Inhibition of chromosome replication in *Mycobacterium smegmatis*: effect of the *rpmH*–*dnaA* promoter region

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In a previous study a functional mycobacterial origin of replication, *oriC*, was isolated on a plasmid. However, it was found that origin function was inhibited by the presence of the adjacent *dnaA* gene or its regulatory region, so that plasmids containing both of these regions next to the origin did not yield transformants. This inhibition could be due either to overexpression of *dnaA* on a plasmid being toxic, the transcription of *dnaA* into the downstream origin topologically inhibiting its function, or to the DnaA boxes upstream of *dnaA* somehow interacting with the DnaA boxes in the origin to prevent its function. To distinguish between these possibilities, plasmids were constructed lacking different parts of the *dnaA* gene: the promoter, the DnaA boxes, or both. Additionally, the putative *dnaA* promoter region was replaced by mycobacterial sequences that exhibit weaker or null promoter activity. The results indicate that the *rpmH*–*dnaA* promoter region, but not the DnaA boxes, is the principal cause of the incompatibility observed and suggest that this region could be playing a role in the inhibition of chromosome replication.

Keywords: *oriC*/replication, *dnaA* promoter, incompatibility, *Mycobacterium*

INTRODUCTION

The genus *Mycobacterium* comprises a wide variety of organisms ranging from rapid-growing saprophytes or opportunists such as *Mycobacterium smegmatis* and *M. fortuitum*, which duplicate in 2–3 h, to slow-growing pathogens like *M. tuberculosis* and *M. bovis*, which have a duplication time of around 18–24 h.

Tuberculosis (TB) remains a major, global public health problem, particularly in low-income countries. In 1993, the World Bank estimated that the disease accounts for more than 25 % of avoidable adult deaths in developing countries. Moreover, the global number of TB cases is expected to continue to increase (Dolin *et al.*, 1994). Better application of current diagnostic, treatment and prevention strategies could lead to a gradual decrease in the disease, but eliminating TB will require new tools and a better understanding of the metabolism of the mycobacteria. An improved knowledge of the repli-

cation process would aid in finding susceptible factors for controlling the mycobacterial cell cycle.

Initiation of DNA replication is a global process, which in bacteria, plasmids and some viruses, involves the binding of initiator proteins to repetitive sequences at the origin of replication, *oriC*. The *oriC* structure, as well as the basic gene order and the primary structures of the encoded proteins in its vicinity, are remarkably conserved among eubacteria. The most important element leading to initiation of bacterial chromosome replication is the DnaA protein. The binding of DnaA to DNA-binding sites (DnaA boxes) has been demonstrated for *Escherichia coli* (Crook *et al.*, 1993), *Bacillus subtilis* (Krause *et al.*, 1997) and *Streptomyces* (Jakimowicz *et al.*, 1998). The DnaA protein binds to DnaA boxes in the *E. coli* *oriC* region, leading to formation of regularly shaped *oriC*–DnaA complexes containing 20–40 DnaA monomers (Bramhill & Kornberg, 1988). The formation of this complex might involve other proteins, such as FIS and IHF (Roth *et al.*, 1994), and lead to DnaA-facilitated strand opening of a region containing three AT-rich 13-mer sequences, preceding the formation of the primosome (Baker *et al.*, 1987; Sekimizu *et al.*, 1988; Marszalek & Kaguni, 1994).

Abbreviations: FIS, factor for inversion stimulation; GFP, green fluorescent protein; IHF, integration host factor protein; OADC, oleic acid albumin dextrose complex.

In vivo studies indicate that the DnaA protein also controls transcriptional events in the *oriC* region. This could lead to a strongly increased negative supercoiling of the 13-mer region up to the time when the cell will be ready to initiate replication and thus facilitate strand opening (Asai *et al.*, 1992).

Autonomous replicating sequences (*ars*'s) on plasmids have been isolated from different species of bacteria, such as *E. coli* (Yasuda & Hirota, 1977), *Pseudomonas putida*, *P. aeruginosa* (Yee & Smith, 1990), *B. subtilis* (Moriya *et al.*, 1992), *Streptomyces lividans* (Zakrzewska-Czerwinska & Schrempf, 1992), *Spiroplasma citri* (Ye *et al.*, 1994), *M. smegmatis* (Salazar *et al.*, 1996; Qin *et al.*, 1997) and recently, in *M. tuberculosis*, *M. bovis* (Qin *et al.*, 1999) and *M. avium* (Madiraju *et al.*, 1999). In Gram-negative bacteria, isolated *ars*'s contain either the region upstream of *dnaA* or a DnaA box region downstream of *rnpA*. In contrast, in Gram-positive bacteria *oriC* always includes the intergenic *dnaA*–*dnaN* region, although DnaA boxes exist in two noncoding regions upstream and downstream of the *dnaA* gene. An exception is *B. subtilis*, where both upstream and downstream DnaA box regions are required *in cis* for initiation of replication, suggesting that in this bacterium, the initiation of replication involves formation of a loop between the upstream and downstream DnaA boxes, mediated by the DnaA protein (Moriya *et al.*, 1992, 1999).

