Active Sites of the Multi-subunit RNA Polymerases of Eubacteria and Chloroplasts are Similar in Structure and Function

Peramachi Palanivelu*

Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai – 625 021, Tamil Nadu, India; ppmkupp@gmail.com

Abstract

Objectives: To analyze and compare the Multi-subunit (MSU) DNA dependent RNA Polymerases (RNAPs) of eubacteria and chloroplasts and to identify the extent of conservations between them with special reference to the active sites. Methods: The advanced version of Clustal Omega was used for protein sequence analysis of the MSU DNA dependent RNAPs from eubacteria and chloroplasts. The conserved motifs identified by the bioinformatics analysis and the biochemical and Site-directed Mutagenesis (SDM) experiments and X-ray crystallographic analysis data available on the eubacterial MSU RNAPs are used to derive and analyze active site regions of the MSU RNAPs of chloroplasts. Findings: Multiple Sequence Alignment (MSA) of RNAPs from both the sources showed many highly conserved motifs among them. The possible catalytic regions in the catalytic subunits β and β' of eubacteria and their counterparts, viz. β, β' and in chloroplasts RNAPs consist of an absolutely conserved catalytic amino acid R, in contrast to a K as reported for DNA polymerases and Single Subunit (SSU) RNAPs. Besides, the invariant ‘gatekeeper/DNA template binding’ YG pair is also found to be absolutely conserved in the MSU RNAPs of chloroplasts, as reported in SSU, MSU RNAPs and DNA polymerases. The eubacterial β, the initiation subunit, is highly homologous to β subunit of chloroplast MSU RNAPs, i.e., the eubacterial and chloroplast β subunits exhibit very similar active site motifs, catalytic regions and distance conservations between the template binding YG pair and the catalytic R. However, the bacterial β' elongation subunit is not completely similar to the β' elongation subunit of chloroplasts, but partly similar to the β' and β'' subunits of chloroplast RNAPs. Interestingly, MSA analysis shows that the active sites are shared between β' and β'' in the MSU RNAPs of chloroplasts, i.e., the metal binding site is found in the β' subunit whereas the catalytic regions are located in β'' subunit of chloroplast MSU RNAPs. Another interesting finding is, in the elongation subunits, i.e., in the eubacterial β' and the chloroplast β'' catalytic subunits, the proposed catalytic R is placed at double the distance, i.e., -16 amino acids downstream from the YG pair, in contrast to SSU RNAPs and DNA polymerases where the distance is only 8 amino acids downstream from the YG pair. An invariant Zn$^{2+}$ binding motif reported in the eubacterial elongation subunit, viz., β' is found in the β'' subunits of chloroplasts. Applications: Analysis of MSU RNAPs of chloroplasts assumes greater importance as it is the one that transcribes the foreign genes in chloroplast transformation experiments.

Keywords: Chloroplast Multi-subunit RNA Polymerases, Distance Conservations, Eubacterial RNA Polymerases, Metal Binding Regions, Multi-subunit RNA Polymerases, Plastid Encoded Polymerases, Polymerase Active Sites

1. Introduction

RNAPs (EC 2.7.7.6) are key enzymes, found in all living cells that perform the flow of genetic information from DNA → RNA. Thus, they play a vital role in the control of gene expression at the transcription level. Interestingly, the sequences, common structural framework and functions of these MSU DNA dependent RNA
polymerases are universally conserved in bacteria, fungi, plants and animals. Understanding the overall structure, mechanism and regulation of the RNAPs are the primary goal of molecular biologists since its discovery. Though RNAPs are found in all species across all three kingdoms, their number and composition vary across taxa. For example, viruses contain mainly two types of RNAPs, viz. DNA dependent RNAPs and RNA dependent RNAPs. Both eubacteria and archaebacteria contain a single type of RNAP of Multi-subunit type. In eubacteria, the MSU RNAP core enzyme is composed of 5 subunits, while eukaryotes contain at least five distinct types of RNAPs (I-V), which are also MSU enzymes but made up of 12-14 subunits. In spite of such differences, there are striking similarities among the transcriptional mechanisms by various types of RNAPs. Chloroplasts in higher plants use two different types of RNAPs, one is the nuclear-encoded SSU type and the other one is the chloroplast-encoded (PEP) MSU type. The MSU RNAPs of eubacteria and PEP are basically similar in structure and functions. Large volumes of protein sequence data are available for most of the prokaryotic and chloroplast MSU RNAPs. Understanding the biological principles buried in these sequencing data is a significant challenge for scientists. A great deal of information is available on the MSU RNAPs of eubacteria. However, data on the conserved motifs and active site analyses of the chloroplast MSU RNAPs are very limited. Analyses of chloroplast MSU RNAPs assume greater importance as it is the one that transcribes the foreign genes in chloroplast transformation experiments.

2. Types of DNA Dependent MSU RNAPs

There are at least 4 different types of MSU RNAPs in living cells, viz.

- DNA dependent MSU RNAP of eubacteria.
- DNA dependent MSU RNAP of archaebacteria.
- DNA dependent MSU RNAP of chloroplasts (Plastid encoded).
- DNA dependent MSU RNAPs of eukaryotes.

In my earlier communications, SSU RNAPs (viral types) and MSU RNAPs from eubacteria were analyzed in detail and reported. In this communication, MSU RNAPs of chloroplasts (prokaryotic type, plastid-encoded) are analyzed for their conserved motifs, active sites, metal binding regions. Based on MSA data and with the biochemical, SDM and X-ray crystallographic data available from their counterpart MSU enzyme from eubacteria, a consensus model for the initiation and elongation cycles are proposed.

