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Mutations in the CCGTTCACA DnaA Box of *Mycobacterium tuberculosis oriC* That Abolish Replication of *oriC* Plasmids Are Tolerated on the Chromosome

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The origin of replication (*oriC*) region in some clinical strains of *Mycobacterium tuberculosis* is a hot spot for IS*6110* elements. To understand how clinical strains with insertions in *oriC* can replicate their DNA, we characterized the *oriC* regions of some clinical strains. Using a plasmid-based *oriC*-dependent replication assay, we showed that IS*6110* insertions that disrupted the DnaA box sequence CCGTTCACA abolished *oriC* activity in *M. tuberculosis*. Furthermore, by using a surface plasmon resonance technique we showed that purified *M. tuberculosis* DnaA protein binds native but not mutant DnaA box sequence, suggesting that stable interactions of the DnaA protein with the CCGTTCACA DnaA box are crucial for replication of *oriC* plasmids in vivo. Replacement by homologous recombination of the CCGTTCACA DnaA box sequence of the laboratory strain *M. tuberculosis* H37Ra with a mutant sequence did not result in nonviability. Together, these results suggest that *M. tuberculosis* strains have evolved mechanisms to tolerate mutations in the *oriC* region and that functional requirements for *M. tuberculosis oriC* replication are different for chromosomes and plasmids.

Mycobacterium tuberculosis, the causative agent of tuberculosis, has infected more than one-third of the world's population and accounts for 3,000,000 deaths each year. *M. tuberculosis* grows slowly, with a doubling time of about 24 h. The genetic and biochemical factors that are responsible for the slow growth rate of *M. tuberculosis* are unknown. Earlier studies designed to understand the replication initiation process in *M. tuberculosis* revealed that the *dnaA-dnaN* intergenic region functions as *oriC* (17). The corresponding region in other species of mycobacteria has also been shown to function as *oriC* in native hosts (10, 17, 19, 20). The *oriC* region is A-T rich and contains several putative DnaA boxes, defined as sequences with one to three mismatches to the consensus sequence TT (G/C) TCC ACA (17). Deletions in the *oriC* region abolish *oriC* activity (17, 18), and point mutations in the DnaA box sequences of *Mycobacterium smegmatis oriC* severely decreased *oriC* activity (18), indicating the importance of both the integrity of *oriC* and the sequence of the DnaA boxes in mycobacterial replication initiation.

The *M. tuberculosis* H37Rv genome contains 16 copies of IS*6110* and several other insertion sequences (5). Recent IS*6110* sequence mapping data for clinical strains of *M. tuberculosis* revealed that the *dnaA-dnaN* intergenic region is a hot spot for IS*6110* insertion (7). While many of these insertions were located outside the DnaA boxes, some disrupted one putative DnaA box with the sequence CCGTTCACA. Interestingly, no other DnaA boxes in the *oriC* of *M. tuberculosis* were found to be disrupted by IS*6110* sequences. The IS*6110* insertion in the DnaA box was designated as A-4 (8). The deletion and point mutation data of the mycobacterial *oriC* (17, 18) raise questions as to whether the CCGTTCACA DnaA box is essential for *oriC* replication and, if so, how clinical strains of *M. tuberculosis* with IS*6110* insertions in *oriC* initiate chromosomal DNA replication. To get insights into the replication initiation process in *M. tuberculosis,* we have characterized the *oriC* regions from selected clinical strains and asked two specific questions: (i) do mutations (insertions) in the *oriC* region of clinical strains interfere with the autonomous replication activity of respective *oriC* plasmids? (ii) Are mutations in the DnaA box of the *oriC* region tolerated on the chromosome of a laboratory strain of *M. tuberculosis*? Our results indicate that mutations in the A-4 DnaA box which abolish replication of plasmids are tolerated on the chromosome, suggesting that functional requirements for *M. tuberculosis oriC* are different for chromosomes and plasmids.

MATERIALS AND METHODS

Strains. *Escherichia coli* Top10 (Invitrogen) and a laboratory strain of *M. tuberculosis* H37Ra were used in the present study. Middlebrook 7H9 medium supplemented with oleic acid-dextrose-sodium chloride-catalase was used to culture *M. tuberculosis* strains. When required, transformants were selected on agar plates supplemented with kanamycin (20 μg/ml), sucrose (0.2%), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 50 μg/ml). Genomic DNA prepared from the following clinical strains of *M. tuberculosis* was used to amplify the *oriC* region: TN670 and TN6333 (A-1 insertion), TN5110 (A-4 insertion), and TN3990 (A-10 insertion). TN670 and TN6333 are two different but related *M. tuberculosis* strains (8). The IS*6110* mapping analyses revealed that the two *M. tuberculosis* strains could have diverged recently following the unique insertion of IS*6110* in *oriC* (8).

Transformation experiments. (i) *oriC* plasmids containing IS*6110* insertions.

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OriC activity was defined as the ability of a DNA fragment from *M. tuberculosis* to render autonomous replication to *E. coli* plasmids in an *M. tuberculosis* host (10, 17, 18). DNA fragments containing the *dnaA-dnaN* intergenic region from clinical strains were amplified by PCR, cloned into pZErO 2.1 vector (Invitrogen), and used in transformation experiments. These DNA fragments were 814 bp in length and contained IS*6110* sequences either outside the DnaA boxes at two different locations designated as A-1 (pMQ387 and pMQ388) and A-10 (pMQ371) or inside the CCGTTCACA DnaA box (pMQ373). As a control, a pMQ219 plasmid construct carrying a corresponding 814-bp DNA fragment from *M. tuberculosis* H37Rv was used (17).

