern.

Bending studies with the L-H site were then performed (Fig. 4D and E). Permutation assays were done in the same way as for the H site. For comparison, the sequence of presentation of restriction fragments was reversed so that the H site had the same orientation as is shown in Fig. 1A. Two concentrations of RepB were used (250 and 500 nM for the studies whose results are shown in Fig. 4D and E, respectively). The naming of the complexes thus formed was done on the basis of the nomenclature described in the Fig. 1 legend. The plots of RL against the relative location of the insert (Fig. 4F) revealed that, in the...
In the case of C1, the bending was nominal but symmetrical. In the case of C2, bending increased significantly (Fig. 4F) but the bend center was clearly not symmetrically located, as evident from the fact that the maximum bend occurred when the L-H fragment was placed in the extreme left position. The most likely possible location of the bend center would be about 10 to 20 bp downstream of the H site and not at the center of the L-H site, as would be expected from a symmetric bend. When saturating amounts of RepB (500 nM) were used, the asymmetric trajectory of the DNA became more pronounced (Fig. 4F). Under this condition, the extent to which the DNA bends was found to be about 66°. In the case of C3, the bending pattern was more or less similar to the bending of C2.

**DNA melting induced by RepB.** The bending experiments, as well as the ligation assays, suggest that the origin may be wrapped around RepB. Such a wrapping is likely to be associated with localized melting. To detect RepB-dependent formation of single-stranded stretches, a KMnO₄ footprinting method was applied. This reagent is known to oxidize mainly T residues (and also C residues, though to a lesser extent) within either single-stranded regions or severely distorted regions (4). The results of the experiments showed (Fig. 5A, B, and C) that under saturating conditions, significant opening up of the origin occurs, as indicated by the presence of hyperreactive bands on both strands (indicated by arrows).

**RepB forms multimers.** The ability of RepB to form multimers was studied using SEC (Fig. 6A). The results showed that, though the monomeric species is the most abundant, a sizable fraction existed as dimers and higher oligomers. That the SEC peaks do indeed correspond to RepB was confirmed by collecting the fractions and analyzing them by Western blotting (Fig. 6B). When the fractions corresponding to peak II were pooled and rerun, both peaks were obtained (data not shown). The results, therefore, indicate that in solution, monomeric RepB is in equilibrium with various multimeric forms. The phenomenon of multimerization was further investigated using glutaraldehyde cross-linking assays. Consistent with the SEC results, various cross-linked species were obtained. The dimer was the major cross-linked species, followed by the trimer (Fig. 6C).

**DISCUSSION**

The present study was undertaken to examine the conformational changes in the pAL5000 origin following RepB binding. Saturation binding of RepB to the origin resulted in the formation of three complexes, C1, C2, and C3. The complexes were specific, since an unrelated probe did not give any binding. Moreover, even at the highest concentration of RepB (800 nM), binding to the L site was feeble, although under the same conditions, binding to the H site was strong. Upon careful examination, it was found that the retarded band C2 and also, possibly, C3 migrated as closely spaced doublets. Although the reason for this is unknown, the two subspecies could represent conformational variants of the origin complex.

The efficient formation of the higher-order complexes C2...
and C3 required that the L and H sites be in phase relative to each other. Such an arrangement is probably necessary for a loop to be formed. That such a looped structure is indeed formed is evident from the circularization assays. In these assays, it was found that, as in the case of several other DNA binding proteins, such as HU, CAP, and Sso7d (9, 10, 17), increasing concentrations of RepB suppressed multimer formation but promoted the formation of a circular monomer. Interestingly, even in the absence of RepB, an ExoIII-resistant circle was formed, but its radius is large, indicating that the origin may inherently possess a moderate degree of curvature. That phasing is also important from the replication point of view was demonstrated. The insertion of 5 bp resulted in a sixfold drop in transformation efficiency relative to the efficiency in the wild type, whereas the insertion of 10 bp resulted in the restoration of normal levels of activity. In fact, the 10-bp insertion gave about twofold-higher transformation activity than the wild type. It is hypothesized that an in-phase increase in distance between the L and H sites may make the DNA more flexible, thereby allowing the origin to function better.

