Published in final edited form as:
Biochim Biophys Acta. 2013 January ; 1829(1): 105–115. doi:10.1016/j.bbagrm.2012.08.007.

The Spt4-Spt5 complex: a multi-faceted regulator of transcription elongation

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Abstract

In all domains of life, elongating RNA polymerases require the assistance of accessory factors to maintain their processivity and regulate their rate. Among these elongation factors, the Spt5/NusG factors stand out. Members of this protein family appear to be the only transcription accessory proteins that are universally conserved across all domains of life. In archaea and eukaryotes, Spt5 associates with a second protein, Spt4. In addition to regulating elongation, the eukaryotic Spt4-Spt5 complex appears to couple chromatin modification states and RNA processing to transcription elongation. This review discusses the experimental bases for our current understanding of Spt4-Spt5 function and recent studies that are beginning to elucidate the structure of Spt4-Spt5/RNA polymerase complexes and mechanism of Spt4-Spt5 action.

Keywords

Spt4; Spt5; DSIF; NELF; P-TEFb; transcription elongation

1. The Spt4-Spt5 complex, a universally conserved transcription elongation factor

Transcription elongation by RNA polymerase II (RNAPII) is a dynamic and highly regulated step in the gene expression cycle. *In vitro*, RNAPII is capable of transcribing naked DNA at rates that rival those observed *in vivo* [1]. However, RNAPII cannot efficiently transcribe chromatin templates *in vitro*, suggesting that transcription elongation accessory factors and/or chromatin remodeling plays an essential role in facilitating elongation *in vivo* [1, 2]. Over the past decade, a wealth of work has established that both of these mechanisms are employed to facilitate transcription elongation (reviewed in [3]). Furthermore, it is now clear that in addition to being a target for regulation, elongating RNAPII also serves as an interaction platform for factors that couple elongation to chromatin remodeling and RNA processing activities.

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The multisubunit RNA polymerases that are responsible for the bulk of transcription across the three domains of life are conserved at the levels of sequence, structure and mechanism [4–7]. Genomic analysis suggests that other than RNA polymerase subunits, the only transcription regulator universally conserved across all domains of life is the Spt5/NusG family of proteins [8]. Thus, it is likely that the function encoded by these proteins is ancient, *i.e.*, that it was already present prior to divergence of the eukaryotic, archaeal and bacterial lineages. Consistent with the idea that they play a central role in transcription, Spt5/NusG proteins are essential for life and they regulate transcription elongation in all 3 domains of life. Furthermore, in eukaryotes they also coordinate elongation with chromatin states and pre-mRNA processing.

In the past several years, a confluence of structural, biochemical, genetic and genome-wide approaches have considerably expanded our understanding of Spt4-Spt5 function. This review will describe the experimental bases for our current conception of Spt4-Spt5 function and will also point out outstanding issues regarding the structure and mechanisms of Spt4-Spt5 in transcription elongation.

2. Discovery of Spt4-Spt5 in yeast and humans

SPT4 and *SPT5* were originally discovered in a genetic screen, performed in *S. cerevisiae,* for mutations that suppressed the transcriptional defects caused by particular set of insertions in the promoter of the *HIS4* gene [9]. Subsequent analysis showed that *spt4* and *spt5* mutations shared this phenotype with several histone mutations, that the *spt4* and *spt5* mutations affected transcription, and that *spt5* mutations could genetically suppress mutations affecting the Snf/Swi complex [10–12]. Collectively, these observations suggested that Spt4 and Spt5 might influence chromatin and promoter function.

A pair of studies published in 1998 indicated that Spt4 and Spt5 form a protein complex that regulates transcription elongation. In the first, selection for genetic suppressors of a cold-sensitive *spt5* allele yielded *S. cerevisiae* strains with mutations in catalytic subunits of RNAPII [13]. One of these polymerase mutations was known to decrease the rate of elongation by RNA polymerase II and several others were predicted to do so, suggesting that Spt5's function is related to the rate of elongation. Consistent with this, *spt4* and *spt5* mutations exhibited growth defects when combined with deletions in elongation factor TFIIS, which functions to rescue RNAPII from transcription arrest [14], suggesting that *spt4* and *spt5* mutations increase the probability that RNAPII will undergo transcription arrest. Treatment of cells with 6-azauracil or mycophenolic acid, inhibitors of nucleotide biosynthesis that may interfere with elongation rate and processivity of RNA polymerase II [15, 16], suppressed the growth defects of the cold-sensitive *spt5* mutant. The suppression of the *spt5* cold-sensitive defects in RNAPII or inhibitors of elongation was allele-specific; although other *spt4* and *spt5* mutants display enhanced growth defects when combined with deletions of the gene for TFIIS, 6-azauracil and mycophenolic acid enhances rather than suppresses their growth defects and they are not phenotypically suppressed by elongation defective mutations in RNAPII ([13]; GAH, unpublished). Mutations that cause cold-sensitivity have been implicated in defective assembly of protein complexes, defects in protein activity and in defects in proteins that interact with RNA [17]. Thus, in the *spt5* cold sensitive mutant, a decreased rate of transcription elongation may allow the defective Spt5 protein more time to execute its function or to associate with an interaction partner. Overall, these observations suggest that Spt4-Spt5 acts during transcription elongation, that it may prevent pausing or arrest of the elongating RNAPII, and that Spt4-Spt5 function and RNA polymerase II elongation rate must be coordinated for normal growth.

