**In-Vitro** Helix Opening of *M. tuberculosis* oriC by DnaA Occurs at Precise Location and Is Inhibited by IciA Like Protein

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Abstract

**Background:** *Mycobacterium tuberculosis* (*M.tb*), the pathogen that causes tuberculosis, is capable of staying asymptomatically in a latent form, persisting for years in very low replicating state, before getting reactivated to cause active infection. It is therefore important to study *M.tb* chromosome replication, specifically its initiation and regulation. While the region between dnaA and dnaN gene is capable of autonomous replication, little is known about the interaction between DnaA initiator protein, oriC origin of replication sequences and their negative effectors of replication.

**Methodology/Principal Findings:** By KMnO₄ mapping assays the sequences involved in open complex formation within oriC, mediated by *M.tb* DnaA protein, were mapped to position −500 to −518 with respect to the dnaN gene. Contrary to *E. coli*, the *M.tb* DnaA in the presence of non-hydrolysable analogue of ATP (ATPγS) was unable to participate in helix opening thereby pointing to the importance of ATP hydrolysis. Interestingly, ATPase activity in the presence of supercoiled template was higher than that observed for DnaA box alone. *M.tb* rRv1985c, a homologue of *E.coli* IciA (Inhibitor of chromosomal initiation) protein, could inhibit DnaA-mediated in-vitro helix opening by specifically binding to A+T rich region of oriC, provided the open complex formation had not initiated. rlcI could also inhibit in-vitro replication of plasmid carrying the *M.tb* origin of replication.

**Conclusions/Significance:** These results have a bearing on the functional role of the important regulator of *M.tb* chromosomal replication belonging to the LysR family of bacterial regulatory proteins in the context of latency.

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Introduction

Replication in eubacteria is initiated when DnaA, an initiator protein, binds to DnaA boxes located within the origin of replication (oriC) sequence [1]. Initiation of replication in *E. coli* proceeds with the binding of DnaA protein to oriC [2] and leads to opening of 13-mer region, which is followed by entry of DnaB helicase to form the prepriming complex [3]. In many bacteria either or both the 3’ and 5’ flanking regions of the dnaA gene exhibit oriC activity, thereby conferring the ability to replicate autonomously. In *Bacillus subtilis*, both the 5’ and 3’ flanking regions of dnaA act as oriC [4], whereas in *Mycobacterium tuberculosis* (*M.tb*), *M. bovis* [5] and *M. smegmatis* [6,7,8], only the 3’ flanking region provides oriC function. There are five DnaA-binding sites in the oriC region of *E. coli*, referred to as R boxes, to which both active ATP-DnaA and inactive ADP-DnaA proteins bind with equal affinity [9,10]. There are additional initiator binding sites in the oriC, region referred to as I sites, to which only DnaA-ATP can bind [11].

DnaA protein binds with nearly equal affinity to ATP and ADP. In *E. coli* the function of ATP appears to be allosteric and the non-hydrolysable analogue ATPγS can replace ATP in helix unwinding [12]. For opening of the DNA duplex multiple DnaA proteins, complexed with ATP, bind to oriC and melt the DNA unwinding element (DUE). ADP bound form of DnaA is inactive for replication initiation, forming an important level of regulation at the origin.

The *E. coli* IciA protein (Inhibitor of Chromosome Initiation) blocks initiation at very early stage *in-vitro* by binding specifically to A+T rich region of oriC [12,13]. Binding of IciA blocks the opening of A+T rich region mediated by DnaA and HU (Histone like protein) or integration host factor (IHF) protein and this inhibition of strand opening by IciA does not affect binding of DnaA and IHF (or HU) protein to their respective binding sites [14]. IciA contains helix turn helix motif at the N terminal region and shows homology to LysR family of prokaryotic transcription regulators [12]. IciA has also been implicated in binding to A+T rich regions within the plasmid *on* sequence and the copy number of the F plasmid is increased in *iciA* deletion mutant [15]. IciA also shows higher binding preference for curved DNA [16]. Further, IciA is involved in regulation of *mdc* gene encoding ribonucleoside
diphosphate reductase [17], activating dnaA gene [18] and has recently been shown to also regulate the yggA gene encoding the arginine exporter [19].

M.tb maintains itself in two physiologically distinct growth states – an active replicative state and a non-replicative persistent state [20]. In persistent state, the bacterium is metabolically active, but shows no multiplication for extended periods, only to revive later and multiply to cause infection [21]. The genetic elements responsible for the replication process in M.tb, specifically its initiation and regulation, are not known. In M.tb, the DNA fragments bearing the dnaA-dnaN intergenic region function as oriC [5]. Upon comparison of the oriC region of E. coli, M.tb and B. subtilis (Figure 1A) it appears that E. coli has three A+T rich 13 mers [1], B. subtilis has a 27 mer [4] which is exclusively rich in A+T residues, but M.tb has only one A+T rich 15 mer region [5,22]. It should also be noted that E. coli has only 5 DnaA boxes (Figure 1B) whereas M.tb has 13 such boxes. In addition, both E. coli and B. subtilis have DnaA-ATP boxes (Figure 1A), however in M.tb such boxes are not present [23]. One more unusual observation reported for M.tb is the requirement of hydrolysis of ATP for rapid oligomerization of DnaA on oriC [23]. It should also be noted that E. coli possesses only five DnaA boxes, whereas M.tb has 13 presumptive DnaA box sequences that bear little sequence similarity to any of the E. coli DnaA boxes [5,8]. DnaA protein of mycobacteria has been shown to bind to at least some of these boxes [24,25]. These studies suggest that the replication origin site in M.tb is very complex thereby making it interesting to study the mechanism of DNA replication and its regulation in M.tb.