The phasing and looping assays strongly suggest that origin bends. Circular permutation assays were thus performed to study RepB-induced origin bending. The results indicate two levels of bending: (i) a local curvature which is induced in the isolated H site and (ii) a long-range curvature induced in the L-H combination. The long-range curvature was asymmetric and maximally affected the sequences 3'/H to the H site. There are some examples of asymmetric bending where the bend center is not exactly in the center but at the edges, such as in the case of the site-specific recombinase of yeast FLP (15). The nearest similarity that we could trace in the literature was the case of Sp1’s binding to its target site, where it was observed that 5’-end positioning resulted in

FIG. 5. Chemical foot-printing using KMnO₄. (A and B) Autoradiograms showing KMnO₄ reactivity of the top and bottom strands, respectively, either in the absence (lanes 2) or in the presence (400 and 800 nM; lanes 3 and 4) of RepB. Lanes 1 represent A+G ladders. RepB-dependent KMnO₄-hypersensitive sites are shown by arrows in the autoradiograms (A and B) and in the origin sequence (C). The L and H sites are indicated by white and black bars.

and C3 required that the L and H sites be in phase relative to each other. Such an arrangement is probably necessary for a loop to be formed. That such a looped structure is indeed formed is evident from the circularization assays. In these assays, it was found that, as in the case of several other DNA binding proteins, such as HU, CAP, and Sso7d (9, 10, 17), increasing concentrations of RepB suppressed multimer formation but promoted the formation of a circular monomer. Interestingly, even in the absence of RepB, an ExoIII-resistant circle was formed, but its radius is large, indicating that the origin may inherently possess a moderate degree of curvature. That phasing is also important from the replication point of view was demonstrated. The insertion of 5 bp resulted in a sixfold drop in transformation efficiency relative to the efficiency in the wild type, whereas the insertion of 10 bp resulted in the restoration of normal levels of activity. In fact, the 10-bp insertion gave about twofold-higher transformation activity than the wild type. It is hypothesized that an in-phase increase in distance between the L and H sites may make the DNA more flexible, thereby allowing the origin to function better.

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stronger bends than 3'-end positioning, indicating a displacement of the bend center towards the 3'-end of the GC box, which is the SpI binding element (21). However, in none of the cases is a situation encountered where the central positioning shows a bend less than or equal to the terminal positioning. An interesting feature observed in this study is the ability of RepB to form multimers. This may explain the formation of multimeric complexes observed in EMSA and, also, the associated bending events. Replication initiator proteins are known to form multimers. The best example is DnaA, which is responsible for the initiation of replication from E. coli oriC (20). It has been postulated that, in the case of oriC, the DNA is wrapped around the DnaA multimeric structure and that this helps in the opening up of the origin (7). A similar situation possibly exists here, with a multimeric RepB acting as a scaffold for the formation of a “wrapped” complex, the net result being the opening up of the origin, as evident from the KMnO$_4$ oxidation experiment.

In keeping with the observations presented in this study, it is proposed that RepB binds as a dimer to the H site and a monomer at the L site (Fig. 7). Since RepB has a propensity to form trimers, the H-bound dimer and L-bound monomer are likely to interact with each other, forming a trimeric core around which the DNA would appear to be wrapped. If the concentration of RepB increases, then the core may expand and become tetrameric. Wrapping is possible because the RepB binding induces curvature. However, this bending is asymmetric and possibly involves secondary contacts between the origin-bound RepB and the DNA on the 3' side of H. The situation may be similar to that of RNA polymerase complexes, where it has been found that, under certain circumstances, DNA can loop back and touch the backside of the promoter-bound RNA polymerase (6). The ultimate consequence of such complex movements in the origin of pAL5000 is the opening up of the origin.

Plasmid origins of replication constitute attractive models for studying bending and looping mechanisms. As of now, most of the studies in this area have been done with E. coli plasmids. The present investigation, which is the only one of its kind in the case of the pAL5000 family, should throw more light on the replication mechanisms of plasmids hosted by Actinomycetes bacteria.

FIG. 7. Model depicting RepB-origin interaction. The white, black, hatched, and dotted regions represent L, H, spacer, and flanking sequences, respectively. The loop is created in such a way that the DNA flanking the 3' end of the H site folds back and makes secondary contacts with the H-site-bound RepB. The KMnO$_4$-oxidizable sites are indicated by bubbles.

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