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Review

RNA polymerase – The third class of primases

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Abstract. Multisubunit RNA polymerase transcribes DNA in all living organisms. RNA polymerase is also known to synthesize DNA replication primers in some replication systems, a function that is commonly performed by primases. There are two unrelated types of primases, the bacterial and eukaryal-archaeal types; RNA polymerase has no evolutionary relationship to either type. Here we discuss the mechanism of primer synthesis by RNA polymerase and compare it to mechanisms used by primases of both types as well as to the mechanisms used by RNA polymerase during transcription.

Keywords. RNA polymerase, primase, initiation, elongation, origin, primer, replication.

Introduction

Precise duplication of information coded by genomic DNA is the main prerequisite of life. This duplication is carried out by specialized enzymes, DNA polymerases. However, no DNA polymerase can initiate DNA synthesis *de novo.* Such an apparent deficiency might have evolved to increase processivity of these enzymes whose job is to replicate genomes of thousands and millions of bases long. The processivity would be impaired by the sequence-specific interactions with template, which is needed for specific initiation of *de novo* synthesis. All DNA polymerases require an accessible and properly positioned hydroxyl group for attaching a deoxyribonucleotide. In other words, DNA synthesis needs to be primed. Although "ready to use" primers, such as protein primers [1], tRNA molecules, or nicks in the DNA strand are used to initiate replication in some systems, in the vast majority of cases DNA replication is primed by specialized enzymes called primases. Primases are DNA-dependent RNA polymerases that synthesize oligoribonucleotides that remain annealed to template DNA and are used by DNA polymerases as primers.

The evolutionary origins of DNA replication proteins are obscure. Although some components are conserved within all living organisms, several major players, including primases, are different in eukaryotes-archaea and bacteria, indicating that these essential components of present-day replication machines could have emerged after the split of bacteria from eukaryotes and archaea [2]. The eukaryotic-archaeal and bacterial superfamilies of primases [3] share no structural or sequence similarity. There is also a great divergence within each superfamily, leading to differences in some aspects of primer synthesis. However, the basic mechanism is the same within a group, and there are also significant con-

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vergent similarities between mechanisms used by enzymes from different groups.

Most cellular RNA is synthesized by multisubunit RNA polymerases (RNAPs), enzymes responsible for transcription. Amazingly, some replicons rely on RNAPs, rather than primases, for initiation of replication of their genomes. Replication priming by RNAP was discovered more than 30 years ago, but until recently remained poorly understood despite a dramatic progress in mechanistic and structural understanding of transcription. In this review, we discuss RNAP-dependent replication priming and compare it to priming by eukaryotic-archaeal- and bacterial-type primases and to functioning of RNAP in transcription. We focus on bacterial RNAP, the only enzyme of its class known to prime replication. Given the exceptionally high level of conservation of RNAP structure and mechanism, the absence of reports on involvement of eukaryal and archaeal RNAPs in replication priming most likely reflects the lack of precedent rather than true absence of such mechanism in nature.

The functioning of RNAP as a primase was first observed by Arthur Kornberg while setting up a defined *in vitro* replication system [4, 5]. In fact for a brief time, RNAP was thought to be the only primase in the cell until a dedicated primase, DnaG, was found, also by Kornberg and colleagues [6–8]. However, RNAP was still shown to be essential for initiation of replication of the M13 phage leading to the formation of double-stranded form of its genome from a single-stranded form present in phage virions [4]. In this system, RNAP was shown to synthesize a primer of defined length at a specific position of the genome [9–11]. Later, additional examples of replication primer synthesis by RNAP were discovered [12, 13]. Although all currently known examples are limited to plasmids and bacteriophages, the possibility of RNAP participation in replication of cellular genomes could not be dismissed (see below).