Figure 1. A) Alignment of A+T rich regions from E. coli, M. tuberculosis and B. subtilis. These regions were aligned using adjacent DnaA box (shaded arrow) to A+T rich regions. Shaded boxes represent A+T rich cluster of E. coli, M. tuberculosis and B. subtilis respectively. Underlined regions in E. coli and B. subtilis represent potential DnaA-ATP boxes. L, M and R represent left, right and middle 13-mers. B) Illustration showing the organization of oriC region of M.tb and E. coli. AT represent AT rich region (rectangle) and the arrows represent DnaA boxes. The direction of arrows represents the orientation of these boxes.

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Given the clinical significance of persistence within the macrophages, it is important to identify and characterize the events involved in *M. tb* replication initiation and the negative effectors of replication initiation. We describe the interaction between *M. tb* DnaA protein and the *M. tb* oriC, including mapping the nucleotide sequences involved in DNA opening, and the requirement of ATP hydrolysis in this process. We additionally show the ability of *M. tb* IciA like protein, coded by *Rv1985c*, to block DnaA mediated helix opening and the eventual DNA replication by specifically interacting with A+T rich sequences present within the oriC region.

**Results**

DnaA protein shows higher ATPase activity in the presence of supercoiled template

In order to determine the preference, if any, of *M. tb* DnaA for a given form of DNA template, DnaA protein activity was measured in terms of ATPase activity. Recombinant DnaA protein expressed in *E. coli* was refolded after its purification under denaturing conditions and assayed for ATPase activity. ATPase activity was assayed either in the absence of DNA, or in the presence of linear DNA, or supercoiled pUC_OriMtb, or non-specific supercoiled template pBSK II. As could be seen from the densimetric scanning of the gel, ATPase activity in the presence of DnaA box (Figure 2, lanes 5–8) is expectedly higher than in the absence of DNA (Figure 2, lanes 1–4). However, ATPase activity increases significantly in the presence of supercoiled pUC_OriMtb (lanes 9–12) and pBSK II (lanes 13–16) (Table 1). The ATPase activity is a direct function of the concentration of rDnaA protein with maximal activity at 0.8 μM after which it stabilizes. These results while confirming that the refolded rDnaA protein is enzymatically active, also confirm that DnaA has very weak intrinsic ATPase activity which however increases in the presence of supercoiled DNA independent of whether *M. tb* oriC is present or not.

Open complex is formed near the A+T rich repeat

oriC region of *M. tb* is very complex and is different from its *E. coli* counterpart (Figure 1). The *M. tb* oriC has 13 imperfect DnaA boxes, which bear little sequence homology to *E. coli* DnaA boxes

**Figure 2.** A) DnaA protein (2 μM) was incubated with 16 nM [γ-32P]ATP for 30 min at 0°C in buffer C. Lanes 1–4: 0, 0.4, 0.8 and 1.2 μM respectively, of DnaA protein without any DNA; lanes 5–8: 0, 0.4, 0.8 and 1.2 μM respectively of DnaA protein with DnaA box; lanes 9–12: 0, 0.4, 0.8 and 1.2 μM respectively of DnaA protein with 550 ng of pUC_OriMtbs and lanes 13–16: 0, 0.4, 0.8 and 1.2 μM respectively of DnaA protein with 550 ng of pBSK II. B) The phosphate released in ATPase assay was quantified using Typhoon Variable Mode Imager and Image Quant Software (Amersham).

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Table 1. Bacterial strains, plasmids and oligonucleotide primers used in the current study. Nucleotides in bold represent the restriction enzyme sequence appended to the primers to enable directional cloning in pET28a/pUC18 vector.

| Bacterial Strains | Relevant characteristics | Source/ref. |
|-------------------|--------------------------|-------------|
| Strains           |                          |             |
| E. coli DH5α      | supE44 lacU169 (ΔlacZYA15) hisD17 recA1 endA1 and endA1 gyrA96 thi-1 recA1β | Invitrogen |
| E. coli BL21(DE3) | F' ampT hsdS(rB- mB-) gal dcm (DE3) | Invitrogen |
| E. coli BL21(DE3) | F' ampT hsdS(rB- mB-) gal dcm (DE3) plYS1 (CamR) | Invitrogen |
| M. bovis BCG pasteure | Vaccine Strain, Pasteur Institute | AstraZeneca, India |