(ii) *oriC* containing a *Bgl*II mutation in the DnaA box (pJor2). A *Bgl*II sequence in the CCGTTCACA DnaA box at the A-4 site was created by using a PCR mutagenesis protocol (25) with the pMQ219 plasmid as a template. The oligonucleotide primers A (AGATCT**AACGGAACCGCCGGGACTG**), which binds to the DnaA box and its upstream sequence at the A-4 site, and B (AGATCT**ACAACCCACGCCTCATCC**), which binds to the *oriC* sequence downstream of the A-4 insertion site, were used. The *Bgl*II site is underlined, and nucleotide sequence complementary to the *oriC* sequence is shown in boldface type. Restriction digestion of the amplified product with *Bgl*II followed by self-ligation resulted in the production of a mutant pMQ219 plasmid containing *Bgl*II sequence at the A-4 insertion site.

(iii) *oriC* containing mutations in the IS*6110* sequence. Two plasmids designated pQ373D and pQM373D and containing mutations in different regions of the IS*6110* sequence were constructed. Digestion of pMQ373 with *Sma*I and *Sca*I enzymes followed by self-ligation produced pQ373D, which lacked an approximately 900-bp internal DNA fragment of the IS*6110* sequence. A derivative of this plasmid lacking the left inverted repeat (IR) was constructed by PCR using primer A (see above) and C (AGATCT**CAGTTCTTGGAAAGGATGGG**), which binds to the IS*6110* sequence downstream of the left IR. The *Bgl*II site is underlined and the nucleotide sequence complementary to the IS*6110* sequence is shown in boldface. PCR amplification and restriction digestion of the amplified product with *Bgl*II followed by self-ligation resulted in the production of a recombinant plasmid, pQM373D, lacking the left IR. The nucleotide sequence of the *oriC* region was verified in both constructs to ensure that no mutations were created during the plasmid construction.

Recombination experiments. The chromosomal *oriC* sequence was replaced with a mutant *oriC* sequence by homologous recombination, following a two-step strategy (16). First, suicidal recombination delivery vectors carrying mutant *oriC* sequences were transformed into *M. tuberculosis* to produce a single-crossover (SCO) strain. Next, double crossovers (DCOs) were selected to yield mutant strains. Two suicidal recombination delivery vectors containing mutant *oriC* sequences, designated pJOR4 and pJOR14 and each containing different lengths of homologous sequences flanking the *Bgl*II site, were constructed. For the construction of pJOR4, an 814-bp *oriC* DNA fragment containing the *Bgl*II mutation in the A-4 DnaA box was cloned into p2NIL, a nonreplicating mycobacterial vector (16). In the next step, a 6.1-kb *Pac*I marker gene cassette carrying the genes *lacZ* (for blue color), *aph* (responsible for resistance to kanamycin), and *sacB* (responsible for sensitivity to sucrose) was released from a pGOAL17 vector (16) and cloned into the above vector. This construct contained approximately 300 and 500 bp of homologous DNA flanking the 5′ and 3′ ends of the *Bgl*II site, respectively. The pJOR14 vector was constructed by PCR using a 5-kb *M. tuberculosis dnaA* region (Fig. 1) as a template. First, a 2,060-bp 5′ DNA fragment upstream of the A-4 site was amplified using the primers 5′-AACTG CAGCCCGGCAACCGCTTCAGGGC-3′ and 5′-GAAGATCTGAACGGAA CCGCCG-GGACTG-3′. Similarly, the 1,360-bp 3′ DNA fragment downstream of the A-4 site was amplified with primers 5′-GCGGATCCGTCCAACATCAT CGGCACCCG-3′ and 5′-GAAGATCTACAACCCACGCCTCATCC-3′. The *Bam*HI, *Pst*I, and *Bgl*II restriction endonuclease sites used to facilitate cloning are underlined. In the next step, a 1.3-kb DNA fragment containing the 3′ flanking sequence was released by *Bgl*II-*Nde*I digestion and fused with the 2-kb 5′ flanking region at the *Bgl*II site. The 3.4-kb DNA fragment containing the *Bgl*II mutation in the A-4 site was cloned as a *Bam*HI-*Pst*I fragment into p2NIL, and the selection cassette from pGOAL17 was inserted as described above (see Fig. 4). The SCOs were selected on agar plates containing kanamycin and X-Gal and their sensitivity to sucrose was confirmed. To promote selection of DCO events, SCOs were streaked on antibiotic-free plates, resuspended in broth, and spread on agar plates containing sucrose and X-Gal. Potential DCOs were confirmed as white colonies that were sucrose resistant and kanamycin sensitive. The presence of the *Bgl*II mutation was verified by restriction digestion of genomic DNA followed by Southern hybridization.

Surface plasmon resonance (SPR) experiments and DnaA protein. Binding of *M. tuberculosis* DnaA protein to oligonucleotides containing DnaA boxes was examined using a BIAcore X instrument. The recombinant *M. tuberculosis* DnaA protein was purified as a His-DnaA fusion protein on nickel affinity columns as described previously (27). A biotinylated oligonucleotide with a single DnaA box flanked on either side by its native *oriC* sequence (5′-CGGCGGTT-**CCGTTCA CA**ACCCACGC-3′) was synthesized, annealed to complementary oligonucleotide, and used to immobilize the streptavidin-coated sensor chip. The DnaA box sequence is shown in bold letters. The CCGTTCACA DnaA box disrupted with the *Bgl*II sequence at the same location as the A-4 insertion (5′-CGGCGGTT CCGTTCAGATCTACAAC-3′), was also synthesized, annealed to complementary strand, and used as a control. The *Bgl*II site is underlined. In addition, oligonucleotide with a scrambled sequence (5′-AAGTAAGTATATAGTTTAA GTAAGT-3′) was synthesized and used as a second control. Biotinylated oligonucleotides were captured on streptavidin-coated SA sensor chips, different concentrations of DnaA protein were injected over the sensor chip surface, and both association and dissociation were recorded.