Recognition of the primer synthesis start site

While RNAP operates on double-stranded (ds) DNA (during transcription), to allow template-directed RNA synthesis it locally melts the DNA helix to form a transcription bubble of about 12–14 melted base pairs. RNAP initiates transcription at specific sites called promoters. The catalytically proficient, elongation-competent RNAP core enzyme does not recognize promoters. Binding of one of the several specificity subunits σ converts the core into the holoenzyme, which can recognize promoters and initiate transcription (for review see [14]). In the context of the holoenzyme, σ recognizes two conserved upstream DNA elements positioned around registers −10 and −35 relative to the start of transcription, and nucleates the melting of the DNA at position −11 (Fig. 1). The melting is next propagated towards the transcription start site (for review see [15]). Melting positions dsDNA downstream of the transcription start site into the so-called downstream DNA-binding channel formed by structural elements of the RNAP core; however; this interaction is nonspecific. The precise location of the transcription start site is primarily determined by the distance from the promoter elements recognized by the σ subunit. However, because of a strong preference to use purine nucleotides (nt) for transcription initiation, transcription start sites in some promoters can be shifted one to two nt upstream or downstream of the preferred position (*i.e.*, one defined by the proper distance from the −10 element).

In contrast to "normal" transcription, most cases of RNAP-catalyzed replication priming involve single-stranded (ss) DNA (for review see [12]). However, to be recognized by RNAP single-stranded origins of replication (*ori*) form pseudo double-stranded structures. In this review we focus on initiation of replication of phage M13, currently the best-understood case of priming by RNAP (Fig. 1). Early work demonstrated that priming in this system requires the holoenzyme form of RNAP of *E. coli*, the host bacterium for the M13 phage. The requirement for the σ subunit was rationalized by proposing that the *ori* forms two partly double-stranded hairpins each containing sequences that resemble basal promoter elements sequences [16–18]. The *ori* was, therefore, thought to function in essence as a "normal" double-stranded promoter. However, later investigations revealed that *ori* recognition (but not primer synthesis) can be carried out by both the holo and the core enzyme [19]. This was a highly surprising finding, since RNAP core was thought to lack the ability to recognize specific DNA sequences. The minimal *ori* fragment specifically recognized by RNAP (both core and holo) consists of an ~17-bp-long imperfect hairpin (Fig. 1). The structure of the *ori* (particularly the loop of the hairpin and mismatch bulges in the stem) may be more important for recognition than the sequence *per se* (N.Z., unpublished). In a way, it is probably more correct to say that the *ori* hairpin recognizes RNAP, *i.e.*, that the structures of the origin evolved as aptamers that tightly bind to a specific site of RNAP. The complete partially double-stranded *ori* structure is much larger than the minimal structure that is capable of RNAP binding. The additional sequences and/or structures may stabilize the structure recognized by RNAP but may also improve the recognition by RNAP through interactions with the σ subunit or

