Deletion of the Gene \textit{rpoZ}, Encoding the \( \omega \) Subunit of RNA Polymerase, in \textit{Mycobacterium smegmatis} Results in Fragmentation of the \( \beta' \) Subunit in the Enzyme Assembly

Renjith Mathew,\(^1\) Madhugiri Ramakanth,\(^2\) and Dipankar Chatterji\(^1\)*

\textbf{Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India;\(^1\)} and Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India\(^2\)

Received 14 April 2005/Accepted 21 June 2005

A deletion mutation in the gene \textit{rpoZ} of \textit{Mycobacterium smegmatis} causes reduced growth rate and a change in colony morphology. During purification of RNA polymerase from the mutant strain, the \( \beta' \) subunit undergoes fragmentation but the fragments remain associated with the enzyme and maintain it in an active state until the whole destabilized assembly breaks down in the final step of purification. Complementation of the mutant strain with an integrated copy of the wild-type \textit{rpoZ} brings back the wild-type colony morphology and improves the growth rate and activity of the enzyme, and the integrity of the \( \beta' \) subunit remains unaffected.

DNA-dependent RNA polymerase (RNAP) is the central enzyme involved in gene expression and also constitutes a major target for genetic regulation (7, 8, 31). The bacterial RNAP core enzyme consists of four subunits: alpha (\( \alpha \)), beta (\( \beta \)), beta’ (\( \beta' \)) and omega (\( \omega \)) (21, 36). The \( \omega \) subunit is the least well studied among all the subunits, though the subunit encoded by the \textit{rpoZ} gene was proposed to be an integral part of the core RNAP several years ago (5, 10). In \textit{Escherichia coli} \( \omega \) is not found to be necessary for survival of the bacterium under laboratory conditions (12). At the same time \( \omega \) homologues are present in the sequenced genomes of free-living bacteria, suggesting an important and conserved role for the protein (23).

It was identified in our laboratory that \( \omega \) is required for the restoration of denatured core RNAP to its functionally active form (25). Further, we showed that the enzyme purified from an \textit{E. coli} strain lacking \( \omega \) recruits large amounts of GroEL (26) and removal of GroEL results in a completely inactive core RNAP which lacks the ability to even associate with \( \beta' \) (24). Subsequently, it was demonstrated that \( \omega \) binds to the \( \beta' \) subunit and promotes RNAP assembly by facilitating the association of \( \beta' \) with the previous step of the assembly, \( \alpha_2\beta \) (13).

The X-ray crystal structure of \textit{Thermus aquaticus} RNAP determined at 3.3 Å resolution (36) and subsequent analysis of the \( \omega-\beta' \) interface by Minakhin et al. (23) identified the conserved regions of \( \beta' \) which \( \omega \) binds with in a manner that reduces the configurational entropy of \( \beta' \) and facilitates its interaction with the \( \alpha_2\beta \) subassembly. Further experiments in our laboratory showed that the C-terminal tail of the \( \omega \) subunit is constrained in the presence of \( \beta' \) (14).

In spite of this well established evidence, a number of recent observations about the \( \omega \) subunit in different organisms warrant suspecting functional roles for this protein which are not clearly elucidated yet. In \textit{Streptomyces kasugaensis}, it was observed that a mutation in the gene encoding the \( \omega \) subunit resulted in characteristic pleiotropic effects (19). The Kranz laboratory recently demonstrated that presence of \( \omega \) was a prerequisite to obtain an active in vitro assembly of \textit{Rhodobacter capsulatus} RNAP (30). In a completely different scenario, Periago et al. (29) demonstrated induction of YloH, the \( \omega \) subunit of \textit{Bacillus cereus}, by heat stress, suggesting a role for the subunit in stress adaptation of the transcription machinery.