RESULTS AND DISCUSSION

IS*6110* insertions located outside the DnaA boxes do not affect *oriC* activity. We showed earlier that an 814-bp *M. tuberculosis oriC* DNA fragment containing the 527-bp *dnaA-dnaN* intergenic region, 177 bp of the 3′ end of the *dnaA* gene, and 110 bp of the 5′ end of the *dnaN* gene functioned as *oriC* in *M. tuberculosis* hosts. Furthermore, we showed that plasmids containing less than 814 bp of *oriC* DNA were unstable, whereas those containing more than 814 bp of *oriC* DNA, i.e., the *dnaA-dnaN* intergenic region and different lengths of the upstream *dnaA* gene including the *rpmH-dnaA* intergenic region, did not increase the transformation efficiency of the *oriC* plasmids (17). We also found that plasmids containing the 5-kb *M. tuberculosis dnaA* region, i.e., the *rpmH, dnaA,* and *dnaN* genes and their intergenic regions, exhibited *oriC* activity (17). However, the majority of the recovered plasmids contained deletions in the *rpmH-dnaA* intergenic region and the *dnaA*-coding region. Presumably, the *rpmH-dna*A intergenic region may interfere with *oriC* activity in plasmid context. To test whether DNA fragments derived from clinical strains containing IS*6110* insertions in the *dnaA-dnaN* intergenic region support *oriC* activity, appropriate plasmid constructs were transformed into the laboratory strain of *M. tuberculosis*. Plasmid constructs bearing IS*6110* insertions outside the DnaA box sequences designated A-1 (pMQ387 and pMQ388) and A-10 (pMQ371) supported *oriC* activity like that of the control plasmids without insertions (pMQ219; Fig. 1), but the construct designated A-4 (pMQ373) did not (Fig. 1). The transformation efficiency of these positive plasmids was comparable to that of the control ($\approx 0.4 \times 10^4$ transformants/μg of input plasmid DNA).

To investigate if the observed *oriC* activities of the pMQ371, pMQ387, and pMQ388 plasmids are due to accumulation of compensatory mutations in the *oriC* region or due to changes in the IS*6110* site of insertion following transformation and growth, plasmids from the stable transformants were isolated and DNA was analyzed using several restriction enzymes. In addition, the nucleotide sequences of the entire 814-bp *oriC* DNA fragment (minus the IS*6110* sequence) of the input as well as the recovered plasmids were determined. Both the restriction digestion patterns and nucleotide sequence of the *oriC* region, including the sites of insertion of IS*6110* of the input as well as the recovered plasmids, were found to be identical (data not shown). Together, these results suggest that the presence of IS*6110* insertions outside the DnaA box, i.e., A-1 and A-10, do not affect *oriC* activity, whereas those in the