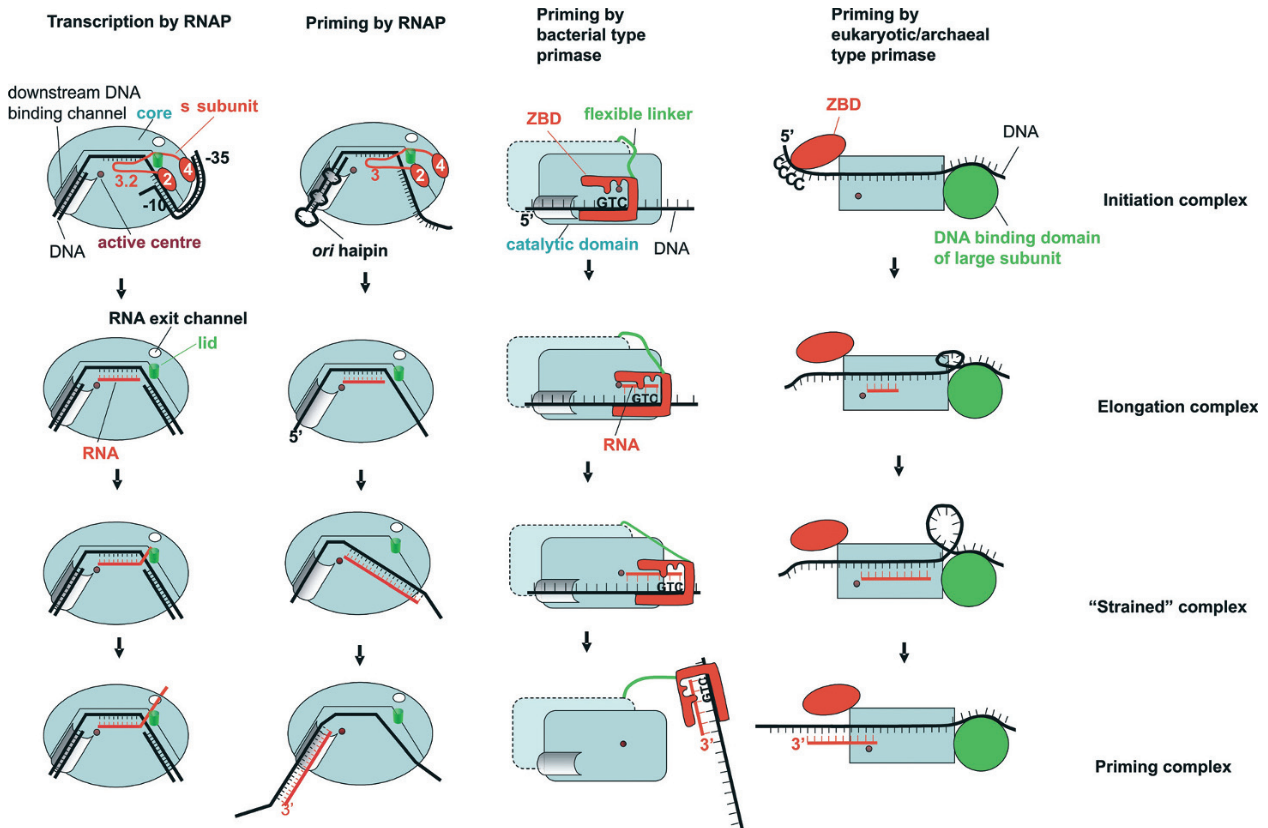

Figure 1. Transcription and primer synthesis by RNAP, and hypothetical mechanisms of primer synthesis by bacterial-type and eukaryotic-archaeal-type primases. Schemes represent the sequences of events from formation of the initiation complex to the formation of the priming complex (except for transcription). The main players of the mechanisms are shown in color and labeled. See text for details. For the complexes of RNA polymerase during transcription the non-template strand of the transcription bubble is omitted for clarity.

additional domains of the core. Since RNAP has a high affinity to the ssDNA, the ~5000-base-long genome of the phage must be covered with ssDNA-binding protein (SSB), to, on the one hand, exclude RNAP from ssDNA and, on the other hand, expose the partially double-stranded origin. In this way, SSB strongly increases the specificity of RNAP binding to the *ori*. *In vitro* synthesis of primer from a minimal *ori* fragment occurs without SSB and is, in fact, inhibited by it [18, 19].

The minimal M13 origin hairpin is bound in the RNAP core channel normally occupied by dsDNA downstream of the transcription initiation start site [19]. Such a binding mode positions the single-stranded 3′ end of the hairpin in the active center of RNAP, making it possible to initiate the RNA synthesis (Fig. 1). Thus, in contrast to transcription initiation from promoters, which depends on specific interactions of the σ subunit with DNA elements upstream of the RNAP active center, *ori* recognition is accomplished by RNAP core contacts with DNA downstream of the active center.

In contrast to RNAP recognition of the M13 *ori,* primases recognize ssDNA generated by the action of helicases that are often associated with the primases, which allows localizing the primase activity to functional replication forks. Since primer synthesis must occur frequently during lagging strand synthesis, sequences recognized by primases are short. For bacterial-type primases, the recognition sites are 3 nt long (5′-CTG-3′ for *E. coli* DnaG primase), and are positioned around the site of the synthesis start [20, 21]. During the lagging strand synthesis, several primase molecules are bound to the hexameric ring of the helicase. The helicase generates ssDNA that emerges from the hole of the ring and is scanned by primases for the presence of the recognition site sequence [22]. The catalytic domain of bacterial-type primases possesses only nonspecific DNA-binding activity and cannot recognize this sequence [23]. The recognition site binding is specified by a flexibly linked Zn-binding domain (ZBD). Curiously, site recognition for a given catalytic domain is performed *in trans* by the ZBD of a neighbor primase bound to the helicase ring [23, 24]. The function of ZBD in primer synthesis start site recognition resembles the role of the σ subunit in the case of promoter recognition by RNAP. The cooperation between (at

least) two primase molecules that are needed for primer synthesis initiation presumably decreases the possibility of aberrant primase function outside of the replication fork.