| Plasmids          |                          |             |
|-------------------|--------------------------|-------------|
| pUC18             | Ampicillin resistant; multicopy plasmid with a ColE1 – type replicon | Fermentas |
| pET28a            | Expression Vector (kanamycin resistant) | Novagen |
| pETDnaA           | pET28A derivative carrying M.tb DnaA (Rv0001) | This work |
| pETIciA           | pET28A derivative carrying M.tb IciA (Rv1885c) | This work |
| pUC_OriMtb        | pUC18 derived carrying intergenic region between dnaA and dnaN | This work |
| pBSK II           | Ampicillin resistant multicopy plasmid with a ColE1 type origin sequence | Stratagene |

| Oligonucleotide primers |                          |             |
|-------------------------|--------------------------|-------------|
| IciAF                   | GGAATTCATATGTTGACCTGACGCTTGA |             |
| IciAR                   | GCAAGCTTTCAACCGGCGGCGGCGGC |             |
| DnaAF                   | GGAATTCATATGACCGATACCAGCGGTTTC |             |
| DnaAR                   | GCAAGCTTCTAGCGCTTGGAGGCGTGAC |             |
| MtboOrF                 | GCAAGCTTCGGATACGCGCTGTTCTT |             |
| MtboOrR                 | GCGGATCCGCCAGAACATGAGCGTGA |             |
| DnaA box 8-9fw          | ACCAGACTGTCCTCCAAAATCGCACACCTCT |             |
| DnaA box 8-9Rv          | AGAGGGGTGTCAGTTGGGGACAGTCTGTT |             |
| Ori F1                  | TTCTCCGGACAAATCTTTAAAAATCTCTCTA |             |
| Ori R1                  | TAGAAGAGTAAAAATAGAAGGTGCTGGAAGAA |             |
| SeqOriR1                | TCTTGGTCGAGGTACGATGCGGATG |             |
| SeqOriR2                | ACCGCGGGGACTGATGGA |             |
| SeqOriR3                | GTTTTCCAGTCAGCACG |             |

and also lack distinct A+T rich nucleotide repeat which is however present both in E. coli and B. subtilis at the 3’ end of dnaA gene, and is thought to be the site for helix opening. Given this complexity of M.tb oriC, DNA sequences involved in open complex formation were therefore mapped by primer extension analyses by KMnO₄ probing. Permanaganate is a very strong oxidant and thus reacts with the base moiety of DNA. Unlike DNase I, KMnO₄ generally does not modify naked double stranded DNA. However KMnO₄ selectively oxidizes unpaired pyrimidines, especially thymine residues, in single stranded DNA and in helically distorted duplex DNA. The most reactive site of the attack is 5, 6 double bond of the thymine ring. This attack can occur either from above or below the plane of the ring. But in native B form DNA this kind of attack is strongly hindered. The susceptible bond lies within the stacked array of bases under the DNA backbone within the major groove of DNA. Thus out of plane attack is just not possible as it is hindered by both the backbone and the adjacent bases. This accounts for the high selectivity of KMnO₄ for single stranded DNA. The initial stable product of the attack on thymine is glycol (diol form). Oxidized pyrimidines prevent primer extension by the DNA polymerase beyond the modified residues. This technique is routinely used for the study of replication complexes.