Salazar *et al.* (1996) reported that with *oriC* plasmids of *M. smegmatis*, containing both upstream and downstream DnaA box regions *in cis*, transformants could not be obtained in this bacterium, even at a low copy number. Since this incompatibility was partially relieved when a small deletion that inactivated the *dnaA* gene was introduced, or when the upstream *dnaA* region and 5' end of the *dnaA* gene were deleted, it was suggested that the incompatibility could be due to overproduction of the DnaA protein.

In this work, a series of *oriC* plasmids of *M. smegmatis* was constructed in which the upstream *dnaA* region (*rpmH*–*dnaA* promoter and DnaA boxes) was deleted or exchanged with sequences that expressed a different promoter activity (weak or null) or by sequences that did not contain a consensus DnaA box motif. A short sequence (270 bp) containing the strong *rpmH*–*dnaA* promoter was found to exert an inhibitory effect on *oriC* activity. The three DnaA boxes upstream of *dnaA* exhibited a limited effect on *ori* activity. When the *rpmH*–*dnaA* promoter with the *dnaA* gene was cloned *in trans*, a stronger incompatibility was observed.

METHODS

Bacterial strain, plasmids and media. *E. coli* XL-1 Blue (Stratagene) was used for routine cloning and plasmid amplification. *M. smegmatis* high-frequency electroporation strain mc²155 was grown in Middlebrook 7H9 or 7H10 broth supplemented with 0.5% (v/v) glycerol and 10% OADC. Tween 80 (0.05%) was added to liquid media. Aliquots of 0.1–0.5 µg plasmid DNA were used in electroporation experi-

ments as described previously (Salazar *et al.*, 1996). The transformation efficiencies for each plasmid are shown as the number of transformants per µg DNA, based on the mean from at least four independent electroporations. Colony transformants were counted after incubation at 37 °C for 72–120 h. As control, plasmids pOM11 and pIJ963 were electroporated. Carbenicillin (Cb; 50 µg ml⁻¹), kanamycin (Km; 25 µg ml⁻¹) and hygromycin (Hyg; 50 µg ml⁻¹) were added to the culture media as appropriate.

The plasmids used in this study are listed in Table 1. The *ori* plasmids pOS239, pOS242 and pOS245 were described previously (Salazar *et al.*, 1996). For the construction of pOS239K and pOS242K, the *aph* gene (Km^r) from pYUB53 (Jacobs *et al.*, 1996) (1.3 kb fragment) was cloned in the *SalI* site of pUC19 (pUC19Km^r). *Bam*HI–*Kpn*I fragment from pOS239 and *Sma*I fragment from pOS242, 3.3 kb and 2.65 kb, respectively, were cloned in pUC19Km^r. pOS246 and pOS246K were constructed by deletion (cut, blunted and religated) of the 270 bp *Hind*III–*Eco*RI region, containing P_{*dnaA*} (–35 and –10 sequences), from pOS239 and pOS239K, respectively. Similarly, pOS247 was constructed by deletion of the 113 bp *Eco*RI–*Nco*I region (containing DnaA boxes) from pOS239. pOS248 was constructed by deletion of P_{*dnaA*} and the DnaA boxes (383 bp *Hind*III–*Nco*I fragment) from pOS239. For the construction of pOS249, the fragment containing the promoter region of a sigma factor of *M. smegmatis* was amplified by PCR (fragment A, Table 2) and cloned into the *Hind*III/*Eco*RI site of pOS239. In a similar way, pOS250 was constructed by the cloning of a PCR fragment containing the promoter of the *gyrBA* gene of *M. smegmatis* (fragment B, Table 2) into pOS239. The promoter of the *dnaA* gene and/or DnaA boxes region upstream of *dnaA* were replaced by sequences that did not exhibit promoter activity nor contained consensus sequences for DnaA boxes, by cloning PCR amplification fragments containing the *rnpA*–*rpmH* genes of *M. tuberculosis* into pOS239 (fragments C, D and E, respectively; Table 2). In the PCR reactions, DNA of the cosmid pIV101 for *M. smegmatis* and pIV305 for *M. tuberculosis* (Salazar *et al.*, 1996) was used as substrate. To create pOS254, pJEM15 (Timm *et al.*, 1994) was digested with *Cla*I to release the transcriptional termination sequence (Ω fragment composed of a streptomycin–spectinomycin resistance gene flanked by short inverted repeats containing *tT4*), and pOS239 was linearized with the endonuclease *Mlu*I. Both fragments were treated with Klenow DNA polymerase and deoxynucleoside triphosphates, purified from 1% agarose gels and ligated. The resulting plasmid carries an *oriC* with a transcription stop signal precisely 123 nucleotides from the stop of the coding sequence (TGA) of the *dnaA* gene, between the AT-rich region and the first DnaA box on *oriC* of *M. smegmatis*. To screen for deletion or replacement *oriC* mutants, DNA from transformants was amplified with specific primers flanking the upstream *dnaA* region, LS60 (5'-TGGAAGGT-CCGGTTGCCCTTG-3') and SM15 (5'-GGACGATTACCCCTTTGAGG-3'), and the fragments obtained were analysed by sequencing. To clone the *dnaA* gene *in trans*, the *dnaA* gene including its upstream region was amplified by PCR using specific primers LS60P (5'-TTCAGCTGGAAGG-TCCGCTTGCC-3') and SM2P (5'-CTCAGCTGTCAGCG-TTGCGCGCTGGC-3') and DNA from plasmid pOS239 as template. The PCR product was digested with *Pvu*II and cloned in the *Sca*I site of pOS245. Similar constructions were made using DNA from pOS249 and pOS250 as template.