Interestingly, some bacteriophages use bacterial primase to prime the lagging strand replication of their single-stranded genomes. In this case, primase acts without the help from helicase; however, two primase molecules are still involved in origin recognition and primer synthesis [25, 26]. The primer synthesis reaction depends on the presence of SSB. Presumably, SSB and the extensive secondary structure of the origin together ensure that primase binds exclusively at the *ori* site [27]. SSB may also directly participate in the reaction of primer synthesis (for review see [28]).

Eukaryotic-type primases are either less sequence specific than bacterial-type primases, or their specificity is not yet understood. The only obvious sequence requirement appears to be that a base coding for the 5′ end nt of the primer must be a pyrimidine [29]. In addition, the sequence of DNA ~10 nt downstream of the primer start site must be pyrimidine rich [30]. Crystallographic analysis suggests that this sequence is recognized by ZBD of eukaryotic-archaeal-type primases (euZBD). Despite (or perhaps because of) their low specificity, eukaryotic primases have an extended ssDNA-binding surface [31], presumably needed for stabilization of the initiation complex. The smaller ssDNA-binding surface of bacterial-type primases may be compensated by the specificity of their ZBD interaction to the recognition sequence.

Initiation of primer synthesis

On regular promoters, the RNAP σ subunit initiates melting of the double-stranded promoter DNA, while the core finalizes the process leading to the formation of transcription-competent open promoter complex (for review see [15]). However, productive RNA synthesis that would lead to escape from promoter and processive transcript elongation cannot begin immediately: at least *in vitro*, large numbers of short abortive products are synthesized in each productive promoter escape event. Such apparently wasteful abortive synthesis is a common property of all RNA polymerases (the only enzymes capable of *de novo* template-directed synthesis of phosphodiester bonds), and can be regarded as a necessary price for not using a primer. During *de novo* synthesis of the first as well as several following phosphodiester bonds, the synthesis product cannot form an extended stable hybrid. During elongation, such a hybrid stabilizes the 3′ hydroxyl of the product in the active center, increasing the processivity of synthesis. Therefore, during initiation of synthesis, RNAP must form a tight (and specific) complex with the template and substrates to achieve stabilization of reacting groups in the active center. The stabilization is achieved through sequence-specific interactions of the enzyme with the template. However, after the RNA product becomes long enough to form a stable hybrid with the template, stable initiation complex must be destroyed to allow the formation of the elongation complex that is stable (due to the formation of product-template hybrid) and yet sequence nonspecific (to allow processive elongation). If the stable initiation complex fails to be destroyed, further synthesis becomes impossible, preventing promoter escape. In this situation, the product is aborted and the enzyme reinitiates the synthesis without leaving the promoter. Generally, the formation of a stable initiation complex requires participation of additional subunits or domains. Conversely, release or rearrangement of these subunits or domains results in stable initiation complex disruption. For example, during initiation on common promoters, the stabilization is thought to be achieved through sequence-specific interactions of the σ subunit with DNA, which must be destroyed for transition into productive synthesis, and which presumably causes abortive initiation.

The RNAP core enzyme was found to be able to specifically initiate RNA synthesis on the M13 *ori* hairpin or, in the presence of SSB, even on the M13 genome, in the absence of the σ subunit [19]. Despite the absence of σ, the product, a dinucleotide, is synthesized in an abortive manner [19]. Presumably, stabilization of the initiation complex in this case is achieved through "structure-specific" interactions of the downstream DNA-binding channel of the core with the *ori* hairpin.