3. MSU DNA dependent RNAPs of Chloroplasts

3.1 Structural Features of the PEP and its similarity with Eubacterial Enzyme

As the chloroplasts are known to have inherited the PEP from cyanobacteria by endosymbiosis about 1 x 10^9 years ago, it is also composed of the same core subunits, viz. α, β, β′ and β″. However, the rpoC gene coding for the β′ subunit in eubacteria is split into rpoC1 (β′) and rpoC2 (β″) in chloroplasts. Thus, the MSU enzyme from chloroplasts is very similar to the eubacterial counterparts, except having an additional subunit, i.e., β″ in its structure and hence the chloroplasts’ core enzyme is composed of α, β, β′, β″ and ω-subunits (Figure 1) and encoded by rpoA, rpoB, rpoC1, rpoC2 and rpoZ genes, respectively. When a σ subunit (nuclear-encoded) is associated with the core polymerase, the PEP holoenzyme is formed, which can initiate transcription at specific sites on the chloroplast DNA templates. Figure 1 shows a schematic diagram of a chloroplast MSU RNAP. MSA shows that the plastid-encoded MSU RNAP subunits α, β, β″ are homologous and functionally similar to the α, β and β′ subunits of eubacterial RNAPs, respectively.
3.2 PEP and NEP are Structurally and Functionally Different

Chloroplasts contain a circular, double-stranded genome of sizes ranging from 100 to 200 kb and harbour ~120 genes. (For example, the Zea mays chloroplast genome is of 140 kb in size and contains 70 protein-coding genes, 30 tRNAs and 4 rRNAs). Chloroplasts in higher plants use two different types of RNAPs to transcribe all its genes. One is the nuclear-encoded SSU RNAP (NEP), which is homologous and very similar to SSU viral and mitochondrial polymerases and the second one is the plastid-encoded MSU RNAP (PEP), which is structurally and functionally very similar to the eubacterial MSU RNAPs (this communication). The SSU RNAP, encoded by the nucleus is structurally unrelated to PEP and belongs to the “SSU RNAP” protein family which are similar to the RNAPs of bacteriophages T3, T7, SP6, etc. in structure and function. The NEP mainly involves in the transcription of non-photosynthetic housekeeping genes whereas the PEP mainly involves in transcription of the photosynthesis-related genes and tRNAs. This is evident from the knockout mutants of PEP that show an albino phenotype and lack photosynthesis. Thus, both PEP and NEP are essential for transcription of all chloroplast genes but each transcribes a distinct group of genes. The PEP and NEP operate independently. For example, Serino and Maliga have reported that deletion of each of the PEP genes yielded photosynthetically defective plants that lack PEP activity while maintaining transcription specificity from NEP promoters. It is interesting to note that the rpoB operon encoding 3 of the 4 PEP core subunits in all higher plants is solely transcribed by NEP. Genes encoding the core subunits of PEP (rpoA, B, C1 and C2) are located in the chloroplast genome itself, but those encoding the σ factors, which are required for promoter recognition and transcription initiation are located in the nuclear genome.

NEP and PEP use different promoters. However, many plastid genes have both PEP and NEP promoters. Most NEP promoters have the conserved YRTA motif and most of the PEP promoters resemble bacterial ‘σ 70 promoters type’ and typically characterized by ~10 and ~35 consensus sequence motifs. However, a number of PEP promoters lack the ~10 or the ~35 elements, a few even both. Although plastids possess genes for the core subunits of a PEP, this enzyme can only correctly initiate transcription together with nuclear-encoded σ factor(s). Therefore, transcription by PEP in chloroplasts is controlled by the nucleus by providing the σ factor and the NEP. Furthermore, the transcriptional activity of NEP and PEP is also affected by endogenous and exogenous factors.

4. Transcription Process in Chloroplasts and Eubacteria

The genetic material of chloroplasts is organized in the same way as bacterial nucleoids. The core structures of the chloroplast enzyme and its regulatory DNA sequences for transcription exhibit a strong homology with their E. coli counterparts as discussed elsewhere. As the subunit structures of both the MSU RNAPs are very similar with large numbers of highly conserved motifs, the initiation, elongation and termination events should be also very similar to eubacteria as reported. This is further elaborated in this communication.

5. Materials and Methods

A large number of MSU RNAPs from eubacteria and chloroplasts have been isolated, purified, characterized, cloned and sequenced and references therein. Complete nucleic acid and protein sequence data are available for many of these enzymes from different sources. Thus, these data have become valuable tools in analyzing and understanding the structure-function relationships of the chloroplast enzymes. For MSA of various MSU RNAPs, the sequences were retrieved from SWISS-PROT and PUBMED sites and analyzed using Clustal Omega, an accurate, fast and widely accepted algorithm, available on their website. This communication also presents a...
consensus model for initiation and elongation processes for PEPs similar to the eubacterial MSU RNAPs.

6. Results and Discussion

6.1 MSA DNA dependent MSU RNAPs of Chloroplasts from Different Sources

Figures 2-7 shows the MSA of various subunits of MSU RNAPs from chloroplasts and the mix and match analysis with their counterparts of the eubacterial MSU RNAPs. Only the relevant and highly conserved regions are shown (Figures 2-7). The catalytic, template and substrate binding regions are highlighted in yellow. The possible and experimentally proved metal binding regions (eubacteria) and proposed regions are highlighted green. The protein sequences of standard organisms are highlighted in magenta and used for numbering.