Transcriptional fusion to GFP and measurement of the fluorescence. The shuttle plasmid pFPV27 (Valdivia *et al.*, 1996) was used for cloning fragments fused to the *gfp* gene.

Table 1. Plasmids used in this study

Plasmid	Relevant features	Source or reference
pFPV27	Km ^r ; shuttle vector for operon and gene fusion to <i>gfp</i> gene	Valdivia <i>et al.</i> (1996)
pUC19Km ^r	Km ^r ; <i>aph</i> gene into <i>Sall</i> site of pUC19	This work
pOS239	3.3 kb <i>Bam</i> HI– <i>Bgl</i> III fragment containing <i>rpmH</i> – <i>dnaN</i> region of <i>M. smegmatis</i> in pIJ963; Cb ^r Hyg ^r	Salazar <i>et al.</i> (1996)
pOS242	2.65 kb <i>Nco</i> I– <i>Bgl</i> III fragment containing <i>dnaA</i> – <i>dnaN</i> region of <i>M. smegmatis</i> in pIJ963; Cb ^r Hyg ^r	Salazar <i>et al.</i> (1996)
pOS245	1.3 kb fragment containing <i>dnaA</i> – <i>dnaN</i> intergenic region of <i>M. smegmatis</i> in pIJ963; Cb ^r Hyg ^r	Salazar <i>et al.</i> (1996)
pOM11	1.6 kb <i>Hpa</i> I– <i>Eco</i> RV fragment containing <i>ori</i> region of pYUB18 in pIJ963; Cb ^r Hyg ^r	Salazar <i>et al.</i> (1996)
pOS239K	3.3 kb <i>Bam</i> HI– <i>Bgl</i> III fragment containing <i>rpmH</i> – <i>dnaN</i> region from pIV101 in pUC19Km ^r ; Km ^r Hyg ^r	This work
pOS242K	2.65 kb <i>Nco</i> I– <i>Bgl</i> III fragment containing <i>dnaA</i> – <i>dnaN</i> region from pIV101 in pUC19Km ^r ; Km ^r Hyg ^r	This work
pOS246	Deletion of 270 bp <i>Hind</i> III– <i>Eco</i> RI containing P _{<i>dnaA</i>} from pOS239	This work
pOS246K	Deletion of 270 bp <i>Hind</i> III– <i>Eco</i> RI containing P _{<i>dnaA</i>} from pOS239K	This work
pOS247	Deletion of 113 bp <i>Eco</i> RI– <i>Nco</i> I containing DnaA boxes upstream of <i>dnaA</i> from pOS239	This work
pOS248	Deletion of 383 bp <i>Hind</i> III– <i>Nco</i> I containing P _{<i>dnaA</i>} and DnaA boxes upstream of <i>dnaA</i> from pOS239	This work
pOS249	270 bp fragment containing P _{<i>sigM</i>} of <i>M. smegmatis</i> in pOS239	This work
pOS250	265 bp fragment containing P _{<i>gyrBA</i>} of <i>M. smegmatis</i> in pOS239	This work
pOS251	272 bp fragment from <i>rnpA</i> gene region of <i>M. tuberculosis</i> in pOS239	This work
pOS252	122 bp containing <i>rpmH</i> gene of <i>M. tuberculosis</i> in pOS239	This work
pOS253	383 bp containing <i>rnpA</i> – <i>rpmH</i> gene of <i>M. tuberculosis</i> in pOS239	This work
pOS254	Insertion of a transcriptional termination sequence <i>t</i> T4 into <i>Mlu</i> I site of pOS239	This work
pgfp87	395 bp PCR fragment from pOS239 containing P _{<i>dnaA</i>} , cloned in pFPV27	This work
pgfp85	539 bp PCR fragment from pOS239 containing P _{<i>dnaA</i>} and DnaA boxes, cloned in pFPV27	This work
pgfp134	539 bp PCR fragment from pOS252 containing P _{<i>dnaA</i>} , cloned in pFPV27	This work
pgfp71	269 bp PCR fragment from pOS246 upstream of <i>dnaA</i> , cloned in pFPV27	This work
pgfp43	539 bp PCR fragment from pOS249 containing P _{<i>sigM</i>} and DnaA boxes upstream of <i>dnaA</i> in pFPV27	This work
pgfp13	395 bp PCR fragment from pOS249 containing P _{<i>sigM</i>} in pFPV27	This work
pgfp30	539 bp PCR fragment from pOS251 containing <i>rpmH</i> gene region and DnaA boxes in pFPV27	This work
pgfp434	395 bp PCR fragment from pOS251 containing <i>rpmH</i> gene region in pFPV27	This work
pgfp67	539 bp PCR fragment from pOS253 containing <i>rnpA</i> – <i>rpmH</i> gene region in pFPV27	This work