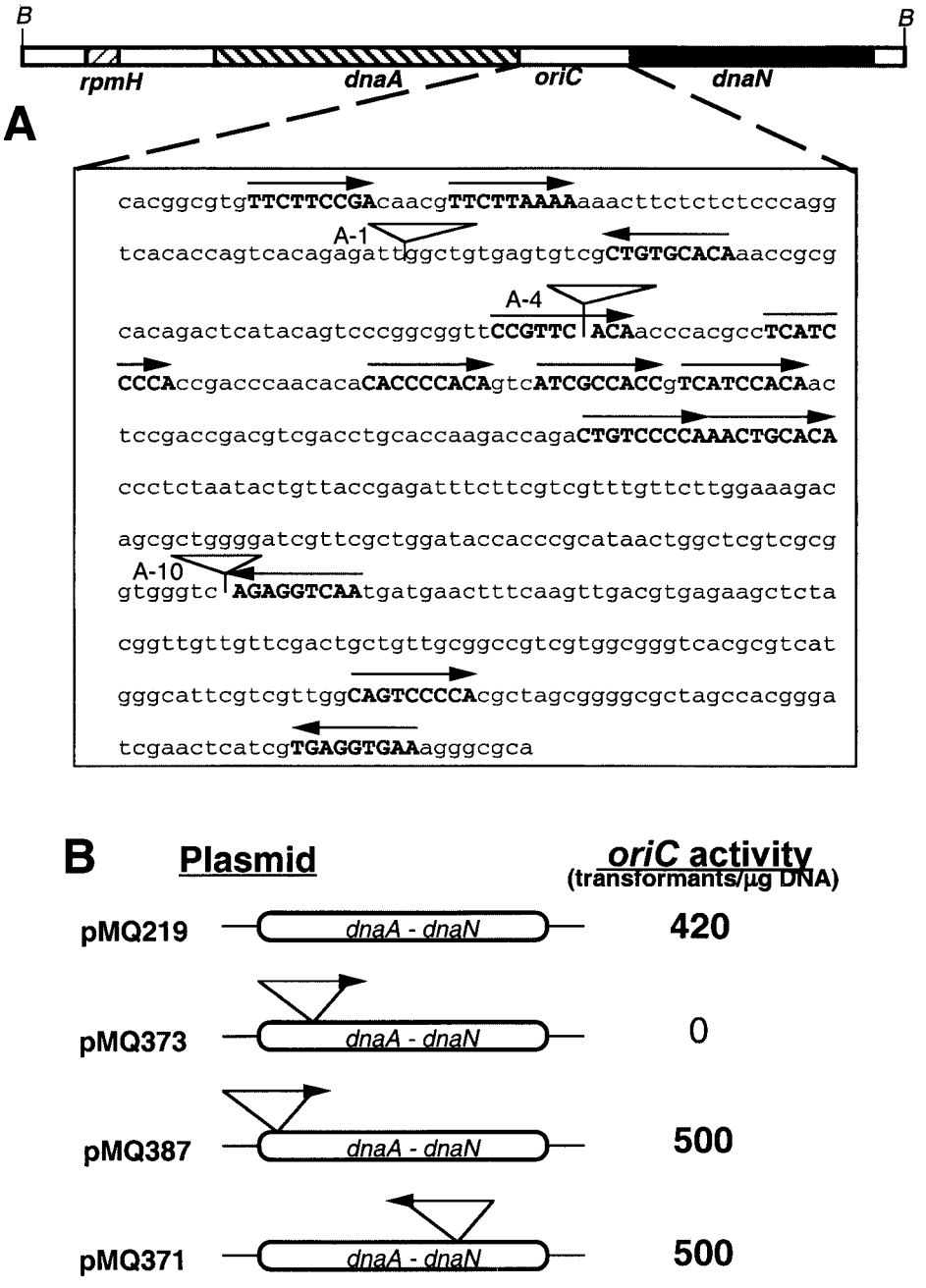


FIG. 1. *oriC* activity of the *dnaA-dnaN* intergenic region containing IS*6110*. (A) Organization of the *dnaA-dnaN* intergenic region. A 5-kb *Bam*HI DNA fragment carrying the *M. tuberculosis dnaA* region (not drawn to scale) and the sequence of the *dnaA-dnaN* intergenic region are shown. B, *Bam*HI site. DnaA boxes are shown in bold capital letters, and the arrows show the orientation of the DnaA boxes. Inverted triangles indicate the location of the IS*6110* insertion sequences at the A-1, A-4, and A-10 sites. (B) *oriC* activity of plasmids containing IS*6110* sequences. Plasmids containing *oriC* sequences derived from the clinical strains were transformed into the laboratory strain *M. tuberculosis* H37Ra, and the ability of the plasmids to transform and replicate as stable autonomous sequences was determined. The *oriC* activity is shown as the total number of transformants obtained per microgram of input DNA. pMQ219 is the control *oriC* plasmid derived from *M. tuberculosis* H37Rv (17). It includes an 814-bp DNA fragment containing the *dnaA-dnaN* intergenic region and its flanking regions. Although not shown, the *oriC* activity of pMQ388 is similar to that of pMQ387.

DnaA box do. Presumably, the integrity of the DnaA box is important for replication of *oriC* plasmids.

The integrity of the CCGTTCACA DnaA box is important for *oriC* activity. The IS*6110* sequence belongs to the IS*3* family of insertion sequences and contains 28 bp of IR flanking the open reading frame for transposase, which recognizes and processes the IR for mobility and transposition (11). Transposition events involving IS*6110* and other repetitive elements have been suggested to furnish the pathogen with an important mechanism for altering gene expression (24). We considered a possibility that the lack of *oriC* activity of pMQ373 plasmids is not due to the disruption of the CCGTTCACA sequence but

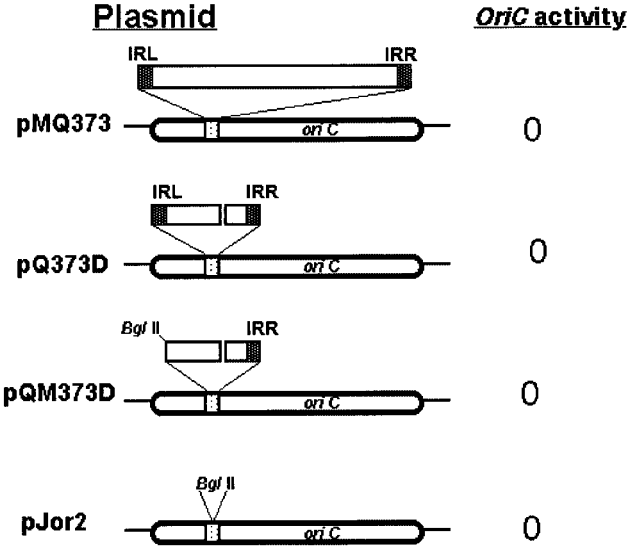


FIG. 2. *oriC* activity of pMQ373 derivatives. *oriC* plasmids with different mutations are shown to the left and the *oriC* activity is shown to the right. Only the CCGTTCACA (DnaA box) sequence is boxed for clarity. The IS*6110* sequence is shown as an inverted triangle above the *oriC* sequence. The gap in IS*6110* represents the internal deletion. The left IR (IRL) and right IR (IRR) are marked as black squares flanking IS*6110*.