6.2 MSA of α Subunits of MSU RNAP from Chloroplasts

MSA analysis of the α subunits of chloroplast MSU RNAPshas shown that they are highly conserved and showed long stretches of conserved motifs among them. Unlike the α subunits of eubacteria, the α subunits chloroplasts MSU RNAPs showed two invariant YG pairs and an LG pair suggesting their possible role in template binding (data not shown).

6.3 MSA of β Subunits MSU RNAP from Chloroplasts

Figure 2 shows the MSA and conserved motifs in the β subunits of MSU RNAPs from chloroplasts. There are large numbers of conserved motifs among them and some are found to be very long stretches (highlighted). The Zea mays β subunit is used as the standard for numbering and highlighted in magenta. As seen in the eubacterial β subunits, the ‘gatekeeper pair’ YG and the catalytic R are strictly conserved, including the distance conservation here also (highlighted). Furthermore, the catalytic R and the YG pair maintain a distance of 7 amino acids as seen in eubacterial β subunits. This strongly suggests that the DNA polymerases, SSU and MSU RNAPs use a similar set of amino acids for the template, substrate binding and catalysis, establishing a structure-function relationship among the nucleotidyl transferases. The immediate downstream amino acid from the catalytic K in DNA polymerases is usually a G or A but in viral RNAPs, it is an R or sometimes a K, in eubacterial MSU RNAP β subunits, it is an invariant D and here it is G/D. An invariant R is found -7/8 amino acids downstream from the catalytic R (Table 1). Unlike the β subunit of eubacterial RNAPs, which possess three YG pairs, the chloroplast β subunit showed an invariant YG pair, an LG pair and two FG pairs suggesting at least three of them may possibly involve in the ‘three-point attachment’ on the template. Other than some of the viral SSU RNAPs, most of the SSU RNAPs use only one template binding YG pair. The DNA polymerases also use only one YG pair for template binding. In only about ten organisms C-terminal region is found to be highly conserved as indicated in Figure 2.

6.4 MSA of β’ Subunits of the MSU RNAPs from Chloroplasts

Figure 3 shows the MSA and conserved motifs in the β’ subunits of MSU RNAPs of chloroplasts. There are about a dozen conserved motifs among them (highlighted) with one or two long stretches around the metal binding site. The Zea mays β’ subunit is used as the standard for numbering and highlighted in magenta. Possible metal binding sites are highlighted in green. As seen in eubacterial β, β’ and chloroplast β subunits, no ‘gatekeeper YG pair’ and the corresponding catalytic R are found in the β’ subunits of chloroplasts RNAPs, suggesting that this subunit may not directly involve in catalysis as the catalytic R and the YG pair is found to be present in the β’ subunit only. However, the absolutely conserved metal binding motif –NADFDGDQMA - is found in this β’ subunit as seen in β’ subunits of eubacteria, suggesting that the active site regions are split or shared between the β and β’ subunits in chloroplast MSU RNAPs. No conserved motif is found in the C-terminal region as seen in the β and β’ subunits of eubacterial MSU RNAPs. In the absence of an YG pair, an FG and two LG pairs are seen (highlighted) in these subunits suggesting a possible interaction with the template by this subunit also. However, the absence of an invariant YG pair and its catalytic R at downstream from the YG pair at the expected distance suggests that this subunit may be playing only a supporting metal binding role in the transcription process. This can be proved
CLUSTAL O (1.2.4) MSA of the β subunits of various chloroplast of MSU RNPs
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Figure 2. MSA of β subunits of various chloroplast MSU RNAPs.
by SDM or deletion mutants. Interestingly, a highly conserved region among the β’ subunits from various sources is observed between amino acids 450 and 513, where the metal binding site is also located. Estimation of RNAP activity in the absence of this β’ subunit will throw more light on the functional role of this subunit in catalysis. The sequence from Euglena shows few variant amino acids in the conserved region (marked in red). It should be noted that *Euglena longa*, a close relative of the photosynthetic model alga, *Euglena gracilis*, possesses an enigmatic non-photosynthetic plastid*

### 6.5 MSA of β” Subunits MSU RNAPs from Chloroplasts

Figure 4 shows the MSA and conserved motifs in β” subunits of MSU RNAPs of chloroplasts. As found earlier, there are a large number of conserved motifs and some are found to be long stretches of conservations (highlighted). The Zea mays β” subunit is used as the standard for numbering and highlighted in magenta. It is obvious from the Figure 4, that the β” subunits are more conserved than the β subunits of chloroplasts. It is interesting to note that the Zn binding motif, with 3 invariant Cs, that is found in all eubacterial elongation β’ subunits is also found at the same distances in the β” elongation subunits of all chloroplast MSU RNAPs (e.g., in E. coli β’ the conserved Cs are found at amino acids 888, 895 and 898; in Zea mays β” 296, 303 and 306) within the catalytic region of β” subunits. X-ray crystallographic analysis has shown that this is the Zn binding motif in the thermophilic bacterium, *Thermus aquaticus*.

The completely conserved Zn binding motif in all eubacterial and chloroplast MSU RNAPs with absolute distance conservation of the Cs, suggests their possible role in the proof-reading activity. As seen in β initiation subunits, the ‘gatekeeper pair’ YG and the catalytic R are found and are strictly conserved, including distance conservation in all
Figure 3. MSA of β’ subunits of various chloroplast MSU RNAPs.