The second study examined the mechanism of action of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which inhibits transcription elongation, but not initiation [18]. Wada and colleagues identified a fraction of a HeLa cell nuclear extract which supported RNAPII transcription *in vitro*, but that was insensitive to DRB[19]. They then purified a protein complex which, when added back to their *in vitro* transcription reactions, restored DRB sensitivity. This complex, DRB Sensitivity Inducing Factor (DSIF), was composed of the human homologs of Spt4 and Spt5. Interestingly, although DSIF was purified as an inhibitor of elongation, it was also found to stimulate elongation over the body of a gene *in vitro*, although only when nucleotides were present at limiting concentrations [19, 20]. As will be discussed in more detail below, Spt4-Spt5's inhibitory activity has only been demonstrated in a few organisms. Thus, the term DSIF will be used here only in reference to systems in which this negative activity is known to occur.

3. Organization of Spt4 and Spt5 proteins

Spt5 is a large, highly conserved, multi-domain protein consisting of an N-terminal acidic domain, a NusG N-terminal (NGN) domain, multiple Kyprides, Ouzounis, Woese (KOW) domains and a set of short repeats at its C-terminus (CTR) (Figure 1A;[21]). Some metazoan and plant Spt5 proteins include an additional stretch of ~100 amino-acids at their extreme C-terminus which may include a poorly conserved KOW domain. Although the NGN domain appears to be unique, KOW domains are a subset of a larger family of domains called Tudor (Figure 1B), which are thought to mediate protein-protein and protein-nucleic acid recognitions [22–24]. Sequences of the repeats within the CTR vary across species, but typically contain residues that can be phosphorylated (tyrosine, serine, threonine). Archaeal Spt5 proteins and their bacterial homologs, NusG proteins, are much smaller than eukaryotic Spt5, consisting of an NGN and single KOW domain. *SPT5/NusG* genes appear to be essential for life in bacteria, yeasts, Drosophila, Zebra fish and mammalian cells [11, 25–29].

In contrast to Spt5, Spt4 is a small zinc finger protein [10]. Spt4 proteins are conserved across the eukaryotic and archaeal lineages, but are not found in bacteria [21]. Mutation of any one of the 4 cysteines of the zinc finger of Spt4 causes severe phenotypes similar to those of null alleles [10, 30, 31]. Although *spt4* null mutants of *S. cerevisiae* are viable [10], *SPT4* may be essential in *Drosophila* [32]. As discussed in a section below, crystallographic studies have shown that Spt4 interacts intimately with the NGN domain of Spt5 in forming the Spt4-Spt5 heterodimeric complex. Consistent with its sequence conservation [33], human *SPT4* can complement *spt4* null mutation in yeast, attesting to functional conservation and probably structural conservation as well [34].

4. Spt4-Spt5 plays pervasive role in transcription elongation

The initial indications that Spt4 and Spt5 form a complex that regulates elongation are now supported by an abundance of other data. The overwhelming majority of Spt4 and Spt5 proteins are found in the Spt4-Spt5 complex [35], and Spt4-Spt5 tightly associates with RNAPII in a transcription-dependent manner [36]. *In vitro* transcription experiments [37, 38] show that this association begins just downstream of the transcription start site. Multi-gene and genome-wide ChIP studies also show that association of Spt4-Spt5 with genes begins downstream of transcription start sites, that it persists until around the site of termination and that it largely mirrors the distribution of RNAPII [39–44]. Consistent with this, staining of *Drosophila* polytene chromosomes shows that Spt5 extensively co-localizes with the elongating, Ser2-phosphorylated form of RNAPII [45, 46]. Furthermore, using a ChIP assay to follow the migration of RNAPII elongation complexes across a long (~8kb) gene in yeast [16], several groups have shown that *spt4* and *spt5* mutations affect the

translocation rate and processivity of the elongating RNAPII *in vivo* [16, 47, 48]. An *in vitro* transcription assay with *spt4*-null yeast whole cell extracts also found a positive role for the yeast factor in RNAPII transcription [49]. More recently, Spt4-Spt5 has been shown to required for normal transcription of a variety of long homopolymeric sequences including the expanded poly-CAG repeats which are found in disease associated variants of the Huntington's gene and several other neurological disease genes [50].

Although genome-wide studies suggest that Spt4-Spt5 likely acts at all genes, in some cases it may also act in a gene-specific manner. DSIF appears to play a role in TAT-mediated regulation of transcription elongation across the HIV genome [37, 38, 51]. In addition, DSIF represses transcription of the NF-κB-responsive A20 gene in an activator and core promoter-dependent fashion [52, 53].