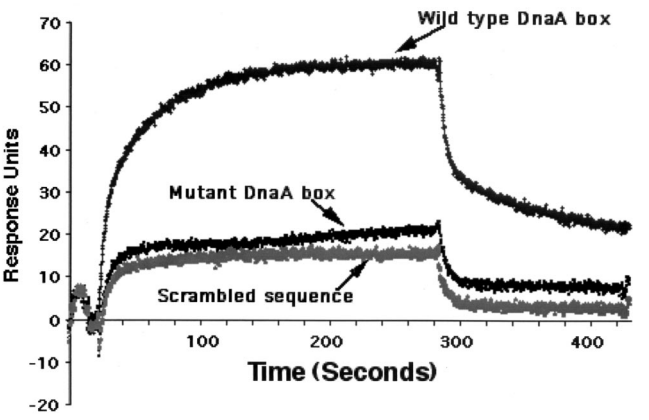


FIG. 3. DnaA protein binding to the DnaA box. Biotinylated oligonucleotides containing either the wild-type DnaA box sequence CCGTTCACA or the mutant sequence CCGTTCagatctACA or a scrambled sequence (see text) were coupled to the streptavidin sensor chip surface. DnaA protein at different concentrations was incubated for 4 min at room temperature in 50 mM Tris acetate buffer (pH 8.0), 0.5 mM magnesium acetate, 0.3 mM EDTA, 10 mM ammonium chloride, with 0.005% Tween 20, 1 mM ATP, and 30 ng of poly(dA-dT) competitor DNA/ml and then injected over the sensor chip surface at a flow rate of 5 μl/min. Total injection time was approximately 5 min. The sensor chip was washed with the above buffer 3 min after the injection to record dissociation. Only results obtained with 125 nM DnaA protein are shown. For the sake of clarity, spikes in the beginning and the end of the injection were removed.

due to some deleterious activity originating from the IS*6110* sequences. To address this question, plasmid constructs containing mutations in the IS*6110* sequences (pQ373D and pQM373D; Fig. 2) were transformed into *M. tuberculosis* strain H37Ra. It is expected that mutations that remove potential promoter and IS*6110* internal sequence would minimize the IS*6110* activity (11). Transformation of the *M. tuberculosis* strain with the above recombinant plasmids failed to produce any viable transformants, suggesting that the lack of plasmid replication is not due to insertion sequence activity affecting the biology of the DnaA box. Next, to investigate whether sequences other than the IS*6110* in the CCGTTCACA DnaA box at the A-4 site are tolerated, we examined the *oriC* activity of the pJor2 plasmid containing the *Bgl*II restriction enzyme sequence at the A-4 site. Consistent with disruption of the DnaA box by IS*6110*, the *Bgl*II insertion containing the *oriC* plasmid, designated pJor2, did not exhibit *oriC* activity (Fig. 2). Together, these results suggest that integrity of the CCGTTC ACA DnaA box is important for *oriC* replication, and mutations or insertions that affect the integrity of the CCGTTCA CA DnaA box abolish *oriC* activity.

The DnaA protein is not proficient in interacting with the mutant DnaA box. Replication is believed to be initiated when the DnaA protein recognizes and binds to the DnaA boxes located in the *oriC* region. The interaction of DnaA with the DnaA boxes is believed to be the first step in triggering the initiation process (7, 15). The lack of replication of pMQ373, pQM373D, pQ373D, and pJor2 plasmids in *M. tuberculosis* hosts could be due to the lack of binding of the DnaA protein to the mutant DnaA boxes. It should be noted that although sequence comparisons identified approximately 13 DnaA box-like sequences in the *oriC* region of *M. tuberculosis,* actual binding of *M. tuberculosis* DnaA protein to any of these boxes has not been confirmed (17). To test whether CCGTTCACA is a DnaA box and to test the above prediction, the *dnaA* gene from *M. tuberculosis* H37Rv was cloned and expressed in *E. coli* from the bacteriophage T7 promoter, and the recombinant protein was purified on nickel affinity columns as a His-DnaA fusion protein (27). Preliminary results indicated that the recombinant protein hydrolyzed ATP, and it bound and reduced the mobility of the *oriC* DNA in agarose gels (27). The ability of the recombinant protein to interact with the CCGTTCACA DnaA box was investigated by SPR using a BIAcore X instrument. Change in mass at the surface of a sensor chip is measured by SPR (4, 13). The major advantage of this technique is that protein-DNA interactions are monitored in real time. Injection of the DnaA protein (125 nM) to the wild-type DnaA box sensor chip surface resulted in a rapid binding within 25 s which then slowed and reached equilibrium by 100 s (Fig. 3). This was followed by a slow dissociation. Further washing with the buffer resulted in only a slow decay of the signal. From these results we infer that CCGTTCACA is an authentic DnaA box. At a similar protein concentration (125 nM; Fig. 3) and even at twofold-higher concentrations (data not shown), the DnaA protein did not stably associate with the mutant DnaA box. The binding profiles of the DnaA to the mutant DnaA box were comparable to that of the control scrambled sequence.