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| Accession   | Species                  |
|-------------|--------------------------|
| SP|P0C506|RPOC1ORYSJ| Oryza sativa subsp. japonica |
| SP|A7M957|RPOC1CUSRE| Cassutareflexa |
| SP|B1X3M9|RPOC1PAUCH| Paulinellachromatophora |
| SP|Q1XDN6|RPOC1PYRYE| Pyrriylezeaeonisis |
| SP|P42080|RPOC1CYAPA| Cyanophoraparadoxa |
| SP|Q4G3A6|RPOC1EMIHU| Emilianiahuxleyi |
| SP|O19897|RPOC1CYACA| Cyanidium caldarium |
| SP|P51251|RPOC1PORPU| Porphyrapurpurea |
| SP|Q6B8R7|RPOC1GRATL| Gracilariatenuestitieata var. liui |
| SP|P56763|RPOC1ARATH| Arabidopsis thaliana |
| SP|P16024|RPOC1MAIZE| Zea mays |
| SP|Q85FM8|RPOC1ADICA| Adiantum capillus-veneris |
| SP|Q85CL6|RPOC1ANTFO| Anthoceros formosae |
| SP|P42079|RPOC1SYNE7| Synechococcus elongatus |
| SP|P11705|RPOC1SPIOL| Spinacia oleracea |
| SP|P14563|RPOC1NOSCO| Nostoc commune |
| SP|Q2MIA9|RPOC1SOLLCC| Solanum lycopersicum |
| SP|Q6ENI3|RPOC1ORYNI| Oryza nivara |
| SP|P46819|RPOC1SINAL| Sinapis alba |
| SP|P58131|RPOC1EUGLO| Euglena longa |
| SP|O78484|RPOC1GUTH| Guillardia theta |
| SP|Q2VE15|RPOC1SOLUT| Solanum tuberosum |
| SP|A6MVX3|RPOC1RHDSA| Rhodomonassalina |
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Figure 4. MSA of β" subunits of various chloroplast MSU RNAPs.
the chloroplast β” subunits. However, in the β” elongation subunit, the catalytic R and the YG pair distance is maintained at 16 amino acids, almost double the distance as compared to the βinitiation subunits. This is in close agreement with the eubacterial elongation subunits. That is, in these β” subunits the catalytic R is placed at 16th position as found in β’ subunits of eubacteria and hence suggests that the eubacterial β’ and chloroplast β” subunits are very similar in their catalytic regions and in distance conservations. This strongly suggests that the DNA polymerases, SSU and MSU RNAPs might use a similar set of amino acids for template, substrate binding and catalysis establishing a structure-function relationship among the DNA polymerases and RNAPs.

The completely conserved R found downstream from the catalytic R at-7/8 position and at +1 from the YG pair may play a role in NTP selection as suggested for other MSU RNAPs (Table 1). Another interesting observation is that the elongation subunits of eubacterial and chloroplast RNAPs possess two YG pairs, suggesting that the two YG pairs might be possibly recognizing both the strands and slide during the transcription process as seen in some viral polymerases. However, the DNA polymerases and most of the SSU RNAPs use only one YG pair. In β” also there are two conserved motifs at the C-terminal region (about 12 amino acids in length) as seen in other β and β’ subunits. However, the completely conserved metal binding motif – NADFDGDQMA - found in all β’ subunits, is conspicuously absent in the β” subunits confirming that the active site regions are split between the β’ and β” subunits in chloroplast MSU RNAPs. This is in contrast to the eubacterial β’ elongation subunits, where both are located in the same subunit. These results suggest that the β’ subunit harbouring the metal binding site may be also essential for the elongation process in chloroplast MSU RNAPs.

7. Mix and Match Analysis

7.1 MSA of the β Subunits of Chloroplast and Eubacterial MSU RNAP

Only the initiation subunits β of eubacteria and chloroplasts and the elongation subunits β’ and β” subunits of eubacteria and chloroplasts, respectively, were analyzed for their conserved motifs by this method.
CLUSTAL O (1.2.4) MSA of the β subunits of eubacteria and chloroplasts
(Mix and Match analysis)
Active Sites of the Multi-subunit RNA Polymerases of Eubacteria and Chloroplasts are Similar in Structure and Function
Figure 5. MSA of the β subunits of eubacteria and chloroplast MSU RNAPs.
Figure 5 shows the ‘mix and match’ MSA analysis of β subunits of eubacterial and chloroplast MSU RNAPs. There are large numbers of conserved motifs and some are found to be long stretches (highlighted) and a small number of diads and triads among them. The catalytic, template and substrate binding motifs are highlighted in yellow. The possible metal binding regions are highlighted in green. The E. coli and Solanum tuberosum (potato) β subunits are used as the standard for numbering and highlighted in magenta. It is interesting to note that the ‘gatekeeper pair’ YG and its catalytic R are strictly conserved, including distance conservation, in all the eubacterial and chloroplast β subunits of the MSU RNAPs and matches exactly. However, only one YG is aligned in the β subunits of both these MSU RNAPs at the expected catalytic region. Additionally, one invariant FG and an LG pair are seen in both the β subunits making the total template binding pairs as 3 as discussed elsewhere. In both the cases, the catalytic R is placed at 7th position downstream from the YG pair which suggests again that the initiation subunit in both the cases are similar in structure and hence likely in function. As discussed earlier, the β subunit makes many aborted transcripts up to 7 nts and once it crosses 7 nts, the initiation transcript fits into the next elongation subunit’s active centre, i.e., the β” subunit and goes for elongation22. This is further confirmed by quantitation of the preinitiation complex by high-resolution gel electrophoresis technique which showed that many oligonucleotides are formed per preinitiation complex, including species as long as hexanucleotide and strikingly, the dinucleotide always represented 50% of the total of all oligonucleotides23. SSU RNAPs and DNA polymerases use the catalytic amino acid K at -8 position5, 19 in contrast to R. Another interesting feature is the immediate upstream amino acid from catalytic R in both the of eubacterial and chloroplast β subunits is the invariant D. A long conserved stretch at the C-terminal regions, i.e., exclusively at the end, is observed both in the eubacterial and chloroplast MSU RNAPs suggesting a possible role in polymerase cycling or termination.
CLUSTAL O (1.2.4) MSA of β′ subunits of eubacterial and chloroplast MSU RNAPs.