Table 2. Primers and amplified fragments used

Amplified fragment*	Primer (5'→3')	Amplified region	Size (bp)
A	SigM8: CCAAGCTTCCTCGCTGTCGGACACCACCG SigM9: CCGGAATTCCTAAACGGTGTCAAGGCACCT	Sigma M promoter from <i>M. smegmatis</i> †	270
B	Gyr1: CCAAGCTTGGTGCAGTCACAACCTGCTGGC Gyr2: CGGAATTCGAGCGAGTTGTGCGCCGTGTCC	Gyrase promoter from <i>M. smegmatis</i> ‡	265
C	RnpA1: CCAAGCTTGACGATCGACCCAGCCACGTGC RpmH1.1: CGGAATTCACGCAAGGGTCGGCGCACGCTC	nt 598–869 from <i>M. tuberculosis</i> ‡	272
D	RpmH1.2: CGGAATTCACCTTTCGCTCGCCGGCTGGAC RpmH2: ATGCCATGGACCAAGGGCAAAAGGACCTTCC	nt 859–980 from <i>M. tuberculosis</i> ‡	122
E	RnpA1: CCAAGCTTGACGATCGACCCAGCCACGTGC RpmH2: ATGCCATGGACCAAGGGCAAAAGGACCTTCC	nt 598–980 from <i>M. tuberculosis</i> ‡	383

* The PCR reactions were done with *Taq* DNA polymerase (PE), 0.2 µM of the specific primers, 200 µM dNTPs, 2 mM MgCl₂ and 10% DMSO in a final volume of 50 µl. Reactions were carried out in 35 cycles of 92 °C for 1 min, annealing temperature for 2 min and 62 °C for 2 min, with a final elongation step at 72 °C for 10 min.

† N. Arraíz and others, unpublished.

‡ GenBank accession number X92504.

The PCR reaction was carried out using the primers LS60B (5'-AAGGATCCGAAGGTCCGCTTGCCCTT-3') and SM15B (5'-GCGGATCCGGACGATTACCCCTTTGAG-3'), or LS60B and SM17E (5'-CTCGAATTCGCCCCGTGCT-3') and DNA from the plasmids pOS239, pOS246, pOS249, pOS250, pOS251, pOS252 and pOS253 as substrate. The resulting fragments containing the upstream *dnaA* region, or only the promoter region, were cloned into pFPV27 (*Bam*HI or *Bam*HI/*Eco*RI site, respectively). The direction of the inserts was determined by digestion with restriction endonucleases and sequencing. *M. smegmatis* mc²155 cells bearing the transcriptional fusion to *gfp* were grown in 7H9 medium containing 25 µg kanamycin ml⁻¹ at 37 °C. Fluorescence and culture density (OD₆₄₀, Molecular Devices Spectra Max 340) were determined from aliquots of the cultures taken at early, mid-exponential and stationary phase. GFP fluorescence was quantified by fluorometry in a Spectrafluor Tecan (Microplate Reader).