For our helix-opening assay increasing amounts of DnaA protein (0.025–0.3 µg) were incubated in presence of 5 mM ATP with supercoiled pUC_OriMtb, as described. Primer SeqOriR1 annealed between position -292 to -320 of template strand (Figure 3A), primer SeqOriR2 annealed between positions -402 to -420 of the template strand (Figure 3C) and primer SeqOriR3 annealed at position of -40 of pUC18 (Figure 3B). Primer extension reaction carried out using SeqOriR1 and SeqOriR2 would therefore enable read outs from bottom (downstream) while SeqOriR3 will give readouts from top (upstream). The extension products were then fractionated on a standard (6% or 15% as shown in the legend) urea sequencing gel (Figure 3A, B and C). Helix opening could be detected in the presence of 0.075 µg (Figure 3A, lane 4) of DnaA protein but barely when 0.025 µg or 0.050 µg (Figure 3A, lanes 2–3) of DnaA was used and this was evident from the presence of extension products (lane 4 of 199 nucleotides(a) and 200 nucleotides(b) corresponding to position −500 and −501 from the start of the dnaN gene. To further pinpoint the extent of helix opening another primer SeqOriR2 was utilized and the extension products were fractionated on 15% urea gel. As can be seen (Figure 3C, lanes 12–16) extension products corresponding to 98, 99, 113 and 116 nucleotides designated as f, e, d and c respectively, could be observed which correspond to position -500, -501, -515, -518 from the start of dnaN gene. Primer SeqOriR3 annealed at position of -40 of pUC18 and generates extension products (Figure 3B, lanes 6–10) of 63(l), 65(k), 66(j), 76(i), 77(h) and 79(g) nucleotides which represent position −518, −515, −514, −504, −503 and...
Figure 3. *M. tb* helix opening by rDnaA occurs near position −500 to −518 within the A+T rich region and this is inhibited by rIciA. pUC_OriMtb was used as a template for helix opening in the presence of increasing amounts of rDnaA with γ²P labeled SeqOriR1 and the primer extension products were fractionated on 6% sequencing gel. (A) SeqOriR1 primer reads pUC_OriMtb from bottom. Lanes 2, 3 and 4 show KMnO₄.
probing in the presence of 25 ng, 50 ng and 75 ng DnaA. These and other primer extension products of various sizes were designated as 200 nt (a), 199 nt (b) and so on and are summarized in Figure 3F. (B) The upstream primer SeqOriR3 reads pUC_OriMtb from the top and anneals at position −40 of pUC18 vector backbone. The different lanes are: lane 6, no DnaA protein; lanes 7–10: 0.075, 0.1, 0.2 and 0.3 μg of rDnaA. Extension products of 79(g), 77(h), 76(i), 66(j), 65(k) and 63(l) nucleotides could be seen. (C) Primer SeqOriR2 (downstream primer) also reads pUC_OriMtb from the 3’-end. After KMnO4 modification and PCR amplification with 9/×P labeled SeqOriR2, the primer extension products were fractionated on a 15% sequencing gel. Lane 12, control DNA where no DnaA protein is added; lanes 13–16: 0.075, 0.1, 0.2 and 0.3 μg of rDnaA. Extension products of 116(c), 113(d), 99(e) and 98(f) nucleotides could be seen. Non-specific extension products (ns) were also seen in all lanes even in control DnaA free lane. 10 bp ladder was used as DNA molecular size marker (lane 1, 5 and 11) and shown on the left. (D) The reaction was carried out using 0.2 μg of DnaA protein. Helix opening was monitored by primer extension using SeqOriR1 on a 6% sequencing gel. The different lanes are: lane 2, without rIciA; lane 3–4: increasing amounts (0.2 μg and 0.4 μg) of IciA protein. Arrows correspond to the extension products of 200 and 199 nucleotides. (E) Primer SeqOriR3 (lanes 5–11) and SeqOriR2 (lanes 13–19) were used to monitor helix inhibition mediated by rIciA. All the lanes from 5–9 and 13–17 have 0.2 μg of DnaA protein; Lanes 6–9 and 14–17 have increasing amounts (0.2, 0.3, 0.4 and 0.5 μg) of IciA protein; lanes 10 and 18 have 0.5 μg of IciA protein; lanes 11 and 19 have no DnaA or IciA protein; and lane 12 represents 10 bp marker. Arrows on the left correspond to extension products becomes sensitive to KMnO4 modification (the reactive pyrimidines are indicated by arrow). (G) KMnO4 reactive pyrimidines within the A+T rich oriC of M. tb. Letters underlined represents various primers. Amplification products obtained by primer SeqOriR1 are marked by [ ] brackets. “c” bracket represents start of primer extension product and “d” bracket represent end of the primer extension product. It could be noted that primer extension stops at T residue which is modified by KMnO4. The small letters “a” and “b” represent 200 nt and 199 nt band. Similarly the IciA protein; lanes 11 and 19 have no DnaA or IciA protein; and lane 12 represents 10 bp marker. Arrows on the left correspond to extension products of 79, 77, 76, 66, 65 and 63 nucleotides with primer SeqOriR3 and 113, 99 and 98 nucleotides with primer SeqOriR2. (F) The nucleotide sequence of the entire oriC region of M. tb. Letters underlined represents various primers. Amplification products obtained by primer SeqOriR1 are marked by [ ] brackets. “c” bracket represents start of primer extension product and “d” bracket represent end of the primer extension product. The modified T residue is indicated by an arrow. (G) KMnO4 reactive pyrimidines within the A+T rich oriC of M. tb. About 19 bp stretch of pUC_OriMtb becomes sensitive to KMnO4 modification (the reactive pyrimidines are indicated by arrow).

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IciA inhibits helix opening

IciA, in addition to other functions, is a known inhibitor of E. coli chromosome replication initiation in-vitro. M. tb ORF Rv1985c displays 35.8% sequence identity to iciA of E. coli. Analysis of secondary structure (data not shown) also demonstrated that both IciA of E. coli and the putative M. tb IciA (Rv1985c) could be possibly functionally similar. Therefore, we analyzed the inhibitory effect of M. tb iciA, if any, on open complex formation. Helix opening reaction was carried out in the presence of increasing concentrations of recombinant purified IciA protein. 200 nM of rDnaA protein was used as this amount was earlier observed to be the most efficient after which there was no further concentration effect. These mapping data, generated with different primers, are summarized in Figure 3F. To conclude, our results reveal that a 19 bp stretch of M. tb oriC becomes sensitive to KMnO4 (Figure 3G) thereby demonstrating, for the first time, that in M. tb the duplex opening occurs near position −500 to −518 (from start of dnaN gene) which lies within the A+T rich region.