Active Sites of the Multi-subunit RNA Polymerases of Eubacteria and Chloroplasts are Similar in Structure and Function
Figure 6  MSA of the $\beta'$ subunits of eubacteria and chloroplast MSU RNAPs.
Active Sites of the Multi-subunit RNA Polymerases of Eubacteria and Chloroplasts are Similar in Structure and Function

| Accession | Organism                          |
|-----------|-----------------------------------|
| sp|P0C506| Oryza sativa subsp. Japonica  |
| sp|Q6EN13| Oryza nivara                      |
| sp|P16024| Zea mays                           |
| sp|A7M957| Cascutareflexa                    |
| sp|Q2MIA9| Solanum lycopersicum              |
| sp|Q2VE15| Solanum tuberosum                 |
| sp|P11705| RPOC1Spinacia oleracea            |
| sp|P56763| Arabidopsis thaliana              |
| sp|P46819| Sinapis alba                      |
| sp|Q85FM8| Adiantum capillus-veneris          |
| sp|Q85CL6| Anthoceros formosae               |
| sp|O19897| Cyanidium caldarium               |
| sp|Q4G3A6| Emiliania huxleyi                 |
| sp|P42080| Cyanophoraparadoxa                |
| sp|Q6B8R7| Gracilaria tenuistipitata         |
| sp|Q1XDN6| Pyropia yezoensis                 |
| sp|P51251| Porphyrapurpurea                  |
| sp|O78484| Guillardia theta                   |
| sp|B1X3M9| Paulinellachromatophora           |
| sp|P42079| Synechococcus elongates           |
| sp|P14563| Nostoc commune                    |
| AEG34223.1| Thermus thermophilus          |
| ASR51305.1| Blastomonas fulva            |
| OXR47930.1| Pusillimonas sp. T2     |
| sp|A7M9Q8| Cronobacter sakazakii             |
| sp|B2TWH4| Shigella boydii serotype 18       |
| sp|P0A8T7| Escherichia coli (strain K12)     |
| tr|A0A237JUP3| Shigella sonnei                 |
| sp|P0A2R5| Salmonella typhi                  |
| tr|A0A0F1RBF2| Enterobacter asburiae     |
| tr|A0A1B3EGW0| Enterobacter cloacae         |
| tr|A0A0F0XM62| Enterobacter kobei        |
| sp|A9MHE9| Salmonella arizonae               |
| tr|A0A232XM43| Salmonella muenchen           |
| tr|B5RFK0| Salmonella gallinarum            |
| sp|P0A2R5| Salmonella typhi;                 |
| tr|A0A078LHA5| Citrobacter koseri            |
| tr|A0A2J2K6S7| Klebsiella michiganensis         |
| tr|A0A0G3RZQ0| Klebsiella oxytoca           |
| tr|A0A1R0FP41| Citrobacter braakii           |
CLUSTAL O (1.2.4) MSA of eubacterial β’ and chloroplast β’ subunits (Mix and Match analysis)

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| Alignment | Sequence Details | Clustal O MSA | Gaps |
|-----------|------------------|--------------|------|
| A001015.1 | A001025 | 80.0% | 0.0% |
| A001035.1 | A001045 | 80.0% | 0.0% |
| A001055.1 | A001065 | 80.0% | 0.0% |
| A001075.1 | A001085 | 80.0% | 0.0% |
| A001095.1 | A001105 | 80.0% | 0.0% |
| A001115.1 | A001125 | 80.0% | 0.0% |

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ASRS1305_1 Blastomonas fulva

| Strain/Species | Description                  | Accession Number | Length |
|----------------|-------------------------------|------------------|--------|
| sp|A7MQQ8|RPOC_CROS8 Cronobacter sakazakii | | |
| tr|A0A0F1RBF2|A0A0F1RBF2_ENTAS Enterobacter asburiae | | |
| sp|B2TWHI4|RPOC_SHIB3 Shigella boydii | | |
| sp|P0A8T7|RPOC_ECOL1 Escherichia coli, K12 | | |
| tr|A0A237JUP3|A0A237JUP3_SHISO Shigella sonnei | | |
| tr|A0A232XM43|A0A232XM43_SALMU Salmonella muenchen | | |
| tr|B5RFK0|B5RFK0_SALG2 Salmonella gallinarum | | |
| sp|P0A2R5|RPOC_SALTI Salmonella typhi | | |

Figure 7. MSA of eubacterial $\beta'$ and chloroplast $\beta''$ subunits of MSU RNAPs.
7.2 MSA of the Eubacterial β’ Subunits with β’ Subunits of Chloroplasts in the MSU RNAPs (Mix and Match Analysis)