Other molecular techniques. Digestions, ligations, filling of protruding ends and plasmid DNA isolation were performed according to standard procedures (Ausubel *et al.*, 1995). Amplified fragments were sequenced with USB Sequenase 2.0 (USB, Amersham) and [³⁵S]dATPαS or with a dye terminator cycle sequencing kit and an ABI377 sequencer (PE Biosystem), using the appropriate primers. PCR amplification reactions were carried out with *Taq* DNA polymerase (Perkin Elmer), according to the manufacturer's recommendations.

Determination of plasmid copy number. *M. smegmatis* mc²155 cells bearing *oriC* plasmids were grown overnight at 37 °C to mid-exponential phase, in 7H9 medium containing 50 µg hygromycin ml⁻¹. Total DNA was then prepared as described by Ausubel *et al.* (1995), linearized by digestion with *Pvu*II and separated by 1% agarose gel electrophoresis. Southern blot analysis was performed with [³²P]dCTP-labelled *oriC* from *M. smegmatis* as probe. The *oriC* probe was obtained by PCR amplification using the primers SM10 (5'-GCCCCCTTCGATAATCCCCGCA-3') and SM11 (5'-C-ACGCTCGGCGGCTGTGGATA-3') and pIV101 cosmid as template. Hybridization was at 65 °C, overnight, and final

wash conditions were 65 °C for 30 min with 0.1 × SSC and 0.1% SDS. DNA bands were scanned and analysed using ALPHA EASE software (Alpha Innotech). The copy number was determined as the ratio between the chromosomal and plasmid band intensities after hybridization.

RESULTS

Contribution of the upstream *dnaA* region to the inhibition of replication

In an attempt to determine the nature of this inhibition, a series of *oriC* plasmids from pOS239 was constructed, eliminating or modifying the *rpmH-dnaA* promoter region (*P_{rpmH-dnaA}*) and/or the DnaA box region upstream of the *dnaA* gene. The plasmid pOS239 carries a 3.3 kb fragment of *M. smegmatis* mc²6, with the *mpA-dnaN* region including the origin of replication. It is not able to remain as an *ars* in *M. smegmatis* mc²155 (Salazar *et al.*, 1996). By cutting with restriction endonucleases and religation of pOS239, the *rpmH-dnaA* promoter region, the three DnaA boxes localized upstream of the *dnaA* gene, or both, were deleted. Similar constructions were made using the pUC19Km^r-cloning vector. All of these constructions were then transformed into *M. smegmatis* mc²155. Transformation frequencies were considered as an expression of the *oriC* activity. The results showed that the inhibitory effect observed in pOS239 was overcome, regardless of the selective marker (hygromycin or kanamycin resistance), when the *P_{rpmH-dnaA}* (pOS246 and pOS246K plasmids) or *P_{rpmH-dnaA}* and DnaA box region (pOS248 plasmid) were removed, but not when only the DnaA box region (pOS247 plasmid) was removed. The plasmids pOS246, pOS246K and pOS248 could efficiently transform *M. smegmatis* with a frequency of 0.3–0.5 × 10⁴ transformants per µg DNA. In contrast, pOS247 showed a

Fig. 1. Schematic representation of the constructs obtained from pOS239. Arrows indicate the orientation of transcription. Dotted white boxes denote the deleted regions. pOS239 and pOS242 were described previously (Salazar et al., 1996). $\oplus$, *dnaA* promoter region; $\diamond$, DnaA boxes region; $\odot$, origin of replication; H, *Hind*III; E, *Eco*RI; N, *Nco*I. Transformation frequencies represent the mean of at least four independent assays; NT, not tested.

Table 3. Efficiency of transformation of *oriC* plasmids

Plasmid	Features	$10^{-4} \times \text{No. of transformants } (\mu\text{g DNA})^{-1*}$
pOS249	Derived from pOS239; replacement of P_{dnaA} (nt 346–616) by 270 bp fragment from P_{sigM} of <i>M. smegmatis</i> †	13.8
pOS250	Derived from pOS239; replacement of P_{dnaA} (nt 346–616) by 265 bp fragment from P_{gyrBA} of <i>M. smegmatis</i> ‡§	2.0
pOS251	Derived from pOS239; replacement of P_{dnaA} (nt 346–616) by 272 bp fragment from <i>rnpA</i> gene region of <i>M. tuberculosis</i> ‡	1.0
pOS252	Derived from pOS239; replacement of DnaA boxes (nt 619–732) by 122 bp fragment from <i>rpmH</i> gene region of <i>M. tuberculosis</i> ‡	~ 0
pOS253	Derived from pOS239; replacement of P_{dnaA} and DnaA boxes (nt 346–732) by 383 bp fragment from <i>rnpA-rpmH</i> gene region of <i>M. tuberculosis</i> ‡	1.0
pOS254	Derived from pOS239; insertion of a transcriptional termination sequence into <i>Mlu</i> I restriction site	~ 0

* Mean of at least four assays.