In the present work we have tried to look into the role of \( \omega \) in \textit{Mycobacterium smegmatis}, which is used as a model organism to investigate basic mycobacterial biology. The gene \textit{rpoZ}, encoding the \( \omega \) subunit in \textit{M. smegmatis}, was identified by comparing the \textit{Mycobacterium tuberculosis} \textit{rpoZ} sequence against the \textit{M. smegmatis} genomic sequence, which was available as contigs at the TIGR website (http://www.tigr.org/). \textit{M. smegmatis} \( \omega \) has 79% and 75% identity with the \textit{M. tuberculosis} and the \textit{Mycobacterium leprae} proteins, respectively.

\textbf{Nucleotide sequence accession number.} The nucleic acid sequence of \textit{M. smegmatis} \textit{rpoZ} has been deposited in GenBank with accession number AY973203.

\textbf{Targeted mutagenesis of \textit{rpoZ} in \textit{M. smegmatis} mc\textsuperscript{2}155.} A recombinant cassette was constructed to delete \textit{rpoZ} from the \textit{M. smegmatis} mc\textsuperscript{2}155 chromosome (Table 1). It consisted of a 953-bp DNA fragment spanning from the 914th base upstream to \textit{rpoZ} to the 39th base downstream of \textit{rpoZ} and a downstream fragment of DNA from the 216th base of \textit{rpoZ} to the 964th base downstream to it with the EcoRI fragment holding the \textit{aph} gene from vector pUC4K between them. After the preparative cloning steps this whole recombinant cassette was transferred to the suicide vector pPR27 (28) to get the final construct, pOKOI. mc\textsuperscript{2}155 was transformed with pOKOI. The \textit{ sacB} mutant gentamicin-susceptible and kanamycin-resistant colonies were selected for further analysis. Disruption of \textit{rpoZ} was verified by Southern hybridization as well as PCR in one of

* Corresponding author. Mailing address: Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India, Phone: 91-80-22932836. Fax: 91-80-23600535. E-mail: dipankar@mbu.isc.ernet.in.
the selected colonies (data not shown) and this strain, mcdrz, was used in further studies.

**mcdrz grows more slowly than mc$^2$155 and possesses a different colony morphology.** The mutant was found to grow at a lower rate than the wild-type strain in Middlebrook 7H9 (MB7H9) broth supplemented with 2% glucose and 0.05% Tween 80 (Fig. 1A). A slow growth phenotype has also been observed for the *E. coli rpoZ* mutant (26) although it has been suspected to be due to a polar effect on the downstream gene *spoT* (12). Here it must be mentioned that mycobacteria do not have *spoT*, as a single gene encodes a bifunctional protein, Rel, which carries out the role of RelA as well as SpoT (1). The appearance of individual colonies grown on MB7H9 agar also varied between the wild type and the mutant, as shown in Fig. 1B. Wild-type bacteria (Fig. 1B, panel 1) formed colonies characteristic of mc$^2$155, with a relatively flat surface and irregular edges. The mutant colonies (Fig. 1B, panel 2) were found to grow to smaller diameters with a drier surface, and viewed from the sides, they appeared as elevated humps. In liquid culture the mutant cells aggregated considerably more than the wild-type cells, even in the presence of 0.05% Tween 80 (data not shown).

**Complementation of mcdrz with the wild-type rpoZ gene.** To ascertain whether the *rpoZ* deletion was responsible for the observed phenotypes, the mc$^2$155 *rpoZ* was amplified from genomic DNA and cloned in pET21b (pETOsm). A transcriptional fusion of the *hsp60* promoter of pMV261 (33) and *rpoZ* along with its translational signals from pET21b was generated (pMOsm). *oriM* was removed from pMOsm and the backbone was ligated with the integrating signal of mycobacteriophage L5 from pDK20 (9). Since mcdrz carried a kanamycin resistance marker in its genome, the hygromycin resistance cassette from plasmid pSDHy was introduced into pMOsm, resulting in phMOsm. mcdrz was transformed with phMOsm and colonies (mcdrzco) were selected in the presence of hygromycin as well as kanamycin. Adequate expression of ω protein was observed during growth of the culture at 37°C by Western blotting of the cell lysate with anti-*M. smegmatis* ω antibodies raised in rabbits. The growth rate of the complemented strain improved and the growth curve followed the wild-type one quite closely (Fig. 1A, mcdrzco), and the mcdrzco colonies had an appearance similar to the wild-type colonies (Fig. 1B, panel 3).