However, despite the ability of RNAP core to recognize *ori*, form initiation complex, and initiate RNA synthesis, no synthesis beyond the first phosphodiester bond is observed, and the σ subunit is still strictly required for primer synthesis. It was shown that in this system, σ region 3.2 interacts with short transcripts and stabilizes them, making their further extension possible [19]. σ thus increases the chances of transition into elongation. This σ-induced stabilization is needed at least until the second phosphodiester bond is synthesized, as the product of second phospodiester bond formation, a trinucleotide, is bound tightly enough to be extended even without σ, solely by the core [19].

During transcription initiation from promoters, the σ subunit was also shown to increase the affinity of the promoter complex to transcription substrates necessary for the synthesis of the first and second phosphodiester bonds [32, 33], although the mechanisms(s) are

not defined. One of the possibilities is that σ region 3 interacts with template DNA, fixing it in the active center and thus helping to fix reactants in the active center. The effect of σ here can also proceed through direct interaction with the initiating nt as suggested by cross-linking data [34]. To summarize, σ is not needed for the initiation complex formation at the M13 *ori*, but it is essential for escape into productive elongation. The biological significance of this function of the σ subunit may be to prevent RNAP core from initiating transcription at the wrong i.e., nonspecific, sites, which would offset the carefully balanced regulation of gene expression in the cell.

The conceptually similar mechanism of stable complex formation during *de novo* initiation of primer synthesis is used by bacterial-type primases. ZBD strongly stimulates the catalytic activity of the catalytic domain, although the mechanism remains obscure [23, 35]. It seems that ZBD in complex with the recognition sequence and nt substrates in the active center of catalytic domain stabilizes the entire structure [36] (Fig. 1). Thus, ZBD has a function that is similar to that of the RNAP σ subunit and reduces the probability of nonspecific primer synthesis. That a stable complex forms before or during the first phosphodiester bond formation reaction is consistent with this reaction being a rate-limiting step at this stage. Failure to destruct the stable complex during transition into elongation leads to abortive synthesis [37].

Despite their low sequence specificity, eukaryotic primases form stable initiation complexes that undergo abortive initiation reaction [38]. Again, such a behavior is consistent with a view of abortive synthesis as a result of the enzyme's failure to escape into elongation. It is suggested that euZBD interacts with DNA (and presumably participates in the recognition of specific sequences in DNA) downstream of the start site and thus contributes to stable initiation complex formation and abortive synthesis [31] (Fig. 1). This is supported by the finding that the deletion of the euZBD resulted in reduced abortive synthesis and the increased transition to elongation. This situation resembles initiation of transcription by RNAP from M13 *ori*, where specific interactions required for initial complex formation also occur downstream of the start site and are presumably responsible for abortive initiation (see above).

Primer synthesis

After the formation of sufficiently long RNA and disruption of contacts with promoter, RNAP escapes into elongation phase, in which it processively extends 3′ end of the nascent RNA. Elongating RNAP uses the nascent RNA as a primer for fast and efficient elongation in the same manner as DNAPs. The only difference is that this RNA is initially synthesized by RNAP itself. Thus, the transcription initiation complex can be regarded as a primase for transcription elongation stage.

During transcription elongation, RNAP can processively synthesize RNAs for thousands of nt. What mechanisms can be responsible for RNAP-catalyzed synthesis of short replication primer of defined length? The normally highly processive transcription elongation complex can pause at defined positions of the template and then continue elongation or terminate transcription, with release of the transcript, RNAP, and DNA. Neither of these events results in formation of a transcript that can serve as a replication primer, i.e., it contains an exposed 3′ end of the transcript annealed to the template DNA. The structure of active and some paused elongation complexes is such that the 3′ end of RNA, while annealed to the DNA template, is buried deep in the RNAP active center cleft and is therefore not accessible (Fig. 1). At some pause sites, RNAP moves backward, the phenomenon called backtracking [39], and the 3′ end of the transcript disengages from the RNAP, is threaded through the RNAP secondary channel and becomes exposed; however, in this case the 3′ end is not annealed to DNA and, therefore, cannot serve as a primer. During termination, the transcription bubble collapses, restoring the double-stranded DNA and releasing the transcript. Thus, again no primer-template structure suitable for replication initiation is formed. How then is it possible for an RNAP transcript synthesized from the M13 *ori* to act as a primer?