ATPase activity is essential for open complex formation

Having mapped the nucleotides (within the oriC region of M. tb) involved in opening of the duplex DNA, we investigated the requirement of ATP hydrolysis and also whether other hydrolysable and poorly hydrolysable analogues of ATP could provide the necessary energy to drive this process. The E. coli DnaA protein has a very weak ATPase activity but the intrinsic ATPase activity of M. tb DnaA promotes rapid oligomerization of DnaA on oriC and both ATP binding and ATP hydrolysis are required for rapid oligomerization of DnaA on oriC [23]. We therefore carried out helix opening reaction with 5 mM of ATP, ADP and ATPγS (Lithium salt). After oxidation with 8 mM KMnO4 the primer extension products were fractionated as usual using 6% urea gel. Only when 5 mM ATP (Figure 4, lane 1), but not when ADP (lane 2) or ATPγS (lane 3) was used as energy donor could DnaA bring about helix opening as could be seen from the appearance of the expected 200/199 nucleotides primer extension product. These results while highlighting the difference between M. tb and other bacteria, directly support the role of ATP in helix opening, which is a prerequisite for replication initiation.

IciA inhibits DNA replication

Having shown the ability of rIciA to inhibit helix opening in-vitro, experiments were designed to assess the ability of rIciA to actually inhibit DNA replication by using a reconstituted replication system. M. bovis BCG fraction II which supports in-vitro replication of DNA from M. bovis BCG actually inhibit DNA replication by using a reconstituted replication system. Fraction II (Figure 5A). Therefore this concentration of fraction II was included 10 min after incubation at 37°C, to allow open complex formation, it failed to inhibit helix opening (Figure S1). These results suggest that once the helix opening has been initiated by the binding of DnaA protein to oriC and the 13-mer region has been opened, IciA protein cannot block formation of the open complex thereby demonstrating that IciA protein can block open complex formation by possibly binding directly to the oriC sequences.

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chromosomal origin, The initiator protein, DnaA, is central for bacterial replication from putative inhibitor of replication (IciA), involved in DNA replication.

In the presence of 0.6 μg of rIciA protein only 10% replication activity could be seen. These results directly point to the ability of rIciA to act as an inhibitor of DNA replication.

IciA binds to A+T rich region of M.tb oriC

The results presented so far clearly suggest that rIciA is able to block helix opening (Figure 3D and E) and consequent DNA replication (Figure 5) only when it encounters the oriC sequence before DnaA protein has initiated helix opening thereby pointing to a possible ori specific DNA binding activity of rIciA protein. M.tb oriC is located within a small patch of A+T rich sequence which was earlier mapped as the site for helix opening (Figure 3A, B and C). Having identified the nucleotides (Figure 3F) involved in in-vitro helix opening, oligonucleotides corresponding to this region were used to determine DNA-protein interaction involving IciA. Electrophoretic mobility shift assays were carried out using this A+T rich oriC element, rIciA and huge excess (1 μg) of poly (dI/ dC). Results clearly show that IciA protein binds to A+T rich region (Figure 6, lanes 2–4). That this binding is specific is clearly evident from homologous and heterologous cold competition assays. Even in 100-fold molar excess of non-specific competitor DNA, the DNA-protein complex is not abrogated (Figure 6, lane 5); whereas the DNA-protein complex completely disappears in the presence of 50 fold (lane 6) and 100 fold (lane 7) molar excess of specific homologous cold competitor DNA. These results demonstrate that IciA specifically binds to A+T rich region of the oriC and the inhibitory effect of IciA on the DNA helix opening (Figure 3D and E) and DNA replication (Figure 5) is a likely consequence of this oriC/IciA interaction.

Discussion

Regulation of DNA replication is a very critical process mediating a switch between active and latent phase of M.tbc. In the present study we focused on two critical proteins, the initiator (DnaA) and a putative inhibitor of replication (IciA), involved in DNA replication. The initiator protein, DnaA, is central for bacterial replication from chromosomal origin, oriC. In E. coli, initiation of replication starts when DnaA specifically recognizes nine base pair consensus sequence, termed DnaA box within the oriC region. E. coli has five such DnaA boxes in the oriC region, but M.tbc oriC region has 13 such DnaA boxes. Also the oriC of M.tbc lacks a distinct A+T rich repeats and the binding of DnaA to all 13 DnaA boxes is not simultaneous. It has been proposed that DnaA first binds to a few high affinity DnaA boxes followed by binding to low affinity DnaA boxes to form a productive DnaA oriC initiation complex [23]. This oligomerization results in a local unwinding of the DNA double helix at −500 and −518 relative to start of dnaA gene.

Earlier studies used P1 nuclease for mapping helix opening of a supercoiled plasmid [1,14] or KMnO₄ probing for distorted B form of DNA [26,27]. We have used potassium permanganate (KMnO₄) probing assay to monitor in-vitro opening of the DNA helix. Using KMnO₄ probing assay we were able to determine the locus/site of opening of the double helix in M.tbc oriC. Our helix unwinding assays.
IciA Inhibits M. tb Replication

Figure 6. rIciA binds specifically to the A+T oligonucleotide derived from the oriC region of M. tuberculosis. Increasing amounts of IciA protein was used in electrophoretic mobility shift assays. The different lanes are: lane 1, 0 ng; lane 2, 250 ng; lane 3, 500 ng; lane 4, 750 ng; lane 5, 100 × cold non specific competitor; lane 6, 50 × and lane 7 100 × of cold homologous competitors. Specific DNA protein complex is indicated by an arrow. The sequence of the oligonucleotide used is given below the gel.