† N. Arraíz and others, unpublished.

‡ GenBank accession number X92504.

§ Casart (1996).

frequency of transformation close to zero (Fig. 1). On rare occasions, some transformant colonies were found with plasmids pOS239 and pOS247. These results indicate that the region that contained $P_{rpmH-dnaA}$ specifically causes the incompatibility observed between *oriC* plasmids and the chromosome of *M. smegmatis*.

To elucidate the mechanism of replication control by P_{dnaA} , a 270 bp *Hind*III–*Eco*RI fragment from pOS239

containing $P_{rpmH-dnaA}$ was replaced by sequences that exhibit a weak or null promoter activity. Assuming that a space conservation between the motifs is important in the replication function, in each case fragments having the same length (Table 2) were introduced into this region. PCR fragments containing the *sigM* promoter of *M. smegmatis* and the coding sequence of the *rnpA* gene of *M. tuberculosis* (fragments A and C, respectively) were cloned into the *Hind*III/*Eco*RI site of pOS239,

Fig. 2. (a) Strategy for cloning of the *dnaA* gene *in trans*: cloning of P_{dnaA} -*dnaA* gene in the *ScaI* site of pOS245. (b) Sizes of *oriC* plasmids isolated from *E. coli*. *BglII*-digested DNA was electrophoresed into 1% agarose gel. Lanes: 1, 1 kb ladder; 2, pOS239; 3, pOS245; 4, P_{dnaA} -*dnaA* gene; 5, P_{sigM} -*dnaA* gene; 6, P_{gyrBA} -*dnaA* gene; 7, P_{dnaA} -*dnaA* gene. Arrows show the lengths of the restriction fragments.

resulting in plasmids pOS249 and pOS251. We have demonstrated that the expression of *sigM* from *M. smegmatis* and *M. bovis* BCG is very low in normal growth conditions (rich medium, 37 °C) (N. Arraíz and others, unpublished, and Fig. 3). In that work, we found that a 488 bp fragment containing the *pknC*-*sigM* intergenic region from *M. smegmatis* shows promoter activity. The fragment C did not show promoter activity (see below). Both of these plasmids are able to transform *M. smegmatis* mc²155, but pOS249 resulted in a stronger *ars* activity (Table 3).

Following a similar methodology and taking advantage of the fact that the DnaA boxes upstream of the *dnaA* gene of *M. smegmatis* are localized in a 113 bp *EcoRI*-*NcoI* segment, the DnaA box region was replaced with a fragment that did not show a consensus motif for DnaA binding (fragment D). The resulting plasmid pOS252, like pOS247, did not yield transformants. However, when the 383 bp fragment containing both P_{dnaA} and DnaA box regions was exchanged with a

Fig. 3. Fluorescence level in *M. smegmatis* bearing transcriptional fusions with *gfp*. Transcriptional fusions were generated as described in Methods. Fluorescence was determined by spectrofluorometry. All measurements were carried out on triplicate cultures. Specific promoter activity is expressed as fluorescence intensity at 535 nm (emission filter), corrected for the fluorescence emission of untransformed cells.

fragment that did not contain any of these motifs (fragment E), *ars* activity was restored (plasmid pOS253; Table 3). These results support the hypothesis that the DnaA box region upstream of the *dnaA* gene is not involved in the incompatibility observed.

To determine if *oriC* function is negatively regulated by the transcription of *dnaA*, two different approaches were adopted, which avoided the topological effect but left *dnaA* expression intact. First, the insertion of the transcription terminator *tT4* at the end of the *dnaA* gene, which prevents *dnaA* transcripts from entering and traversing *oriC*, did not release the inhibitory effect (pOS254; Table 3). On the other hand, while trying to establish a system in which the *dnaA* gene was supplied *in trans*, P_{dnaA} -*dnaA* gene was cloned into the *ScaI* site of pOS245, disrupting the ampicillin-resistance gene (Fig. 2a). Unexpectedly, on several occasions, the restriction analysis of the plasmids isolated from transformed colonies of *E. coli* revealed a deletion instead of the expected lengthening. The same results were also observed when P_{sigM} or P_{gyrB} drove *dnaA* expression (Fig. 2b), or when the *dnaA* gene was cloned in another site on the plasmid (data not shown).