The lack of binding of DnaA protein to the scrambled and mutant DnaA box sequences in vitro and the lack of replication of plasmids containing the mutant DnaA box sequences in vivo suggest that CCGTTCACA is an important DnaA box and that stable interactions of the DnaA protein with this DnaA box are crucial for replication of these plasmids in vivo. The *oriC* sequence (minus the IS*6110* sequence) of the clinical strains is

essentially identical to that of the corresponding sequence of the laboratory strain of *M. tuberculosis*, suggesting that the replication initiation mechanisms are similar in these strains. Since mutations (insertions) in one DnaA box abolished replication of the plasmids in *M. tuberculosis* hosts, similar mutations in the *oriC* region of the clinical strains would be expected to be lethal. In contrast, the clinical strains tolerate the IS*6110* sequences in the *oriC* region. These results are different from those observed with *E. coli oriC* plasmids (6, 9). *E. coli oriC* has five DnaA boxes that are distributed throughout *oriC*. *E. coli* DnaA protein does not interact with any of the mutant DnaA boxes in vitro, although point mutations in DnaA boxes either individually or in combination are tolerated in vivo (1, 6, 9). These results demonstrate that binding of *E. coli* DnaA protein to mutant DnaA boxes is possible in vivo (9). Presumably, such binding involves cooperativity. Recently, Speck et al. (23) showed that the *E. coli* DnaA protein bound cooperatively to mutant DnaA boxes following its binding to adjacent wild-type DnaA boxes. Their results suggested that cooperative DnaA protein interactions are important for *oriC* activity (14, 23).

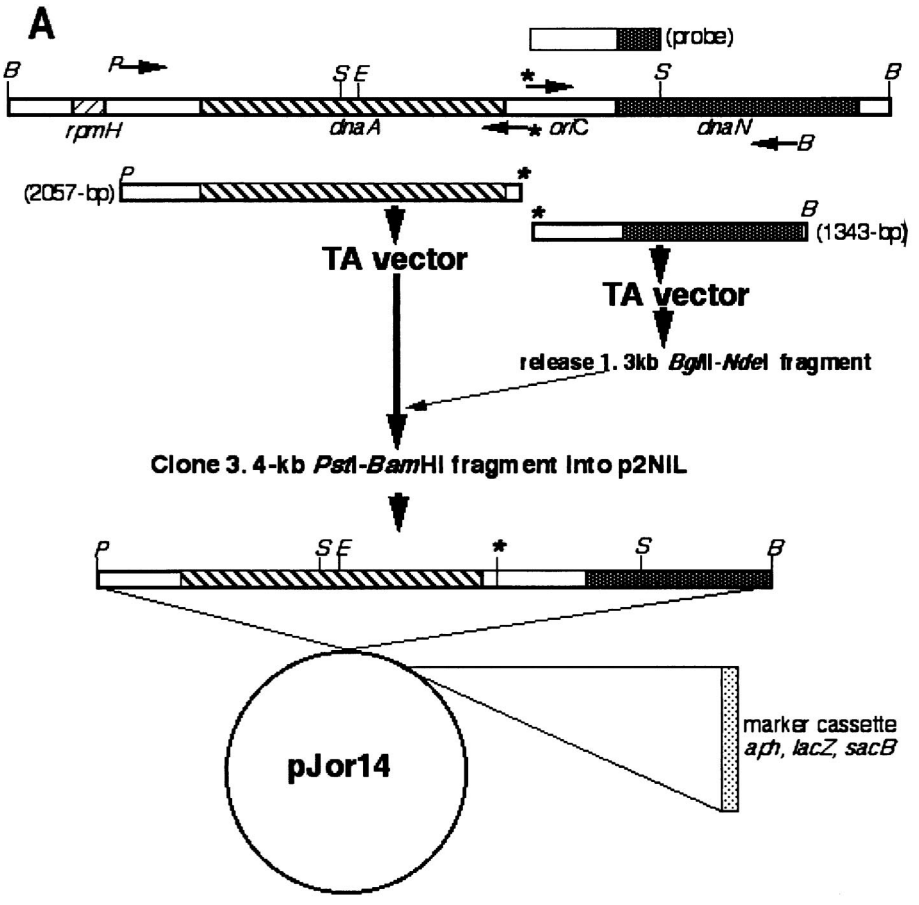
M. tuberculosis oriC, unlike its *E. coli* counterpart, contains 13 DnaA box-like sequences which are distributed in the entire *oriC* (17). Furthermore, the sequences of the designated DnaA boxes of *M. tuberculosis oriC* are clearly different from those of the *E. coli* boxes (17). We showed that the *M. tuberculosis* DnaA protein bound to one DnaA box (Fig. 3). It is unknown whether *M. tuberculosis* DnaA binds to all designated DnaA boxes and whether cooperativity is important for *M. tuberculosis oriC* function. Since cooperativity appears to be a common feature of all the DnaA proteins that have been investigated (12, 14, 21–23), we think that the *M. tuberculosis* DnaA-*oriC* interactions would also involve cooperativity, and such cooperative interactions involving all DnaA boxes are critical for the formation of an effective *oriC* initiation complex. Presumably, nonoptimal binding of *M. tuberculosis* DnaA protein to the mutant DnaA box in vivo could result in a weak cooperativity. This in turn could lead to the formation of a defective initiation complex, thereby affecting the replication of *oriC* plasmids in vivo. Further experiments are required to characterize cooperativity.

***oriC* plasmid constructs containing the *rpmH-dnaA* intergenic region do not support *oriC* activity.** The replication of DNA containing IS*6110* insertions in the *oriC* region of the clinical strains suggests that the clinical strains have evolved mechanisms to tolerate IS*6110* insertions in the DnaA boxes located on the chromosome. One possibility is that the clinical strains with IS*6110* insertions in their *oriC* use alternate replication origins for initiation of chromosomal DNA replication. The *rpmH-dnaA* intergenic region of either *M. tuberculosis* or *Mycobacterium avium* does not function as *oriC* in the respective native hosts (10, 17). Both regions are approximately 65% similar. The *rpmH-dnaA* intergenic region of *M. avium,* however, functions as *oriC* in *M. tuberculosis* hosts. These results suggested that the *rpmH-dnaA* intergenic region is a silent replication origin and its activity could be unmasked in the absence of the functional primary *oriC*, i.e., the *dnaA-dnaN* intergenic region (10). Assuming that the *M. tuberculosis dnaA-dnaN* intergenic region containing the IS*6110* insertions in *oriC* is defective for replication initiation, then one possibility is that the *rpmH-dnaA* intergenic region or possibly other sequences on the chromosome promote replication of DNA.