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reveal that DnaA mediated helix melting occurs just adjacent to a stretch of A residues within the 19 bp core of the oriC.

E. coli oriC also carries I sites, which are specific for DnaA bound to ATP. M. tb oriC however lacks such sites [23] and the orthologues/analogues of E. coli Hda, which stimulate intrinsic ATPase activity of the DnaA are also absent [28]. IHF (integration host factor) and Fis proteins which are involved in DNA bending are absent in M. tb [28]. E. coli has two histone like genes; hax and hufβ, whereas M. tb and M. leprae have only one hu gene denoted as hupB. The M. leprae HU protein has been shown to be associated with adhesion to Schwann cells. These arguably point to the differences in the regulation of replication in M. tb from E. coli. Our results indeed show that only the ATP bound form of DnaA is active for helix unwinding in M. tb which contrasts that observed in E. coli where dATP and the non hydrolysable analog of ATP, ATPγS as well as CTP can substitute for ATP in open complex formation, but not UTP, GTP, dTTP and dCTP [1]. Unlike in E. coli, where ATP functions allosterically [10], in M. tb ATPase activity is also required. That ATP is critical for helix opening in M. tb is further supported by the observation that mutants defective in ATP hydrolysis were not viable [23]. Mutants which can bind ATP, but are unable to hydrolyze, are functionally similar to a situation of DnaA binding to ATPγS.

DnaA – ATP in E. coli is negatively regulated by Hda protein, by a process called RIDA (Regulatory Inactivation of DnaA). Hda and the β sliding clamp subunit (β clamp) of the DNA polymerase promotes hydrolysis of ATP bound to DnaA and thus inactivate DnaA [29]. Another mechanism of regulation of initiation involves the binding of many DnaA molecules to a chromosomal locus, datA, thereby reducing the number of DnaA molecules accessible to oriC [30,31]. Both of these mechanisms perhaps do not operate in M. tb, as both hda gene and datA locus are absent. Therefore, the intrinsic ATPase activity of DnaA of M. tb may be critical in regulating replication in their absence.

The putatively identified M. tb IciA, coded by ORF Rv1985c, inhibits helix opening as seen from KMnO4 probing experiments. By binding specifically to A+T region, as evident from EMSA (Figure 7), rIciA inhibits interaction between DnaA protein at the A+T rich region within the oriC – a process critical for helix opening in a manner similar to that seen in E. coli [14,29]. Binding of rIciA consequently also inhibits in-vitro plasmid replication (Figure 5). DNA replication in-vitro using M. bovis BCG fraction II represents an authentic in-vitro enzyme system for studying replication involving M. tb origin. That rIciA is able to inhibit in-vitro DNA replication in this reconstituted system (Figure 5) clearly points to novel and an important role of IciA in inhibiting M. tb replication.

E. coli iclA null mutants are known to be completely viable and have the same growth rate as of wild type [12]. IciA is therefore not considered as a general replication inhibitor, but is thought to act under certain specific growth conditions. In E. coli, only limited sets of growth conditions have been evaluated and IciA and several other replication origin binding proteins may act as a replication inhibitor during nutrient starvation or during sudden changes in growth rate [15]. M. tb is known to survive for extended periods during the latency phase without any replication. During this phase bacteria sense the surrounding environmental conditions and iclA may have a role in maintaining mycobacterial latency. That IciA may have a role in M. tb latency is indirectly supported by results from E. coli where the concentration of IciA protein...
Increases 4-fold (400 dimers per cell) as cells approach stationary phase [14] and cells which have elevated levels of IciA protein exhibit a growth lag upon transfer to fresh medium [12]. Based on our results we propose a working model for helix inhibition by IciA. The supercoiled template, having A+T rich region and 13 DnaA boxes, in the presence of DnaA protein and ATP binds to these DnaA boxes and causes rapid oligomerization of the supercoiled DNA. This interaction is favored by DNA bending proteins like HU. This is followed by the generation of open complex formation (Figure 7, upper half), so that other components of DNA replication can easily be loaded. Nearly about 19 nucleotides of the oriC region are unwound by DnaA alone, which can easily be detected by KMnO4 sensitivity of this region. The end product of this series of DNA protein interactions during M.tb chromosomal DNA replication signals the advent of the bacterial activation process. In contrast, during dormancy the IciA protein binds to the A+T rich region of the oriC (Figure 7, lower half) and this binding of IciA blocks DnaA dependent helix opening of the A+T rich region, a step critical for chromosomal initiation to occur. Consequently chromosomal DNA replication remains arrested so that M.tb can stay in a dormant state. It is therefore tempting to suggest that IciA could be one of the factor(s) involved in maintaining the latent state of growth of M.tb. Direct evidence for such a role of IciA will come from M.tb iciA knockouts in an infection model and also studies monitoring the steady expression level of M.tb IciA during latency and activation phase, in a clinical setting. While these experiments are underway, we are also investigating the quantitative expression of IciA as a molecular marker for M.tb activation.