Transcriptional fusion to *gfp* in plasmids

To confirm the promoter activity of the fragments used in the experiments described above, each one (fragments A-E; Table 2) was fused to the green fluorescent protein gene (*gfp*) on the plasmid pFVP27 (Valdivia *et al.*, 1996). The results of the fluorescence measured from *M. smegmatis* strains carrying the different plasmids are shown in Fig. 3. The fragment containing P_{dnaA} (plasmid pgfp87) showed promoter activity. It reached the highest level during the exponential growth phase. The promoter activity shown by P_{sigM} (pgfp13) confirmed our previous result (N. Arraíz and others, unpublished):

Fig. 4. Photographs of *M. smegmatis* colonies bearing *oriC* plasmids: (a) pOM11, (b) pOS210, (c) pOS246, (d) pOS253. Bar, 5 mm.

Fig. 5. Copy number of the *oriC* plasmids: 1, mc²155 (control); 2, pOS210; 3, pOS245; 4, pOS246; 5, pOS249; 6, pOS250; 7, pOS251; 8, pOS253. Chr indicates the chromosome band. The values represent the mean ($\pm$ standard error) of at least three different measurements.

P_{sigM} of *M. smegmatis* is a weak promoter and its activity is doubled when the cells reach the stationary phase. In some cases, with P_{dnaA} and P_{sigM} , promoter activity was slightly reduced by the DnaA boxes cloned downstream (plasmids pgfp85 and pgfp43, respectively). The fragments containing a portion of *rnpA*–*rpmH* gene sequences (pgfp30, pgfp434, and pgfp67) showed a fluorescence level indistinguishable from that emitted by *M. smegmatis* cells carrying the vector plasmid, pFVP27.

Phenotype of *M. smegmatis* colonies containing *oriC* plasmids and copy number determination

M. smegmatis mc²155 colonies bearing the *oriC* plasmids were smaller than those carrying the control plasmid, pOM11 (Fig. 4), indicating some incompatibility towards the chromosome. Additionally, differences in colony morphology were found between strains carrying the *oriC* plasmids. When only P_{dnaA} was modified, the resulting colonies were smaller than when

an additional modification in the DnaA boxes region was introduced (compare strains with plasmids pOS253 and pOS246).

The copy number of the *oriC* plasmids was measured as described in Methods. Qin *et al.* (1997) reported for *ars* plasmids of *M. smegmatis* a copy number of two. In this study, variations in the copy number of the *ars* plasmids of *M. smegmatis* were found (Fig. 5), depending of the modification introduced in the upstream *dnaA* region. When P_{dnaA} was removed, or exchanged by a weak promoter, the copy number was close to one (plasmids pOS246 and pOS249). However, when additional modifications were introduced into the DnaA boxes region the copy number was considerably higher [plasmids pOS210 (Salazar *et al.*, 1996), pOS245 and pOS253]. Additionally, in previous work (Salazar *et al.*, 1996) and in this study, it was found that the *oriC* plasmids could transform *M. smegmatis* if the upstream *dnaA* sequence was removed. In the cases where some transformants of pOS239 or pOS252 were found, the recovery of the intact plasmid DNA from these colonies failed and insertions in the chromosome were not detected (data not shown).

DISCUSSION

The upstream region of the *dnaA* gene of mycobacteria is composed of, at least, two conserved motifs: the promoter region with divergent transcription for *rpmH* and *dnaA* genes; and three highly conserved 9 bp sequences constituting the DNA-binding motif for the DnaA protein (Salazar *et al.*, 1996). To understand how the upstream *dnaA* region regulates the replication of *oriC* plasmids of *M. smegmatis*, a large number of derivatives of pOS239 (a non-replicative plasmid that contains the *oriC* region) were constructed. The two recognized motifs, P_{dnaA} and/or DnaA boxes, were modified or deleted. The incompatibility of the chromosome previously observed with pOS239 was eliminated when the P_{dnaA} or all the upstream *dnaA* region was

removed (Fig. 1). These results strongly suggested that it is the *rpmH*–*dnaA* promoter region, and not the DnaA boxes, which exerts the negative effect on replication.

Some additional results were found to be inconsistent with the hypothesis that the direct interaction between the plasmid-borne and chromosomal DnaA boxes, mediated by DnaA protein, could be the cause of the strong incompatibility between the plasmid and chromosomal *oriC*s, as has been suggested to occur in *B. subtilis* (Moriya *et al.*, 1992) and *S. lividians* (Zakrzewska-Czerwinska *et al.*, 1992). (i) No significant variations in the transformation efficiency were observed when the *ars* contained the DnaA boxes upstream of *dnaA* (compare plasmids pOS246 and pOS248 in Fig. 1). (ii) Despite the fact that the *oriC* region from *M. smegmatis* contained seven DnaA box motifs, while the region upstream of *dnaA* contains only three, the *ars* plasmids are highly stable and did not insert into chromosomes (Fig. 5; Qin *et al.*, 1997; Salazar *et al.*, 1996). Nevertheless, the DnaA box region upstream of the *dnaA* gene might be playing some regulatory role. First, an atypical morphology of colonies was found when *M. smegmatis* carried *oriC* plasmids. In cases in which P_{dnaA} and DnaA box regions had been modified simultaneously (plasmid pOS253), the cells generated large colonies similar to the control (Fig. 4). On the other hand, deletion of the DnaA boxes increased the plasmid copy number (Fig. 5). The effect of the DnaA boxes upstream of *dnaA* on replication could be indirect, via repression of transcription from the *dnaA* promoter, as deletion of the DnaA box causes an increase in the *dnaA*–GFP activity (Fig. 3). In *E. coli*, deletion of the DnaA box localized between *dnaA1P* and *dnaA2P* causes an increase in expression from *dnaA1P* and a decrease in the activity of promoter 2P (Atlung *et al.*, 1985; Polaczek & Wright, 1990).