**Intermediate steps of purification of RNA polymerase from mcdrz show reduced transcription activity.** RNA polymerase was purified from *M. smegmatis* strains by a modification of the protocol described by Kumar and Chatterji (20) and transcription activities of the various steps of purification were checked by the nonspecific transcription assay as described by Lowe et al. (22). In brief, the enzyme purification involved precipitating the lysate (Fig. 2, lanes A) with polymin P, following which the proteins were extracted from the pellet by salt and loaded onto Bio-Gel A-1.5m (Fig. 2, lanes B). The active fractions from Bio-Gel were loaded onto heparin-Sepharose (Fig. 2, lanes C), the unsound fraction of proteins was collected (Fig. 2, lanes D) and the bound proteins were eluted with high salt (Fig. 2, lanes E). It was observed that the various steps of purification of the mutant polymerase except the final chromatography eluate were active, but there was a complete breakdown of transcription activity over the final chromatography with heparin-Sepharose. However, the activities of the intermediate steps were considerably less than the corresponding wild-type fractions in all cases except the lysate. The actual specific activity values showed a variation of nearly 30% between preparations, but the values of the intermediary steps of the knockout preparation always remained within 30 to 50% of the corresponding wild-type values (Fig. 2).

**Purification steps of RNA polymerase from mcdrz show lower amounts of associated full-length β′ and increased susceptibility of β′ subunit to proteolytic cleavage.** While the RNAP from the wild type behaved as expected with accumulation of β′ subunit in the course of purification (Fig. 3A), the amount of β′ associated with the knockout polymerase was found to be considerably low from the step of Polymin P pellet extraction (Fig. 3B). Western blotting of the steps of purification of polymerase from the wild type as well as mutant mycobacteria was carried out using antibodies raised against the *M. tuberculosis* β′ subunit in rabbit (Fig. 3C and D, respectively). Purified *M. tuberculosis* β′ protein was immobilized on normal human serum-Sepharose matrix (Pharmacia Biotech) which was used for

### TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tr>
<td><em>M. smegmatis</em></td>
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<td></td>
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<tr>
<td>mc$^2$155</td>
<td>Parental strain</td>
<td>This work</td>
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<tr>
<td>mcdrz</td>
<td>rpoZ mutant</td>
<td>This work</td>
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<tr>
<td>mcdrzco</td>
<td>mcdrz complemented with <em>M. smegmatis rpoZ</em></td>
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<tr>
<th>Plasmids</th>
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<tbody>
<tr>
<td>pUC4K</td>
<td>Source of <em>aph</em> gene</td>
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<tr>
<td>pET21b</td>
<td>Cloning vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pPR27</td>
<td>Suicide vector</td>
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<tr>
<td>pMV261</td>
<td>Cloning vector for <em>hsp60</em> promoter</td>
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<td>pDK20</td>
<td>Source of integration signal of mycobacteriophage L5</td>
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<tr>
<td>pSDHy</td>
<td>Cloning vector for hygromycin cassette release</td>
<td>Our laboratory</td>
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<tr>
<td>pOKO1</td>
<td>Construct for knocking out <em>M. smegmatis rpoZ</em></td>
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</tr>
<tr>
<td>pETOsm</td>
<td>Intermediate cloning step for complementation construct</td>
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<td>pMOsm</td>
<td>Intermediate cloning step for complementation construct</td>
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<tr>
<td>phMOsm</td>
<td>Complementation construct for <em>M. smegmatis rpoZ</em></td>
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FIG. 1. A. Comparison of growth rates of mc<sup>2</sup>155, mcdrz, and mcdrzco in Middlebrook 7H<sub>9</sub> broth supplemented with 2% glucose and 0.05% Tween 80. B. Effect of ω deletion on the appearance of <i>M. smegmatis</i> colonies. mc<sup>2</sup>155 (panel 1), mcdrz (panel 2), and mcdrzco (panel 3) colonies are shown. Colonies were grown on MB7H<sub>9</sub> agar for 18 days. Bars = 5 mm in all cases.
isolating mono-specific antibodies against the \( \beta' \) subunit from the polyclonal rabbit serum. The Western blots showed an increased propensity of the \( \beta' \) subunit to get fragmented in the case of the mutant RNAP. It can be seen that the full-length \( \beta' \) subunit is visible only in the lysate stage, with some lower bands. The full-length band is not apparent in the subsequent steps. Interestingly the fragments are of discrete size, and remain the same throughout the purification until the final step. The higher-molecular-mass fragment ran between 60 kDa and 70 kDa in an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the two fragments of lower molecular masses had mobilities between 40 and 50 kDa. The \( M. smegmatis \) \( \beta' \) subunit being a protein of 146.5 kDa, we do not expect any major cleavage product to be missed in our experiments. These fragments were absent in the final heparin-Sepharose eluate with the bands appearing to come out completely with the unbound fraction of proteins (Fig. 3, panel D, lane 6). On the other hand, the full-length \( \beta' \) band remains intact in the wild type.