Consistent with the idea that Spt5's function arose early in evolution, Spt4-Spt5 also associates with and appears to regulate transcription elongation by RNA polymerase I [54–56], suggesting that Spt4-Spt5 existed prior to divergence of the nuclear RNA polymerases of eukaryotes. In contrast to RNAPII and RNAPI, there is no evidence that Spt4-Spt5 associates with or regulates RNA polymerase III. In addition, the bacterial homolog of Spt5, NusG, also regulates transcription elongation [57–59]. Like Spt5, NusG associates with elongating polymerases *in vitro* [60] and ChIP analysis suggest that NusG associates with the majority of transcription units *in vivo*, joining polymerase downstream of the initiation site [61]. Finally, *in vitro* transcription assays show that the archaeal Spt4-Spt5 complex also associates with RNA polymerase and can stimulate elongation of a 10 subunit (rather than the normal 12) form of RNA polymerase that is less processive than the complete complex under low-nucleotide conditions [62], demonstrating that Spt5 functions are universally conserved.

5. What mechanisms underlie Spt4-Spt5's function?

As is the case for several other elongation factors (reviewed in [63]), including NusG and RfaH [64, 65], Spt4-Spt5 appears to promote elongation by reducing the frequency of transcription pausing or arrest [37, 66]. This may explain the multiple observations that Spt4-Spt5 only promotes transcription elongation *in vitro* when nucleotides are limiting, a condition that increases the frequency of pausing and arrest. These *in vitro* results are consistent with the genetic interactions of mutations affecting both Spt4-Spt5 and TFIIS described above. Although nucleotide concentrations are not generally limiting *in vivo*, Spt4-Spt5 may protect elongation complexes from other triggers of pausing and arrest, such as nucleosomes (see below). Spt4-Spt5 may also facilitate elongation indirectly by interacting with the nascent transcript and by assisting cotranscriptional RNA processing events including 5′ capping (discussed below), which has been suggested to have a positive role in early elongation [67].

Further work on DSIF has yielded new insights into the mechanism of its inhibitory activity in human cells. First, a second multisubunit complex, Negative Elongation Factor (NELF), is also required for DSIF activity [68]. Curiously, although Spt4-Spt5 is found in all eukaryotes, NELF is not found in plant, yeast or nematode genomes [69]. Whether or not Spt4-Spt5 inhibits early elongation in organisms that lack NELF is an open question.

A second set of insights into Spt4-Spt5 function derives from the observation that DRB inhibits P-TEFb, the protein kinase that phosphorylates the serine-2 position of the RNAPII CTD repeats [70]. Consistent with the idea that DRB's effects on transcription are mediated through its inhibition of P-TEFb, flavopiridol, an inhibitor of P-TEFb unrelated to DRB, also inhibits CTD phosphorylation and prevents RNAPII from leaving promoter proximal locations and entering into productive elongation [71, 72]. Furthermore, In addition to

modifying the CTD, P-TEFb and its yeast homolog, Bur1, also phosphorylate a conserved serine (*S. cerevisiae;* [73]) or threonine (*S. Pombe* and humans; [29, 74, 75]) in the C-terminal repeats of Spt5. Deletion analysis of the Spt5 CTR suggests that it is necessary for Spt5's ability to both repress and activate transcription [75, 76]. Phosphorylation of Spt5's CTR prevents its inhibitory function and stimulates its positive function in elongation [29, 77].

The biochemical mechanisms that underlie the function of Spt5's CTR remain to be determined. Genetic interactions suggest a functional overlap between Spt4-Spt5 and the RNAPII CTD [78, 79], DSIF can bind RNAPII independently of the RNAPII CTD [80]. The phosphorylation states of the RNAPII CTD and that of the Spt5 CTR by P-TEFb do not affect RNAPII-DSIF binding [80, 81]. While CTR phosphorylation is critical for DSIF to exert a positive role on elongation, it is not involved in the repressive activity of DSIF/NELF as assayed *in vitro* [29]. Furthermore, in contrast to *bur1* and *spt5* null mutations, deletion of the Spt5 CTR is not a lethal mutation in budding and fission yeast [73, 78, 82, 83], and does not prevent association of human Spt5 with RNAPII [75, 76]. Thus, neither the phosphorylation state of Spt5's CTR nor that of the RNAPII CTD controls Spt4-Spt5/RNAPII association.

Our understanding of transcription elongation and its role in transcriptional regulation has recently been transformed by the discovery that, in at least some eukaryotes, including *Drosophila*, mice and humans, RNAPII is frequently found just downstream from the transcription start site of genes in a transcriptionally engaged, but paused state (reviewed in [84]). Significantly, this 5′ transcriptional pausing depends upon DSIF and NELF, and release from the pause appears to be mediated by P-TEFb in a process that, in at least some cases, involves transcription activators such as c-Myc [43, 85, 86]. In line with such a pause release mechanism, recent studies have identified associations of P-TEFb and other elongation factors with components of the RNAPII coactivator complex, Mediator, and revealed functional involvement of Mediator in the transition of RNAPII from pre-initiation complex (PIC) into the elongation phase of transcription [87, 88]. This model offers a basis for understanding a functional interplay between DSIF and Mediator observed in an earlier biochemical analysis [89]. Yeast genetic data also indicate a role of Mediator in regulating the post-recruitment RNAPII activity on inducible yeast genes such as *CYC1* and *HSP82* [90, 91], although it remains to be characterized whether the mechanisms that withhold RNAPII at these yeast promoters also involves Spt5, the yeast DSIF protein.