To test whether the *rpmH-dnaA* intergenic region functions as *oriC*, two series of experiments were carried out. First, *oriC* activities of plasmid constructs containing a 5-kb *rpmH-dnaA-dnaN* DNA fragment with a *Bgl*II mutation in the CCGTTC ACA DnaA box at the A-4 locus in *M. tuberculosis* hosts were examined. Transformation of *M. tuberculosis* strain H37Ra with mutant *oriC* plasmids did not result in any viable transformants, whereas plasmids containing the entire *dnaA* region did ($\sim 0.4 \times 10^4$ transformants/μg of input plasmid DNA), as previously reported (17). These results are not surprising, because the intact CCGTTCACA DnaA box sequence is essential for replication of *oriC* plasmids (Fig. 1 and 2), and the *M. tuberculosis rpmH-dnaA* intergenic region in a wild-type background does not support *oriC* activity (10, 17). Second, to overcome incompatibility problems, if any, that are associated with *oriC*, the ability of the *M. tuberculosis rpmH-dnaA* intergenic region to function as *oriC* in an *M. avium* host was examined. These experiments did not result in any viable transformants, presumably because of the poor transformation efficiencies of *M. avium* strains (~1 to 10 transformants per μg of input DNA), which are approximately 2 to 3 orders of magnitude less than that of *M. tuberculosis* strains (10).

Mutations in the A-4 DnaA box on the chromosome are tolerated in the laboratory strain of *M. tuberculosis*. In the case of *E. coli* it has been shown that the DnaA box R4, which is essential for replication of *oriC* plasmids, is dispensable for replication at the chromosomal *oriC* (1, 2). Deletion of the R4 DnaA box, however, led to asynchronous initiation of chromosomal replication. More recently, Weigel et al. (26) characterized the chromosomal *oriC* by replacing it with different mutated *oriC* sequences. Their results showed that origins with mutations in the R1 box are nonfunctional whereas those in R2, R3, and R4 are, suggesting that the functionalities of these mutated *oriC* are greater on the chromosome than on a minichromosome (26). Presumably, this may be the case with the *oriC* of the clinical strains of *M. tuberculosis*. To test whether this feature is unique to clinical strains or if other

FIG. 4. Replacement of the wild-type *oriC* sequence with mutant sequence. (A) Construction of pJOR14 recombination delivery vector. A 5-kb *M. tuberculosis dnaA* region, containing the *rpmH, dnaA*, and *dnaN* genes, and *oriC* are shown. The locations of genes are shown as different hatched boxes. The sites of restriction endonuclease enzymes are denoted by single letter codes as follows: B, *Bam*HI; S, *Sma*I; P, *Pst*I; E, *Eco*RI. The locations of primers used to amplify 2- and 1.3-kb DNA fragments are shown as arrowheads. The star above the arrowhead indicates the *Bgl*II site incorporated to create a mutation in the A-4 DnaA box. The amplification products along with their sizes are shown below the *dnaA* region. The DNA probe used in Southern hybridization experiments is shown above the *dnaA* region. The 3.4-kb DNA *Bam*HI-*Pst*I fragment was cloned into p2NIL, and a 6.1-kb *Pac*I marker cassette was inserted to create pJOR14 vector. This vector was used to generate SCOs and, subsequently, DCOs. (B) Southern analyses of the DCOs. Genomic DNA of mycobacterial strains (mutant and wild-type DCOs) was digested with the different enzymes shown, transferred to nitrocellulose membranes, and hybridized with a 600-bp *Sma*I-*Bgl*II fragment. Note that the sizes of the SCO products with *Bam*HI-*Eco*RI enzymes are different. Both wild-type and mutant DCOs were distinguished by *Bgl*II-*Sma*I digestion.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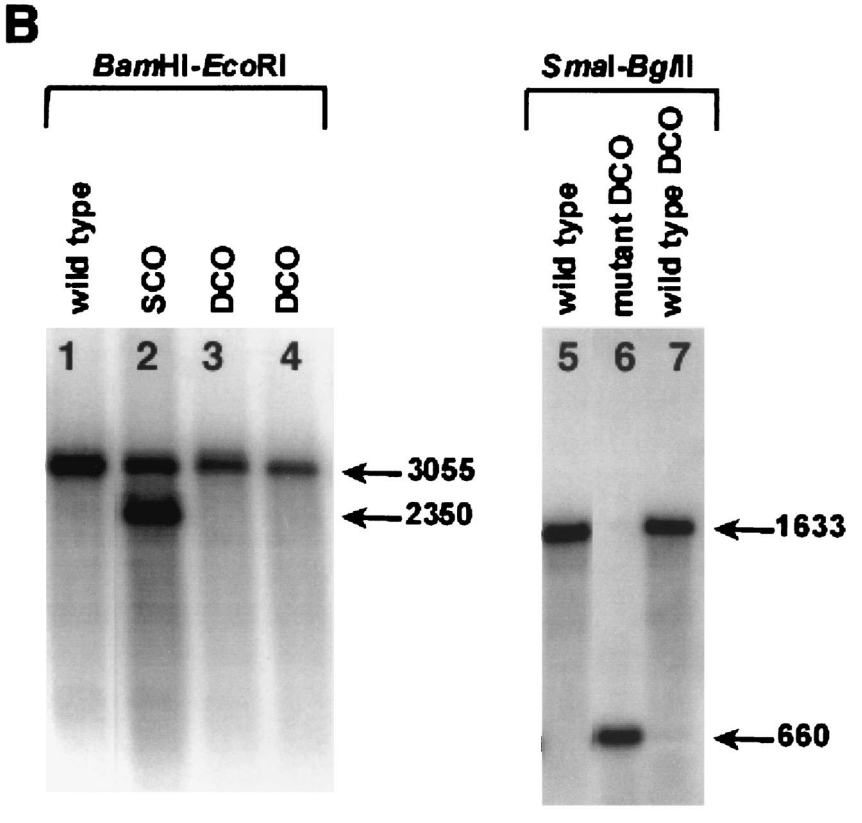
rpmH
dnaA
oriC
dnaN
B
(2057-bp)
P
*
B
(1343-bp)
TA vector
TA vector
release 1.3kb BglII-NdeI fragment
Clone 3.4-kb PstI-BamHI fragment into p2NIL
P
S E
*
S
B
pJor14
marker cassette
aph, lacZ, sacB