During elongation, RNAP moves along the dsDNA maintaining a ~12-bp transcription bubble [40]. The nascent RNA remains largely single-stranded; only eight to nine 3′-terminal bases remain base-paired with the template DNA strand in the transcription bubble, in the main RNAP channel (Fig. 1). The 8–9-bp-long RNA-DNA hybrid is thought to be a prerequisite for stable elongation complex formation [41]. The length of the hybrid is maintained by two factors. First, the DNA duplex that reforms behind RNAP peels RNA from the RNA-DNA hybrid. Second, a small protein domain, the β′ lid, located at the upstream edge of the hybrid helps to disengage RNA from the template DNA [42, 43] (Fig. 1). Single-stranded nascent RNA leaves the transcription complex through an RNA exit channel [42, 44].

During the priming of M13 replication, RNAP synthesizes an 18–20-nt-long primer RNA [11]. Although *ori* is recognized as a hairpin, during the synthesis the double-stranded structure must be

destructed since the "non-template" strand of the hairpin is displaced by the transcript synthesized on the "template" strand. Thus, the synthesis of primer RNA proceeds on ssDNA. During transcription of ssDNA, the first determinant of the RNA-DNA hybrid length, reformation of upstream DNA duplex, is absent. When a growing RNA molecule reaches a length of 8–9 nt, its 5′ end collides with the lid domain (Fig. 1). The lid alone is unable to disrupt the RNA-DNA hybrid, especially if it is G : C rich, as is the case on the M13 *ori.* However, the RNAP catalytic center continues to catalyze phospodiester bond formation and as a result, an overextended hybrid is formed. Since a hybrid longer than 10 bp cannot be accommodated in the RNAPs active cleft due to steric constrains, the upstream edge of the hybrid must be displaced from the cleft (Fig. 1). Presumably, the addition of each "extra" nt at the 3′ end leads to further displacement and accumulation of tension in (and destabilization of) the transcription complex. When the transcript reaches a critical length of 18–20 bases (with correspondingly long hybrid), the complex undergoes a conformational change: the overextended hybrid slides within the RNAP molecule forwards to occupy the downstream DNA-binding channel, which, during initiation of primer RNA synthesis, is occupied by on *ori* hairpin (Fig. 1). As a result of such sliding, the 3′ end of RNA remains annealed to DNA template and becomes accessible to DNAP. It remains unclear whether DNAP recognizes the 3′ end of the RNA in the context of the priming complex with RNAP or whether RNAP is displaced from the complex, similar to situation observed with DnaG [45], before initiation of new DNA synthesis.

Primers synthesized by bacterial-type primases differ in length, from 4 nt for bacteriophage primases to slightly longer than 10 nt for most other primases [28]. Richardson and co-workers [28, 46] suggested that the primer length is determined by the size of the initiation site of the active center, which determines how many bases can be accommodated within the enzyme during extension. However, it was shown that an isolated catalytic domain of bacterial-type primase is capable of processive synthesis of RNAs that are much longer than usual-length primers [35], implying a translocation-like reaction [37]. It was therefore proposed that the interaction of ZBD with the catalytic domain and/or primed template is responsible for cessation of primer synthesis at defined point. It was further suggested that the length of a flexible linker between the catalytic domain and ZBD and the mode of the two domains interaction (*in cis* or *in trans*, i.e., between domains of different primase molecules) may be controlling the primer length [35, 36, 47]. During primer synthesis, ZBD remains bound to the start site [36]. Therefore, as in the case of RNAP-catalyzed primer synthesis, the cessation of primer synthesis is probably caused by a tension accumulated in the priming complex when the primer reaches a certain critical length. This tension is presumably relieved when the elongation complex is disrupted, leading to release of the primed template. The primed template remains bound to the ZBD, and is presented to the DNAP within this complex [23] (Fig. 1).