Given its inherent structural adaptability [92, 93], the Mediator complex seems well suited for relaying induction-specific inputs to regulate the early elongation activities of RNAPII, subsequent to the formation of PIC. As has been shown for NF-κB target genes, induction-specific regulation of RNAPII elongation can be determined by enhancer sequences in conjunction with the type of core promoter, TATA-less (e.g. A20 gene) *vs.* TATA-containing (e.g. IL-8) [94]. In this case, DSIF inhibits A20 gene elongation in the uninduced state with the aid of USF1 binding to an upstream DNA sequence [53].

The relationship between Spt5's positive and negative functions is not clear. Only a few mutations have been reported to selectively remove either the positive or negative functions of Spt5, and these have yet to yield insights into the mechanism(s) of Spt4-Spt5 function [20,29]. Paused polymerases have not been clearly demonstrated in either *Saccharomyces cerevisiae* or *C. elegans*, which lack NELF. However, polymerases do appear to accumulate over promoters in these organisms under conditions of nutrient limitation [95, 96], suggesting the potential for an alternative mechanism for 5′ arrest in organisms that lack NELF. Furthermore, *S. cerevisiae* clearly possesses promoters that constitutively recruit RNAPII, TBP and other basal factors, but are regulated at a step after the assembly of these

basal transcription components [97–99]. In a high-resolution genome-wide mapping, yeast genes with promoter-proximally enriched RNAPII were identified as one of the three major classes of genes that each display a different RNAPII occupancy profile: enriched at promoters, immediately downstream of promoters, or biased toward 3′ ends [100]. These data highlight the importance to understand the detailed mechanism in yeast that underlies the RNAPII withholding at and release from promoter proximal regions.

6. Spt4-Spt5 coordinates transcription elongation with chromatin states

Several sets of observations suggest that Spt4-Spt5 may coordinate chromatin remodeling and histone modification with transcription elongation. First, in addition to the RNAPII mutations mentioned above, genetic suppressors of the cold-sensitive *spt5* allele in *S. cerevisiae* reveal an extensive set of interactions between Spt4-Spt5 and chromatin. These chromatin suppressors of *spt5* include: mutations in the ATP-dependent chromatin remodeler Chd1 [101]; the Paf1 complex [102] which associates with elongating RNAPII and coordinates a variety of histone modifications [103]; substitutions of histone H3 lysines 4 and 36 as well as mutations that inactivate their cognate methyltransferases Set1 and Set2 [48]; and subunits of the Rpd3S histone deacetylase complex [48], whose function depends upon H3K36 methylation [44]. Furthermore, mutations that inactivate the SAGA histone acetyltransferase complex also strongly enhance phenotypes of *spt4* and *spt5* mutations [48].

Interestingly, the chromatin suppressors of the *spt5* cold-sensitive mutation also alleviate the synthetic growth defects observed in *spt4 dst1* and *spt5 dst1* (*DST1* encodes TFIIS) double mutants [48]. Nucleosomes are known to provoke transcription arrest by elongating RNAPII [104]. Thus, one intriguing interpretation of this result is that Spt4-Spt5 protects elongating RNAPII from transcription arrest events provoked by nucleosomes, and that the chromatin suppressors of *spt5* identify perturbations of chromatin that decrease the frequency of nucleosome-provoked transcription arrest. These data suggest that in addition to decreasing the frequency of pausing by RNAPII, Spt4-Spt5 likely also functions to help elongating RNAPII to overcome nucleosomal barriers to elongation. This model suggests that there are two mechanisms for suppression of the *spt5* cold sensitive mutation. In the first, a slow polymerase gives the partially defective Spt5 protein additional time to act before RNAPII collides with a nucleosome, provoking a pause or arrest. In the second model, defective chromatin modification or remodeling lowers the nucleosomal barrier to elongation so that the probability of arrest and the requirement for Spt4-Spt5 function is decreased. Deeper examination of these ideas will likely require *in vitro* analysis of Spt4-Spt5 on chromatin templates.

A second set of observations suggests a mechanism by which Spt4-Spt5 may coordinate transcription with chromatin states. In *S. cerevisiae*, the P-TEFb homolog, Bur1 [105, 106], Spt4 [107] and the Spt5 CTR [73, 83] are required for recruitment of the Paf1 complex to elongating RNAPII, and for methylation of histone H3 lysine 4, one of the histone modifications regulated by the Paf1 complex [103]. Mutations of the Spt5 CTR that substitute alanines for the serine targets of Bur1 also abolish Paf1 recruitment [73]. In addition, Bur1, Spt4, and the Spt5 CTR also regulate association of the Rpd3S complex with chromatin [44]. Thus, like the RNAPII CTD, Spt5's CTR appears to act as a phosphorylation-state dependent regulator of recruitment of transcription-associated chromatin modifying activities. It is appealing to speculate that the dependencies of Rpd3S and Paf1 complex chromatin-association on Spt4-Spt5 reflect direct and phospho-regulated binding of these complexes to the Spt5 CTR. However, forms of Spt5 lacking its CTR or that have been rendered phosphorylation-defective by threonine to alanine substitutions in the CTR can be co-precipitated with the Paf1 complex [77]. Thus the CTR may act indirectly to control factor recruitment.