B
BamHI-EcoRI
SmaI-BglII
wild type
SCO
DCO
DCO
wild type
mutant DCO
wild type DCO
1 2 3 4
5 6 7
3055
2350
1633
660

strains of *M. tuberculosis* can tolerate mutations in the CCGT TCACA DnaA box of *oriC*, we attempted to replace the chromosomal *oriC* of *M. tuberculosis* H37Ra with a mutant *oriC* containing the *Bgl*II mutation in the CCGTTCACA box (pJOR4), using homologous recombination following the two-step gene replacement protocol (16). If an *M. tuberculosis* strain with a mutated *oriC* survives, this would indicate that mutations in the CCGTTCACA DnaA box of *oriC* on the chromosome are tolerated in the laboratory strain as well. Electroporation of *M. tuberculosis* H37Ra with the pJOR4 construct did not result in any viable SCO recombinants. Presumably, the length of homology used, i.e., 300 and 500 bp of homologous DNA to the 5′ and 3′ ends of the *Bgl*II mutation, respectively, in the pJOR4 construct, was not sufficient to promote recombination events.

To promote selection of SCO recombinants, we constructed a pJOR14 recombination delivery vector containing 2 kb upstream and 1.4 kb downstream of homologous sequences flanking the *Bgl*II mutation (Fig. 4A). Electroporation of this vector into *M. tuberculosis* produced two SCOs that were blue, resistant to kanamycin, and sensitive to sucrose. One SCO, designated RGM43, was further processed to select DCO strains that were white, sensitive to kanamycin, and resistant to sucrose. PCR amplification followed by restriction digestion with the *Bgl*II enzyme of the *oriC* region of 10 potential DCOs revealed wild-type and mutant DCO patterns in a 3:7 ratio (data not shown; see below). To further confirm these results, genomic DNA of a potential mutant (RGM46) and wild-type (RGM47) DCO was digested with different enzymes and analyzed by Southern hybridization (Fig. 4B). Digestion with *Bam*HI-*Eco*RI enzymes identified DNA bands corresponding to either a 3-kb fragment for wild type (Fig. 4B, lane 1), both 2.3- and 3-kb fragments for SCO (Fig. 4B, lane 2), and a 3-kb fragment for both wild-type (Fig. 4B, lane 3) and mutant (Fig. 4B, lane 4) DCOs. Since a 6-bp *Bgl*II insertion sequence is not expected to change the size of the DNA band in agarose gels, the fragment sizes obtained from either wild-type or mutant DCOs are of the same size. Digestion of the genomic DNA of the RGM46 and RGM47 strains with *Sma*I-*Bgl*II enzymes followed by Southern hybridization confirmed the presence of a *Bgl*II mutation in the A-4 site of *oriC* in the RGM46 DCO (Fig. 4B, compare lane 6 with lanes 7 and 5). In broth, the RGM46 mutant DCO grew like that of RGM47 and the parent strain, with little or no difference in the doubling time (data not shown).

Together, these results indicate that mutations in the A-4 DnaA box of *oriC*, which abolish replication of plasmids, are tolerated on the chromosome. Presumably, the functional requirements for replication of *oriC* plasmids and chromosomes are also different in *M. tuberculosis*, much like the results reported with *E. coli oriC* systems (1–3, 26). Thus, survival of the clinical strains with IS*6110* insertions in *oriC*, i.e., the *dnaA-dnaN* intergenic region, is due to their ability to tolerate these mutations. The IS*6110* insertions in the *oriC* region of *M. tuberculosis* strains are naturally occurring mutations (8). In this regard, it is pertinent that some clinical strains of *M. tuberculosis* contain two IS*6110* insertions with up to 300-bp deletions in the *oriC* region (e.g., *M. tuberculosis* TN6278 [B. N. Kreiswirth and N. Kurepina, unpublished data]). The deleted region spans the DNA beginning at the A-1 site to the end of A-10 and includes a total of nine presumptive DnaA boxes (see Fig. 1A for locations of DnaA boxes). Further characterization of the *oriC* regions in the clinical strains will enable us to understand how these large deletions in *oriC* are also tolerated in *M. tuberculosis*.

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