Following this observation we tried reconstituting the inactive mcdrz heparin-Sepharose eluate (Fig. 2, lanes E, mcdrz, and Fig. 3D, lane 7) with the unbound fraction of proteins from the same column (Fig. 2, lanes D, mcdrz, and Fig. 3D, lane 6) according to the protocol described by Igarashi and Ishihama for reconstitution of the RNAP (16). Briefly, 500 \( \mu \)g of mcdrz heparin-Sepharose eluate was denatured by dialysis against denaturation buffer. An aliquot of 25 \( \mu \)g was removed and the rest of the enzyme was divided into aliquots of 25 \( \mu \)g each. To these portions, different quantities of the unbound fraction were added. After being kept on ice for 30 min, dialysis was carried out at 4°C against reconstitution buffer. After 150 min of dialysis, multiple-round transcription assays using calf thymus DNA as template were carried out as described by Lowe et al. (22). As controls the 25-\( \mu \)g aliquot of the eluate as well as the unbound fraction in quantities identical to those used in the assay were separately given the same treatments.

It was seen that the heparin-Sepharose unbound fraction, when mixed with the inactive heparin-Sepharose eluate, could bring about an increase in transcription activity in the reconstituted mixture that was considerably more than the sum of the activities of the individual components (Table 2). The values shown here are representative of a set of experiments done with 25 \( \mu \)g of the eluate and 60 \( \mu \)g of the unbound fraction from the same RNAP preparation. Similar reconstitution of the mcdrz eluate was attempted with the unbound fraction from the heparin-Sepharose step of the wild-type RNAP preparation as well, but it did not result in any increase in activity (data not shown).

**RNAP purified from mcdrzco appears similar to the wild-type enzyme.** Purification of RNAP from the complemented strain was carried out following the protocol already explained.
It can be seen from Fig. 4A that the full-length β′ progressively gets enriched as a function of purification of RNAP from mcdrzo, with no loss of β′ by fragmentation (lanes 1 to 7). Lane 8 contains the control wild-type mc²155 RNAP. We also noted that the α subunit remained intact during the RNAP purification from all three strains, with the final heparin-Sepharose eluates having comparable amounts of the α subunit associated, as can be seen from Fig. 4B.