Figure 6 shows the ‘mix and match’ MSA analysis of β’ subunits of eubacterial and chloroplast MSU RNAPs. It is clear from the figure that the metal binding site is absolutely conserved in both the β’ subunits of both the MSU RNAPs (highlighted in green). The template/NTP binding YG pair and its catalytic R placed at -16 is seen only in the β’ subunits of eubacteria (highlighted in yellow) and conspicuously absent in the β’ subunits of chloroplast MSU RNAPs. However, it is interesting to note that this important catalytic motif is found in the β” subunits of chloroplast MSU RNAPs (Figure 7). The C-terminal ends of β’ subunits of eubacteria are highly conserved with the consensus sequence of – GLGGSDND - (highlighted) but no such conservation is seen in the chloroplast β’ subunits (data not shown). The mix and match analysis have revealed that the thermophilic RNAP aligns with the plant RNAPs rather than with the eubacterial RNAPs.

7.3 MSA of the Eubacterial β’ Subunits with the β” Subunits of Chloroplasts (Mix and Match Analysis)

Figure 7 shows the ‘mix and match’ MSA analysis of β’ and β” subunits of eubacterial and chloroplast MSU RNAPs. There are large numbers of conserved motifs and some are found to be long stretches (highlighted) and a small number of diads and triads among them, as seen in the case of β’- β’ matching. The catalytic, template and substrate binding motifs are highlighted in yellow. The possible metal binding amino acids are highlighted in green. The E. coli β’ and maize β” subunits are used as the standard for numbering and highlighted in magenta. The Zn binding motif (from the crystallographic data of E.coli and Thermus aquatics MSU RNAPs is shown in orange. Interestingly, it is completely conserved in both the types of MSU RNAPs elongation subunits suggesting a possible role in Zn mediated proof-reading mechanism for chloroplast MSU RNAPs also, as proposed for DNA polymerases. It is interesting to note that the ‘gatekeeper pair’ YG and the catalytic R are strictly conserved, including distance conservation in all the eubacterial and chloroplast β’ and β”subunits of the MSU RNAPs. In addition to, an additional invariant LG pair is also observed suggesting its possible role in recognizing both the strands as reported for many viral polymerases. Another interesting feature is the immediate downstream amino acid from catalytic R in both the eubacterial and chloroplast and β” subunits is a hydroxyamino acid, S/T, possibly playing a role in substrate binding. In both the cases, the catalytic R is placed at 16th position downstream from the YG pair. This observation is in contrast to the earlier report for DNA polymerases and SSU RNAPs where
it is positioned at -7/8 amino acids only\(^5\). A long stretch of conservation is seen at the C-terminal ends of \(\beta'\) of eubacterial RNAPs (except in *Blastomonas fulva*, an aerobic photosynthetic bacteria) and many of the \(\beta''\) subunits of chloroplast MSU RNAPs with few exceptions.

8. Analysis of the Active Sites of MSU RNAPs of Eubacteria and Chloroplasts

It is clear from the above MSA data, that the chloroplast MSU RNAPs are very much similar in functions to the eubacterial MSU RNAPs, such as the completely conserved substrate, template binding motifs, complete conservation of the catalytic amino acid \(R\) and in the distance conservations between the catalytic \(R\) and the \(YG\) pair in the initiation and elongation subunits. The metal binding region though found in different subunits is completely conserved. Furthermore, their susceptibility to various transcriptional inhibitors is also found to be similar. These points are further elaborated in the following section.

8.1 Substrate, Template Binding and Catalytic Amino Acids are same in Eubacterial and Chloroplast MSU RNAPs

As both the chloroplast and eubacterial MSU RNAPs carry out template-dependent nucleotide polymerization, it might be expected that both the enzymes would be structurally related to each other and hence, might be using similar types of amino acids for substrate, template binding and catalysis. Even though not much data are available on the 3D structures, biochemical, genetic and SDM analyses on the chloroplast MSU RNAPs, the major conclusions arrived in this communication are mainly based on the sequence similarities and completely conserved amino acid motifs in the critical regions between them and from the data available from their eubacterial counterparts. For example, the MSA analysis has shown that the eubacterial \(\beta'\) and chloroplast \(\beta'\) and \(\beta''\) catalytic subunits harbour the mandatory amino acids, viz. the catalytic amino acid \(R\) and the DNA template binding \(YG\) pair, an \(R\) or \(S/T\) for NTP selection (highlighted in yellow) and the completely conserved metal binding sites. This is in close agreement with the earlier report by Palanivelu for the eubacterial MSU DNA dependent RNAPs\(^6\). The interesting observation in chloroplast MSU RNAPs is that both the \(\beta'\) and \(\beta''\) might be possibly participating in the elongation process by sharing the active sites which are accomplished by a single \(\beta'\) subunit in eubacteria. Thus, in the elongation subunit \(\beta''\), the distance between the \(YG\) and catalytic \(R\), is doubled, i.e., it is placed at \(+16\)th position as found in the eubacterial elongation \(\beta'\) subunits\(^6\). The \(+1\) amino acid in this case, is a hydroxyl amino acid \(S/T\) which is placed adjacent to the \(YG\) pair which is reported to be involved in nucleotide discrimination in SSU RNAPs\(^25\).