7. Spt4-Spt5 and pre-mRNA processing

Spt4-Spt5 has also been implicated in a second set of co-transcriptional events, RNA processing. For example, the RNA capping enzymes of budding and fission yeast bind to Spt5 [25, 108]. This interaction, which depends upon Spt5's CTR, may supply an overlapping function in addition to that mediated by RNAPII CTD-phosphorylation and the foot domain of RNAPII that assists the recruitment of capping enzyme [78, 109]. Furthermore, in *S. cerevisiae, spt4* and *spt5* mutations display synthetic growth defects with capping enzyme mutations, cause splicing defects, affect poly-adenylation site choice, and suppress certain mRNA export-defective mutations [108, 110–114]. Spt4-Spt5 interacts with the RNA export factor She2 and attracts She2 to the elongating RNAPII, thereby facilitating the recognition by She2 of the specific bud-localization signal in nascent RNA [115]. Interestingly, *spt4* and *spt5* mutations also alter rRNA processing, consistent with Spt4-Spt5's association with RNA polymerase I [54, 56, 116].

These diverse roles for Spt4-Spt5 in RNA processing in yeast likely reflect similar functions in metazoans. In *Drosophila*, Spt5 interacts with the exosome, suggesting a potential role for Spt4-Spt5 in RNA degradation [117]. A recent RNA-seq analysis showed that knockdown of Spt4 also changes splicing patterns in mammalian cells [50]. Finally, human Spt5 interacts with the capping enzyme and stimulates capping activity *in vitro* [118].

An intriguing set of observations suggests that Spt4-Spt5 may influence RNA processing via direct interactions with pre-mRNAs. First, purified Spt4-Spt5 complexes from yeast bind RNA in a gel mobility assay [55, 119]. Second, using a native gel assay to examine transcription elongation complexes *in vitro*, Price and colleagues found that DSIF is recruited more efficiently to elongation complexes with long RNA transcripts [119]. Subsequently, Gilmour and colleagues used a similar analytical strategy to show that DSIF does not associate with elongation complexes whose nascent RNA transcripts are 18-nucleotide or shorter [120]. They also showed that Spt5 in these complexes could be crosslinked to nascent transcripts that were 22-nucleotide or longer, but not those which were 18 nucleotides long. Although not in agreement with earlier data suggesting that Spt4-Spt5/RNA polymerase interactions are RNAase resistant [108, 121], these data suggest that interactions between Spt5 and RNA are an important determinant of Spt4-Spt5's interactions with RNAPII elongation complex. One intriguing possibility is that, by binding to nascent transcripts in elongation complexes, Spt4-Spt5 restricts the ability of RNAPII to backtrack and arrest, which necessarily requires that the nascent RNA be fed back into the elongation complex as it moves backwards on the template. This mechanism for an anti-pausing and -arrest activity has previously been suggested for U2AF65, which binds RNA, associates with elongating RNAPII and stimulates elongation in vitro under conditions of limiting nucleotides [122]. A more definitive test of this model awaits identification of RNA binding-defective forms of Spt5.

How might Spt4-Spt5 influence pre-mRNA processing? One possibility is that Spt4-Spt5 acts indirectly, influencing processing factor recruitment via its effects on the processivity and rate of RNAPII elongation. Consistent with this possibility, studies of splicing in yeast and metazoan systems show that elongation rate influences alternative splicing patterns (reviewed in [123]), and elongation-defective yeast strains, including *spt4* and *spt5* mutants, show upstream shifts in poly(A) site selection [112–114]. A second possibility is that Spt5's CTR participates directly in recruiting processing factors to elongation complexes. This seems to be the case in capping. Moreover, ChIP analysis indicates a contributing role for the Spt5 CTR in RNA cleavage factor I in yeast [124]. Whether or not recruitment of other pre-mRNA processing factors depends upon Spt5's CTR remains to be determined. However, the observation that *bur1* mutations interfere with the splicing of a significant

subset of yeast genes suggests that this question merits experimental analysis [111]. Finally, observations of the Spt5-RNA interaction suggest that Spt4-Spt5 may influence pre-mRNA processing reactions by binding to nascent transcripts soon after they emerge from the elongating RNAPII and possibly serving as a 'gate keeper' to regulate access of other processing factors to the nascent transcript.