During eukaryotic replication, only primers that are at least 7 nt long are utilized by DNAP [48–50]. In the model of elongating complex of eukaryotic primase, the RNA-DNA hybrid of about 7–14 bp reaches the C terminus of large subunit of the enzyme, and the sterical clash with it may determine the primer length [31], in a way that is reminiscent of primer length control by the β′ lid in the case of RNAP-catalyzed primer synthesis. An alternative model based on the finding that the C terminus of the large subunit has strong ssDNA-binding activity [51] envisions the following scenario (Fig. 1). During primer synthesis, the large subunit, which possesses high affinity to ssDNA, remains bound to the DNA template upstream of the primase active center, leading to accumulation of stress in the complex as the primer length is increased. To release the tension, the RNA-DNA hybrid, upon reaching a certain threshold length, slides forward relative to the primase mainframe, again similar to the situation envisioned for RNAP-catalyzed priming, thus making the annealed 3′ end of RNA accessible to DNAP. The disengagement of the 3′ end of RNA from the active center of the primase is supported by the finding that the primases can further elongate previously synthesized primers, but to do so, they must dissociate from the complex first [52]. In a complex formed after dissociation, there is no more tension between the active center and the DNA-binding domain of the large subunit and primer synthesis can be continued. The cycle can be repeated several times, resulting in RNAs whose lengths equal the integer number of normal primer length [49, 52]. However, primase dissociation is not required for primer utilization by DNAP [48], underscoring a conformational change that occurs at the end of the primer synthesis cycle and exposes the 3′ end of the primer.

Priming on dsDNA

Neither bacterial nor eukaryotic/archaeal primases can synthesize primers on dsDNA, and therefore require the action of helicases. However, in the case of RNAP, priming on dsDNA is possible. Double-

stranded plasmids with ColE1 replicon rely on RNAP for replication priming (for review see [13]). In this case, RNAP synthesizes a 555-nt-long RNA in an apparently "standard" transcription elongation mode. During synthesis, the nascent transcript adopts a secondary structure that makes a part of it, at some point, interact with the template strand of DNA upstream of the transcribing RNAP, thus preventing the reformation of upstream DNA duplex [53]. This eventually leads to cessation of transcription and transformation of the transcription elongation complex into the priming complex. The mechanistic details of this transformation are unknown; however, the absence of the DNA duplex behind RNAP due to the interaction of template DNA with upstream RNA suggests a mechanism similar to the primer complex formation on M13 *ori*.

In general, RNAP complexes similar to the priming complex can occur during transcription of double-stranded genomes. During RNAP movement along the DNA, the DNA-linking number immediately behind RNAP decreases, favoring the melting of DNA behind RNAP, which in turn should favor the formation of an overextended hybrid due to the absence of upstream DNA duplex and subsequent stalling of the elongation complexes. Such complexes, in principal, could participate in the priming of replication [54]. Kornberg and colleagues [55, 56] have shown that *in vitro*, RNAP can catalyze priming of replication from bacterial *oriC*, although this reaction is less efficient than the one catalyzed by the primase. Although it is now thought that RNAP does not participate in primer formation *per se* in this system (although this possibility was never dismissed), it was shown to participate in the regulation of replication initiation [55, 56]. It is suggested that it does so by changing the topology of the DNA at and around the origin during transcription from adjacent promoters, possibly through formation of overextended RNA-DNA hybrid that stimulates the origin unwinding [57]. Although eukaryotic and archaeal RNAPs are not known to initiate replication, theoretically they should be able to do so [58]. Eukaryotic/archaeal RNAP core enzymes are very similar to prokaryotic RNAP in sequence, structure, and function, especially during transcription elongation. Although the structure and sequences of "normal" promoters of archaea and eukaryotes are very different from those used in bacteria, the type of recognition that occurs at the M13 *ori* is certainly possible for eukaryotic and archaeal RNAPs, and transcription from such templates should lead to the same structural constrains, leading to formation of priming-like complexes. Single-stranded origins of replication were reported for some archaeal plasmids, although their ability to function as promoters for cognate RNAPs was not demonstrated yet [59].

Conclusion

While there is no evolutionary relationship between the three different classes of primases, some functional similarities of the basic mechanisms involved in *de novo* template-dependent synthesis of RNA exist. The principles of formation of a primer-template structure that can be recognized by DNAPs are also similar, suggesting that the functional constraints on primase function led to convergent evolution of enzymatic activities performing similar function.

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