Materials and Methods

Molecular cloning

The M. tuberculosis ORF Rv1985c and Rv0001 coding for putative IciA protein and DnaA protein respectively, were PCR

Figure 7. Schematic representation of the mechanism of helix opening by DnaA at oriC and its inhibition by IciA (modified from the model proposed by Bramhill and Kornberg [37] and Madiraju et al [23]). The ATP bound form of replication initiator DnaA protein binds to 13 DnaA boxes (darkly shaded arrow heads pointing the orientation of DnaA boxes) in M.tb oriC located between the dnaA and dnaN gene. Binding of DnaA-ATP complex to the DnaA boxes results in rapid oligomerization leading to the formation of the initiation complex (clockwise direction). Subsequently, the initiation complex gradually opens at A+T rich region which is then acted upon by a host of replication factors which finally lead to DNA replication. When IciA is present before the formation of open complex then it follows another pathway (anticlockwise direction). Here IciA protein binds to the A+T rich region of the oriC. In the presence of the DnaA protein the initiation complex is still formed however it eventually does not lead to the formation of open complex.

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amplified using genomic DNA from H37Rv and primers IciAF, IciAR, DnaAF and DnaAR, carrying specific restriction enzyme sites (Table 1), by Accuqau DNA polymerase (Sigma). The amplicons thus generated were digested with Ndel/HindIII restriction enzymes and cloned into the corresponding sites of pET28a expression vector. The resultant plasmids were labeled as pETIciA and pETDnaA. For cloning intergenic region between pET28a expression vector. The resultant plasmids were labeled as pETIciA and pETDnaA. For cloning intergenic region between pET28a expression vector. The resultant plasmids were labeled as pUC_OriMtb. The authenticity of all constructs was confirmed by restriction analysis and DNA sequencing.

Purification of recombinant His tagged IciA protein

Recombinant putative IciA, coded by M. tb ORF Rv1985c, was purified from the soluble fraction of BL21 (DE3) pLysS cells transformed with pETIciA grown overnight at 18°C and induced with 0.5 mM IPTG at an OD600 of 0.3 for the expression of recombinant protein as described earlier [32,33]. The recombinant protein was purified in buffer containing 20 mM Tris, 300 mM NaCl and 10% glycerol. The purity of the protein was confirmed by SDS PAGE. The concentration of the protein was estimated by BCA (Bichinconic acid) and the purified protein was stored at −20°C until further use.

Purification of recombinant His tagged DnaA protein

Recombinant DnaA protein was purified as described earlier [25] with minor modifications. To prevent the recombinant protein from getting complexed with ATP present in E. coli cytoplasm, which could interfere in the helix unwinding assays, the protein was denatured in buffer A [25 mM Tris acetate (pH 7.5), 250 mM NaCl, 0.1 mM EDTA, 10 mM Magnesium acetate and 10 mM β-mercaptoethanol] containing 8 M urea [23]. This was followed by sequential dialysis in 4 M, 2 M, 1 M and 0.5 M urea in buffer A containing 10% glycerol. The final dialysis buffer A contained 20% glycerol. The refolded DnaA protein, as seen on 10% SDS PAGE, was >95% pure. The protein concentration was estimated by BCA and stored at −20°C until further use.

Preparation of fraction II

In-vitro replication competent fraction II was prepared by growing M. bovis BCG Pasteur in 600 ml of 7H9 media supplemented with OADC and casitone, in 1000 ml roller bottle at 37°C to log phase as described previously [34]. It took around 6–7 days for the cells to reach log phase from 1% primary inoculum. The cells were then harvested and resuspended in buffer B [25 mM, HEPES/KOH (pH 7.6), 0.1 mM EDTA, 2 mM DTT, and 100 mM potassium glutamate] supplemented with 1 mM PMSF. The cells were disrupted by sonication and the supernatant (fraction I) was precipitated by addition of ammonium sulphate cutoff. After an additional 30 min of stirring, the suspension was centrifuged at 4°C for 30 min at 18 000 g. The pellet was resuspended in minimal volume of buffer B (fraction II) of around 600 µl and dialyzed for 50 min at 4°C against 1000 fold excess of buffer B. Protein concentration was estimated by BCA and the replication competent fraction was flash frozen in small aliquots, so as to avoid freeze thaw, and stored at −70°C until further use. Each aliquot was used only once, after subsequent thawing the left over aliquot was discarded.

ATPase activity

Reaction samples were kept on ice in 10 µl of buffer C [50 mM HEPES/KOH (pH 7.6), 0.5 mM Magnesium acetate, 2 mM DTT and 50 mM NaCl] containing 16 mM [γ-32P]ATP and increasing amounts of DnaA protein as mentioned in figure legends. After incubating the samples for 30 min at 0°C, linear DNA carrying the DnaA box or pUC_OriMtb or pBSK II was added and the reactions were further continued at 37°C for 30 min. After this ATPase activity was determined by spotting 1.0 µl aliquot of each sample on Silica gel 60F254 thin layer chromatography plate (TLC). TLC plate was developed with chloroform: methanol: glacial acetic acid (65:15:5, v/v/v), followed by autoradiography and analyzing the image by Typhoon Variable Mode Imager and Image Quant software.