8. Other functions for Spt4-Spt5?

A recent series of papers provide additional support for and insights into roles of Spt5 in regulating RNA processing. In plants, siRNA-mediated DNA methylation and gene silencing depends upon atypical RNA polymerases that are related to, but distinct from RNAPII [125]. One of these, RNA Polymerase V (RNAPV), is required for some, but perhaps not all instances of Ago4-mediated gene silencing [125, 126]. Interestingly, RNAPV associates with an Spt5 homolog, KTF1, also known as Spt5 Like (SPT5L) [127]. Like canonical Spt5, Spt5L is a large protein with a highly charged N-terminus followed by an NGN domain, several KOW domains and C-terminal repeats, in this case containing WG/GW dipeptides [128, 129]. These repeats mediate Spt5L's association with Ago4, and they resemble WG/GW repeats found in other Ago4 binding proteins [128, 129]. ChIP assays demonstrate that Spt5L associates with chromatin, and Spt5L's stability depends upon RNAPV, suggesting that it is recruited to chromatin by and functions in the context of its association with RNAPV [126]. Finally, like canonical Spt5, Spt5L binds RNA *in vitro* and *in vivo* [129].

Spt4-Spt5 has also been implicated in transcription-coupled DNA repair [82, 130, 131], a function it may execute as part of RNAPII elongation complexes. Immunoglobulin class-switch recombination and somatic hypermutation also requires mammalian Spt4-Spt5 [42, 132]. Mechanistically, Spt4-Spt5 facilitates the targeting of this pathway by specifically interacting with activation-induced cytidine deaminase (AID), a key enzyme that initiates these processes, and recruits AID to transcription sites where RNAPII dwells (or stalls). Since AID does not directly interact with RNAPII, Spt4-Spt5 most likely functions as an adaptor between AID and the RNAPII apparatus [42]. In addition, *spt4* mutations affect chromosome segregation [30], and Spt4-Spt5 is required to restrict spread of the histone H3 variant Cse4 away from centromeres [31]. Interestingly, ChIP studies show that Spt4-Spt5 can be found in the vicinity of centromeres, and that this localization may not depend upon RNAPII [31].

As with other functions of Spt4-Spt5, it is likely that its ability to facilitate co-transcriptional events is an ancient function; in bacteria, NusG is required for Rho-dependent termination [133], and may couple transcription and translation via an interaction of its KOW domain and ribosomal protein S10 [134].

9. Structure of the Spt4-Spt5 complex

Several groups have recently made progress on determining the structure of the Spt4-Spt5 complex. Guo *et al.* solved the x-ray crystal structure of an Spt4-Spt5 NGN domain fusion protein [135]. This fusion protein formed domain-swapped dimers in which the Spt4 domain from one monomeric unit of the dimer bound to the NGN domain found in the other monomer. The central feature of the Spt4-Spt5 interface is a large beta-sheet formed by alignment of 4 anti-parallel strands from each of Spt4 and Spt5, creating a large hydrophobic surface [135]. Surrounding this surface are alpha helices, which contribute charged and polar interactions that appear to hold Spt4 and Spt5 in register. Mutations targeting predicted contacts between Spt4 and Spt5 disrupted Spt4-Spt5 interactions both *in vitro* and *in vivo*, suggesting that the structure provided an accurate model of *in vivo* interactions of Spt4 and Spt5.

Subsequent structural analysis of the human [136, 137] and several archaeal [62, 138–140] Spt4-Spt5 complexes support the model of Guo *et al.* The structures of these different Spt4-Spt5 complexes superimpose on top of one another with very small deviations (Figure 1C–D). Importantly, one of the archaeal Spt4-Spt5 structures includes the single KOW domain found in archaeal members of the Spt5 protein family [138], and an NMR structure of the fifth KOW domain of human Spt5 displays a similar structure (PDB ID: 2E70).

10. Structural analysis of Spt4-Spt5/RNA polymerase interactions

It is likely that all of the diverse functions of Spt4-Spt5 are mediated in the context of its physical interactions with RNAPII. Protein mapping analysis in the human system showed that RNAPII binding involves a middle region of Spt5 encompassing the multiple KOW domains, while Spt4 binding involves the NGN domain of Spt5 [75, 76]. The RNAPII–interacting domains of Spt4-Spt5 were further mapped in archaea by Werner and collaborators. They found that in addition to binding Spt4, the Spt5 NGN domain also interacts with RNA polymerase, and is required for the transcription stimulation function of Spt4-Spt5 *in vitro* [62]. Furthermore, they observed that the affinity of the Spt5 NGN alone for polymerase was not as strong as that of the intact Spt4-Spt5 complex and that the NGN's affinity for RNA polymerase can be enhanced by including either Spt4 or recombinant Spt5 proteins with both the NGN and KOW domains in the binding reactions [62]. Thus, although direct Spt4/polymerase interactions have not been observed, Spt4 may influence Spt5/polymerase interactions.

Spt4 may also regulate the stability of Spt5. Spt5 proteins levels drop to about 1/3 of normal in yeast cells that lack Spt4 [82] and Spt4 enhances the thermal stability of archaeal Spt5 [62]. These data may explain the temperature sensitivity of some yeast *spt4* mutants [30]. Collectively, these data indicate a distributed, multi-domain interaction between Spt5 and the polymerase that is modulated by Spt4. The complexity of the interface drives home the point that we do not yet fully understand the regulation and functional importance of Spt4-Spt5/polymerase interactions.