The possibility that *oriC* function is negatively regulated *in cis* by the transcripts initiated from upstream of *dnaA* that enter and traverse *oriC* was discarded (plasmid pOS254). However, topological effects exerted by *dnaA* gene expression were not demonstrated, because a strong incompatibility in *E. coli* was found when this gene was supplied *in trans*, regardless of whether a strong or weak promoter drove the transcription of the *dnaA* gene. The significance of this result is not clear but the *dnaA* and *rpmH* promoters of *M. smegmatis* and *M. tuberculosis* are very well expressed in *E. coli* (data not shown). These genes show a good consensus σ^{70} promoter sequence (L. Salazar and others, unpublished data). It is possible that transcription from upstream of *dnaA* interfered with replication initiation from the origin of replication of *E. coli* on the plasmid. Or alternatively, an intra-molecular interaction may have occurred between both DnaA boxes on the plasmid mediated by DnaA protein. Moriya *et al.* (1988) observed that in the transformant colonies obtained when Region B of *B. subtilis* was cloned into a low-copy-number plasmid, the DNA plasmid isolated showed drastic changes in the structure of the inserted B region. The B region of *B. subtilis* is equivalent to the DnaA box region upstream of *dnaA* in *M. smegmatis*.

In spite of this, the incompatibility was overcome when P_{dnaA} was deleted. The introduction of the weak promoter led to a transformation frequency 26 times higher (Table 2), suggesting that some transcription from upstream of *dnaA* contributed to replication of the *oriC* plasmid, perhaps because the amount of DnaA protein is a limiting factor (Moriya *et al.*, 1990; von Meyenburg & Hansen, 1987). However, excess of DnaA seems to be toxic. The *dnaA* promoter of mycobacteria is relatively strong in relation to those governing other essential genes and *dnaA* transcription is coupled with growth (L. Salazar and others, unpublished; Fig. 3). In *B. subtilis* (Ogasawara *et al.*, 1991) the presence of an intact *dnaA* gene inhibited growth, and the cloning of the *dnaA* gene of *S. lividians*, using high- or medium-high-copy-numbers vectors, led to very poor growth and loss of the inserts (Zakrzewska-Czerwinska *et al.*, 1994).

The results found in the present study clearly indicate that the region upstream of the *dnaA* gene is playing some regulatory role in chromosome replication. They suggest that P_{dnaA} exerts a negative effect by overexpression of DnaA protein, and DnaA boxes seem to be implicated in the regulation of the copy number or stability.

Whether the *dnaA* regulatory region has a role in the cell cycle regulation of initiation at the origin of replication in eubacteria is still unknown. Polaczek (1998) discussed the possibility that the regulatory sequences within and surrounding the *dnaA* promoter region of *E. coli* constitute an evolutionary fossil of no significant physiological relevance, because there is not convincing experimental support that demonstrates a fine-tuned expression of the *dnaA* gene. However, Speck *et al.* (1999) recently showed an unequivocal autoregulation of *dnaA* in *E. coli* through interaction between the DnaA protein and the *dnaA* promoter region. Additional functions have been assigned to the *dnaA* promoter region. Moriya *et al.* (1992) described that in *B. subtilis*, the *dnaA* promoter region is required for autonomous replication in plasmids and that the *ars* activity is affected by neighbouring sequences. Removal of the promoter region of *rpmH* from the *oriC* plasmid resulted in stronger *ars* activity. The *rpmH* gene encodes the 50S ribosomal protein L34. The activity of the *rpmH* promoter of *M. smegmatis* is 1.5–3.5 times higher than that exhibited by the *dnaA* promoter (data not shown). Therefore, it is possible that the divergent transcription from *rpmH* and *dnaA* exerts topological effects, or that the strong promoter activity of *rpmH* will inhibit replication of the *oriC* plasmid. Experiments to determine the contributions of the *rpmH* promoter are in progress.

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