Helix opening assay and KMnO4 probing

The standard helix opening assay [25 µl] was carried out in a buffer containing 40 mM HEPES-KOH (pH 7.5), 8 mM Magnesium acetate, 50 mM potassium glutamate, 1 µg poly dI/dC, 30% v/v glycerol, 320 µg/ml BSA and 550 ng supercoiled template [pUC_OriMtb], with indicated amounts of DnaA and IciA (Rv1985) protein and 5.0 mM of either ATP or ADP or ATP8S (Lithium salt). The reaction mix was incubated for 30 min on ice followed by 20 min at 37°C. KmO4 was then added to a final concentration of 10 mM, and the reaction was further continued for 2 min at 37°C. The reaction was stopped by the addition of stop buffer (1.75 M β mercaptoethanol and 50 mM EDTA) and samples were transferred to ice. 40 µl of phenol was then added and the samples were vortexed and centrifuged at 6000 rpm for 5 min. The supernatant was then passed through SephadexG50 spin column to purify the DNA template for use in primer extension reaction.

Primer extension

10 µl of the primer extension mix included 200 µM each dNTPs, 0.04 µM 32P end labeled primer [SeqOriR1, SeqOriR2 or SeqOriR3 (Table 1)] 0.5 mM MgCl2, 2% DMSO and 0.5 Units Taq DNA polymerase (SIGNAL). The mixture was subjected to primer extension (SeqOriR1) in a thermocycler for 30 cycles: 94°C for 1 min, 92°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min except for 5 min in the last amplification cycle. All the conditions for primers SeqOriR2 and SeqOriR3 were identical, except that annealing was carried out at 48°C and amplification at 72°C for 40 sec. The reactions were stopped by adding 2 µl of formamide sequencing dyc (95% Formamide, 10 mM NaOH, 0.05% Bromophenol blue and 0.05%Xylene Cyanol FF). The samples were heat denatured for 5 min at 95°C and subjected to 6% (or 15%) polyacrylamide gel electrophoresis containing 7 M urea. The gels were dried and analyzed by Typhoon Variable Mode Imager and Image Quant software.

Assay for DNA replication

The standard reaction (20 µl), as described earlier [34], contained 40 mM HEPES-KOH (pH 7.6), 6 mM ATP, 500 µM of each GTP, CTP and UTP, 21.6 mM Creatine phosphate (Fluka), 50 µg/ml BSA, 100 µM each of dGTP, dCTP and dTTP, 50 µM dATP, 200 cpm/molar of total deoxynucleotide [z 32P]dATP, 11 mM Magnesium acetate, 35 µg Creatine Kinase (Sigma), 550 ng supercoiled plasmid DNA/pUC_OriMtb and 7% PEG 10,000. All reactions were assembled on ice and started by the addition of 10–80 µg of protein (Fraction II or rIciA or both as indicated in figure legends) and incubating at 30°C for 30 min. Total nucleotide incorporation was measured by determining
radioactivity retained after 10% trichloroacetic acid precipitation on nylon membrane through dot blot apparatus [BioRad]. All the reactions were quantitated by Typhoon Variable Mode Imager and Image Quant software.

**Electrophoretic mobility shift assays**

For electrophoretic mobility shift assays, synthetic complementary oligodeoxyribonucleotides OriF1 and OriR1 (Table 1) were annealed and 5’ end labeled using T4 Polynucleotide Kinase as described earlier [35,36]. The 32P-labelled oligonucleotides were incubated with increasing concentration of IciA protein, at 30°C in binding buffer D [10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 µg/ml BSA, 1 µg poly dl/cC and 20% glycerol] for 30 min and the DNA-protein complex was fractionated on 3% native PAGE [0.25 mM Tris/borate/0.25 mM EDTA] at 150 V, 4°C for 2–3 hrs. The gels were dried and analyzed by Typhoon Variable Mode Imager and Image Quant software.

**Supporting Information**

Figure S1 IciA cannot inhibit helix unwinding once DnaA has already opened the DNA double helix. The reaction was carried out using 0.2 µg of DnaA protein. Helix opening was monitored by primer extension using SeqOriR1 on a 6% sequencing gel. The different lanes are: lane 3, With DnaA but without rIciA; lane 4: With DnaA and IciA (0.4 µg), but IciA was added after 10 min the addition of DnaA at 37°C. Arrows correspond to the extension products of 200 and 199 nucleotides. Lane 1 and 2 show 50 bp and 10 bp marker.

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**Author Contributions**

Conceived and designed the experiments: SK SEH. Performed the experiments: SK AF. Analyzed the data: SK SEH. Contributed reagents/materials/analysis tools: SEH. Wrote the paper: SK SEH.

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