Further structural and mutagenesis data from the bacterial and archaeal systems have identified the Clamp domain of RNA polymerase as the binding target of the NusG/Spt5 proteins [61, 62, 141]. More precisely, this interface is formed between a concave patch of hydrophobic residues on the NGN domain of NusG/Spt5 and the coiled-coil (CC) motif of the Clamp domain of the largest subunit of RNA polymerases. These structural features are also found in eukaryotic Spt5 and RNAPII, suggesting a universally conserved interaction surface.

A detailed description of the mechanisms by which Spt4-Spt5 executes its diverse functions will require a clear understanding of the structure of the entire Spt4-Spt5/RNAPII complex. However, Spt5's multi-domain structure and the likely unstructured nature of its N- and C-termini present obstacles to obtaining crystals of this large complex. Still, clever alternatives to obtaining tertiary information have been taken successfully with the archaeal homologs. Recent reviews describe much of the structural and biochemical findings in both the archaeal and bacterial systems with a reasonable extrapolation to the eukaryotic complex [142, 143]. As such, we will provide a brief summary of the main features in the current model for tertiary organization of the Spt4-Spt55 complex with RNAPII.

Initial clues to the organization of Spt5/NusG interactions with RNA polymerase came from extensive biochemical and molecular genetic analyses of RfaH, an unusual bacterial member of the NusG family. Unlike NusG, which operates at all genes, RfaH is recruited to particular genes by binding to a specific DNA sequence in the non-transcribed strand of target genes [144]. Artsimovitch and colleagues identified a potential RfaH/RNA

polymerase interaction surface in a hydrophobic pocket of the NGN domain of RfaH and in the conserved CC motif of the RNA polymerase clamp domain [141]. Mutations in these surfaces appear to disrupt RfaH/RNA polymerase interactions [141, 145, 146]. These data led to a model in which NusG spans the central cleft of elongating RNA polymerase, sealing nucleic acids in the elongation complex and thereby promoting processivity. Consistent with this model, RfaH makes a second, possibly dynamic, contact with RNA polymerase at the gate loop (GL) element of the β subunit on the other side of the central cleft, so that it completely seals the central cleft [147]. Interestingly, although the RfaH/GL interaction does not appear to make a significant contribution to the strength of the RfaH/RNA polymerase interaction, it is essential for RfaH's (and NusG's) ability to prevent pausing [145]. Importantly, the GL, CC domain of the Clamp and the hydrophobic pocket of Spt5 are all conserved features in archaea and eukaryotes.

This view of the association of NusG with RNA polymerase has subsequently been supported by structural studies of Spt5/RNA polymerase interactions in archaea. To circumvent the difficulty of crystallizing a complete Spt5/polymerase complex, Cramer's group crystallized a *Pyrococcus furiosus* archaeal complex formed between Spt4-Spt5 and elements of the Clamp domain of the cognate RNA polymerase [139]. Their structure, determined to 3.3 Å resolution, verified the predicted Spt5-Clamp interface described above and, when superimposed over multiple aligned sequences of NGN of various organisms, verified the conservation of this interface. Based on this conservation and the available 3-D structure of another archaeal (*Sulfolobus sulfataricus*) RNA polymerase, the researchers formulated a 3-D model of the archaeal Spt5/polymerase complex. Applying the same modeling procedure, they also modeled the yeast Spt4-Spt5/RNAPII (Figure. 2A) and bacterial NusG/polymerase (Figure. 2B) complexes.

In these semi-crystallographic models, the NGN/Spt4 complex (Figure 2A, *red*) binds to the Clamp (*blue*) near the tip of the CC motif (*cyan*) of Clamp, and sits over the main nucleic acid cleft in the polymerase, enclosing the DNA/RNA hybrid in RNAPII (Figure 2A). As with the bacterial complex [141, 147] these models place the NGN domain near the non-coding DNA strand in transcription bubble. This raises the possibility that Spt5/NusG may promote polymerase processivity by helping maintain the downstream edge of the transcription bubble by withholding the non-coding DNA strand through non sequence-specific interactions. Consistent with this idea, RfaH can be crosslinked to the non-transcribed strand in elongation complexes [144]. Although the X-ray data did not provide information on the location of the sole KOW domain in archaeal Spt5, a consideration of the linker length from NGN to KOW would argue against a placement of the KOW anywhere near the RNA exit pore on RNAPII [139].

The crystallographic data described above are in general agreement with a low-resolution (13 Å) structure determined by cryo-EM single particle analysis of a complete Spt4/5-polymerase complex also from *Pyrococcus furiosus* [138]. However, the EM data indicated a shift of the NGN/Spt4 structure (Figure 2A, *magenta*) toward the opposite side of the nucleic acid cleft, making contacts with domains that are correspondingly named Protrusion (*light green*) and Lobe (*dark green*) in the RNAPII structure. While the KOW was not observed in the crystal structure, the EM map revealed a region of density (Figure 2A, *cross-hashed*) that could be docked with a KOW structure. This places the archaeal KOW near the upstream end of the transcription bubble, but its weak connection to the NGN/Spt4 density dampens the confidence of its identification.