8.2 The Presence of Invariant Catalytic \(R\) and \(YG\) Pair and their Distance Conservations are same in both the Enzymes

It has been found that almost all DNA polymerases and SSU RNAPs use a \(K\) for catalysis, i.e., in the initial proton transfer reactions\(^5\). However, in all MSU RNAPs there is no \(K\) but an \(R\) at the expected distance from the template binding \(YG\) pair (Table 1). However, a detailed analysis has shown that all prokaryotic DNA polymerases-II also use an invariant \(R\) in catalysis with similar distance conservations\(^5\); interestingly, an enzyme is associated \(3'\to5'\) exonuclease activity along with primase activity. Table 1 shows the invariant \(YG\) pair and its catalytic \(R\) in \(\beta\), \(\beta'\) and \(\beta''\) subunits of eubacterial and chloroplast MSU RNAPs, respectively (Figures 2-7).

It is interesting to note that the invariant \(YG\) pair appears to be specific for polymerases using DNA as the template and also for the prokaryotic MSU RNAPs, as it is not reported in RNA dependent RNAPs where they use RNA as the template\(^25\). In fact, the two \(YG\) pairs found in
T7 polymerase are required for the enzyme activity and interestingly, exhibit no activity when single-stranded DNA was used as the substrate. Three invariant Cs between catalytic R and YG pair in the elongation subunits in eubacterial β' is implicated in Zn binding and also found at the same distances in chloroplast β'' (Table 1), suggesting a similar function. Both the initiation and elongation subunits show an invariant R at -7/8 from the catalytic R and adjacent (downstream) to YG pair in both the MSU RNAPs but absent in β' subunits of eubacteria (Table 1).

8.3 Metal Binding Sites in MSU RNAPs of Eubacteria and Chloroplasts are same

The metal binding site (Mg$^{2+}$ binding site) arrived at by the MSA analysis of eubacterial MSU RNAPs was also confirmed X-ray crystallographic analysis and also by SDM experiments. Interestingly, the same Mg$^{2+}$ binding site that is found in both the initiation subunits, viz. in the β subunits of eubacteria and chloroplasts, suggests a similar role in both. Furthermore, similarities are also found in the metal binding sites in the elongation subunits of both the MSU RNAPs, i.e., a Mg$^{2+}$ and a Zn$^{2+}$ binding motifs are very similar in both the elongation subunits of eubacteria and chloroplasts (Table 2). Crystallographic analysis of the T. aquaticus RNAP have shown that the Mg atom is chelated at an absolutely conserved -NADFDGD motif in the β' subunits. Interestingly, the same sequence is absolutely conserved in the β' subunits of chloroplast RNAPs, further confirming an identical function in both the RNAPs. The Zn binding motif that is identified by X-ray crystallographic method in the eubacterial β' is also identified but in the chloroplast β'' subunits, suggesting a similar function. In E. coli β' subunit, the amino acids 814, 888 and 895 are shown to be involved in Zn binding (Table 2). The Zn binding motif that is found in elongation subunits β' and β'' may play a role in the Zn mediated proof-reading in these RNAPs as suggested for DNA polymerases.

8.4 Both the Eubacterial and Chloroplast MSU RNAPs are Similar in their Susceptibility to various Transcriptional Inhibitors

Both chloroplast and eubacterial MSU RNAPs are inhibited by the same types of transcriptional inhibitors, further suggesting structural and functional similarities between them. In fact, in vitro and in vivo studies with rifampicin, a well-known inhibitor of bacterial MSU RNAPs, have also been shown to inhibit the chloroplast RNAPs (but interestingly, did not inhibit the NEP). Further support is provided by the sensitivity of the chloroplast RNAPs to tagetitoxin, a phytotoxin isolated from the plant pathogenic bacterium, Pseudomonas syringae pv. Tagetis also inhibits eubacterial transcription. (Unlike rifampicin which inhibits the initiation step in transcription, the tagetitoxin inhibits both the initiation and elongation stages in both the types of MSU enzymes, as its target is the NTP loading site). These results confirm a high degree of conservation between the plastid-encoded and eubacterial MSU RNAPs. Moreover, the bacterial 'stringent control' mechanism which enables them to adapt to nutrient-limting stress conditions, use the effector molecule, guanosine-5'-diphosphate-3'.

Table 1. Amino acids around the catalytic amino acid K/R and the YG pair in DNA polymerases and in SSU and MSU RNAPs

| 1. DNA polymerases | 2. SSU RNAPs | 3. Chloroplast SSU RNAPs (Zea mays) |
|--------------------|-------------|-----------------------------------|
| α subunit          | Conserved YG pair(s) found | α subunit Conserved YG pair(s) found |
| β subunit          | -R*ERAGFEVRD*VHPTHY*GRV- | β subunit -GR*TAFSRSRDHIPS*GRI- |
| β' subunit         | -ENSVDAVKVRSVVSC$_{11}$DTDFGVC$_{11}$AHCG$_{11}$G | β' subunit No conserved YG pair with the catalytic R |
| β'' subunit        | -FR*AQPISIRT*PFTEC$_{14}$RSTSWC$_{14}$QLC$_{14}$Y$_{14}$GRS- | β'' subunit |

NB: The MSU RNAPs which use R in the catalytic site is shown in bold. The invariant R, at -6/7 is not found in β' subunits of eubacteria but found at -7 in the chloroplast MSU RNAPs.
-diphosphate (ppGpp), also showed a similar function in chloroplasts. Interestingly, the ppGpp inhibits the transcriptional activity of PEP through direct binding onto the β'subunit 33. Thus, under stress conditions, PEPs are also under the control of bacterial-like stringent response, mediated by ppGpp. The above evidences further support that the two MSU RNAPs are very similar in structure and function.