Even though the transcription assay profile (Fig. 2, mcdrzo) showed a recovery of the RNAP activity upon rpoZ integration in strain mcdrzo in comparison to the null strain mcdrz, the RNAP activity remained significantly less than that of the wild type. We reasoned that since the purification of RNAP from the complemented strain was never as clean as that from the wild-type strain (data not shown), the specific activity values, which we have plotted in Fig. 2, go quite off the mark. Taking this into account, we measured the amount of β′ present in the final eluate of mc²155 and mcdrzo (Fig. 3C, lane 7 and Fig. 4A, lane 7, respectively) with quantitative Western blots using enhanced chemiluminescence. The specific activities were normalized for the intensity of the β′ band present in the same amounts of both the proteins and the wild-type value was plotted as 100%. Upon doing this, the mcdrzo enzyme activity value rose to 76.42% of the wild-type value (data not shown).

Our experiments elucidate the key role played by ω in the assembly and structural stability of the M. smegmatis RNAP. In addition to binding and helping the assembly process, the ω subunit also seems to be physically protecting the β′ subunit in the case we have studied. We believe that in the absence of ω, the newly exposed regions of β′ form preferential sites for proteases present in the system. The mutant RNAP retains activity during intermediate steps of purification, in spite of considerably reduced amounts of associated full-length β′. Core peptides within multisubunit proteins are believed to have mosaic structures with separate functional domains, each being constituted by noncontiguous segments in the primary structure (27). Proteolytic or recombinant fragments of a number of enzymes as well as enzyme subunits are known to reassociate in vitro to reconstitute activity (3, 4, 6, 11, 18, 34, 35). This has been shown clearly in the case of the β subunit of E. coli RNAP (32). The β′ subunit of E. coli RNAP also has been shown to possess such mosaic architecture (17). The β′ equivalents of chloroplasts and some archaeabacteria are split into two polypeptides (2). Sequence analyses show that the rpoC genes encoding the β′ subunit homologues contain long evolutionarily nonconserved regions (15). Generally split sites that allow functional assembly of the enzyme occur in regions with poorly conserved sequence homology among homologues.

We find it tempting to hypothesize that the ω subunit in M. smegmatis, while binding to β′, also protects such a region or regions in the β′ sequence which in the absence of ω get exposed to proteolytic cleavage. ω has been shown to bind to regions of β′ which are spread far apart over its primary sequence (13, 23). The split domains remain bound to the rest of the enzyme and are able to carry out transcription, albeit at a reduced level of activity. But the cleavage probably results in physical separation of different functional domains and hence in structural destabilization of the enzyme. This provokes a breakdown of the enzyme during affinity purification over heparin-Sepharose. The loss of the smaller fragments of β′ with the unbound fraction of proteins from heparin explains the lack of activity of the eluate. These conclusions are corroborated by reconstitution of the transcription activity by mixing and reconstituting the inactive heparin-Sepharose eluate with the unbound fraction from the column. The question that still remains is how an RNAP that is compromised by the absence of ω carries out transcription adequately within the cell.

In conclusion, our results show that the role of ω in folding the β′ subunit not only aids the enzyme assembly, but also protects the larger subunit from degradation. The phenotypes that we observe for the mutant also suggest other possible functional roles for ω in M. smegmatis, a question that we are pursuing further.

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<tr>
<th>Eluate</th>
<th>Sp act (nmol [3H]UTP incorporated/mg of protein/h)</th>
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<tr>
<td>mcdrz heparin-Sepharose</td>
<td>0.599 ± 0.158</td>
</tr>
<tr>
<td>mcdrz heparin-Sepharose unbound fraction</td>
<td>0.72 ± 0.184</td>
</tr>
<tr>
<td>Mixture of bound and unbound mcdrz heparin-Sepharose</td>
<td>6.88 ± 0.401</td>
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* Reconstitution of polymerase activity by addition of mcdrz heparin-Sepharose unbound fraction to the eluate; 25 µg of the heparin-Sepharose eluate was reconstituted with 60 µg of the unbound fraction. Each value is the mean and standard deviation of three independent reconstitution assays done with the same protein fractions. Specific activity was calculated, taking into account the total protein present in the mixture.
REFERENCES
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Renjith Mathew, Madhugiri Ramakanth, and Dipankar Chatterji

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India, and Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India