8.5 Promoter Sequences and Promoter Recognition Events by both the Enzymes are Similar

The eubacterial and plastid promoters are found to be very similar in their structure and functions. For example, both the MSU RNAPs recognize σ 70 type promoters, i.e., their promoters have the same type of −10 and −35 consensus sequence elements. Moreover, both the promoters contain similar sequences at -35 (TTGACA) and -10 (TATAAT), which are recognized by σ subunit (for PEPs, the σ subunit is nuclear encoded). Furthermore, the E. coli RNAP is able to faithfully recognize the PEP promoters 22,34 and thus further corroborating similarity in their structure and function in promoter recognition and transcription initiation events.

8.6 The β, β’ and β” in PEPs Possibly Work in Tandem as in Eubacteria

As proposed for the MSU RNAPs of eubacteria, the β, β’ and β” in PEPs also possibly work in tandem as shown in Figure 8. The only difference is the β’ subunit in chloroplast MSU RNAPs is actually needed for the Mg²⁺ binding at the NTP loading site during elongation. Figure 8 shows a consensus model of the PEP subunits from promoter recognition to initiation and elongation events during the transcription process in chloroplasts.

While the σ subunit recognizes and binds to the promoter, the β subunit binds to the TSS and initiates transcription. It has been found in eubacterial MSU RNAPs that the initiation by the β subunits is not smooth and it makes many aborted transcripts of sizes 2-7 nucleotides (nts) before the elongation step takes over 22,29. It has been shown that the eubacterial MSU RNAPs depend on this short RNA/DNA hybrids for stability and further processivity, as the Ternary Elongation Complex (TEC) with RNA/DNA hybrids of less than 8-bp display markedly less stability than those that are 8 bp or longer 35. The experimental evidence corroborates the MSA findings that the distance between the catalytic R and the YG pair is 7 amino acids and is strictly conserved in all β subunits of eubacteria as well as in chloroplasts. This consensus model is further confirmed in E. coli MSU RNAP by quantitation of the preinitiation complex by high-resolution gel electrophoresis technique which showed that many oligonucleotides are formed per preinitiation complex, up to hexanucleotides and only longer transcripts escape the cycling reaction and go for elongation 23. MSA analysis shows that the heptanucleotides fit into the elongation subunit active site and further elongated and possibly not detected in high-resolution gel electrophoresis technique.

Table 2. Similarities in the metal binding sites of MSU RNAPs from eubacteria and chloroplasts

| Subunit/Organism      | Metal binding site                | Method and Reference            |
|-----------------------|-----------------------------------|--------------------------------|
| β eubacteria (E. coli)| - LEHDDAN- & -GYNFEDS* - (Mg²⁺)  | MSA (This communication)        |
| β’ eubacteria (E. coli)| YNADFDGDQM- (Mg²⁺) & X-ray crystallographic data |                     |
| β chloroplasts (Zea mays)| -IEHNDAN- & -GYNFEDA* - (Mg²⁺)  | MSA (This communication)        |
| β’ chloroplasts (Zea mays)| -FNADFDDGDQM* - (Mg²⁺)          | MSA (This communication)        |
| β” chloroplasts (Zea mays)| -RS*PLTC*RSTSWIC²⁷Y¹⁶G-(Zn²⁺)  | MSA (This communication)        |

*The possible metal binding site is arrived at by MSA and SDM*

NB: The β’ subunits of eubacteria contain both the Mg²⁺ and Zn²⁺ binding sites on the same subunit whereas in chloroplasts Mg²⁺ binding site is located in β’ subunit and the Zn²⁺ binding site is found on the β’’ subunit. In both the cases, the Zn²⁺ binding site is built within the catalytic region with the precise distance conservations suggesting a possible role in the Zn-mediated proof-reading mechanism during elongation the process.
Active Sites of the Multi-subunit RNA Polymerases of Eubacteria and Chloroplasts are Similar in Structure and Function

9. Mechanism of Nucleotide Polymerization in the MSU RNAPs of Chloroplasts

MSA of both the MSU RNAPs have shown that they possess the same amino acid motifs, catalytic amino acid and metal binding regions and hence it is highly likely both use the same catalytic mechanism for nucleotidyl transfers as exemplified by Palanivelu⁶. Thus, as shown for the eubacterial MSU RNAPs, a minimal number of steps in the catalytic cycle of chloroplast RNAPs also consists of NTP selection, Watson-Crick base pairing with the complementary nucleotides with the template DNA, catalysis, pyrophosphate release and translocation⁵,¹⁶ and references therein. Polymerization reactions in PEPs should also be very similar in both the initiation and elongation subunits as the use same amino acids, catalytic and metal binding motifs.

10. Conclusions

MSA have shown that in both the MSU RNAPs of eubacteria and chloroplasts, the active sites, catalytic amino acids and metal binding regions are absolutely conserved both in the initiation and elongation subunits. Furthermore, same distance conservations are also observed among the initiation and elongation subunits of both the enzymes. Large numbers of highly conserved monos, diads, triads may play an important role in folding the proteins to the correct 3D structure. Therefore, it is suggested that the MSU RNAPs of chloroplasts may also follow very similar polymerization and proof-reading mechanisms as proposed for eubacteria. MSA data and the available experimental data show that both the eubacterial and chloroplast MSU RNAPs would have possibly evolved from a common ancestor.

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