Could the difference between the crystallographic and EM results reflect different aspects of the same complex? A closer examination of the EM structure indicates that the site of NGN contact on the Lobe domain is located at the tip of Lobe, which shares similarity to the

flexible GL element in the bacterial RNA polymerase. Two possible mechanisms consistent with the current data of GL activity can be envisaged: (i) the flexible GL structure may serve as a dynamic gate to 'seal' off the gap left at the top of the nucleic acid cleft after the binding of an NGN domain-containing elongation factor; and/or (ii) the edge of the bound NGN domain may contact the GL in a dynamic fashion to close the remaining space and/or alter the intrinsic activity of RNA polymerase [147]. Either of these scenarios will yield a population-averaged structure similar to the EM image of Klein et al. in which the protein mass will appear to be continuous. In this sense, the GL, Clamp and NGN domains may associate in multiple modes, resulting in visualization of complexes with ambiguous structures due to superposition of multiple related but subtly distinct structures, a result obtained often in structural analysis of transcription and signaling networks [109, 148, 149].

11. Mechanistic Insights

The current structural model for Spt4-Spt5 bound to RNA polymerase offers a simple mechanism to explain their functions in transcription elongation. The binding of Spt4-Spt5 to the RNAPII elongation complexes will completely encircle the DNA template and RNA/DNA hybrid. The NGN domain may also affect the dynamics of the Clamp. The Clamp is a major structural element that RNAPII uses to engage DNA and to hold the 8–9 base pair DNA/RNA hybrid before the strands separate [150]. Five loop structures (named Switch 1–5) are located near the base of this mobile domain and are seen in the ternary complex making intimate interactions with nucleic acids adjacent to the NTP addition site [150, 151]. Mutations of Clamp residues conceivably impact transcription functions such as the processivity during early stages of elongation, as has been demonstrated for a Switch-2 region mutant [152]. Either by directly restraining the DNA and RNA in the cleft, or allosterically affecting the Switch conformations, or combined, these mechanisms may altogether prevent disassembly of elongation complexes and thus ensure processivity.

Spt4-Spt5 appears to interact exclusively with structural elements on the surface of RNAPII and not directly probe the active site of the polymerase. The best current data indicate the Spt4-Spt5's closest point of contact with the substrate is its interactions with the non-coding DNA strand, and this is located over the top of the cleft, away from the catalytic center. If Spt4-Spt5 exerts a conformational influence on RNAPII active site, it is most likely achieved via allosteric mechanisms. This stands in contrast to the direct mechanism used by elongation factor TFIIS (or SII). Extensive biochemical and structural analyses show that TFIIS binds RNAPII and inserts its Zn-ribbon domain (domain III) into the secondary channel (also called Funnel) of the polymerase, and, in an almost acrobatic fashion, projects its β hairpin of Zn-ribbon domain close to the polymerase catalytic site [153]. The net result of this binding is a precise placement of three charged groups (R287, D290 and E291) at the tip of the hairpin right next to the polymerase catalytic center where phosphodiester bond formation (and its reversal hydrolysis) occurs. This structural model satisfactorily explains the TFIIS function in stimulating the intrinsic RNA cleavage activity and proofreading function of RNAPII [154, 155]. Chiefly, the charged loop-tip residues of TFIIS augment the RNAPII catalytic center in its capacity to stabilize the second Mg ion and also a water molecule that attacks the scissile phosphodiester bond [156, 157]. Additionally, the inserted TFIIS hairpin appears to help maintain the trigger-loop of the largest subunit in a catalytically functional conformation.

Interestingly, like Spt4-Spt5, the general transcription factor TFIIF also appears to use indirect mechanisms to regulate polymerase elongation. (Whether its other functions during PIC assembly involve direct interactions with partner factors such as TFIIB is currently unclear.) Stimulation of elongation has been well demonstrated for metazoan TFIIF [158–160], although not for its fungal counterpart. Biochemical mapping using protein cleavage

and crosslinking-coupled mass spectrometry has demarcated TFIIF's binding sites on RNAPII: the two large subunits of yeast TFIIF interact with the Rpb2 Lobe and Protrusion domains that form one side of the DNA/RNA cleft [161, 162]. The binding area seems to extend toward the Rpb1 Jaw as revealed in the latest structural visualization using EM [163]. All the binding sites that have been elucidated to date are distributed on the surfaces that are immediately outside of the DNA/RNA cleft; current data do not indicate a close approach of any TFIIF element into the cleft or catalytic center. This binding strategy likely explains the functional competition of TFIIF with the newly identified negative regulator Gdown1 of mammals [163–165]. Since mammalian TFIIF also functionally competes with DSIF [166], the question arises: does TFIIF also compete with Spt4-Spt5 for a common binding site(s)? Observing functional interactions between a TFIIF mutation and a Switch-2 mutation, Ponticelli and coworkers proposed an allosteric mechanism in which TFIIF can indirectly affect the conformation around the polymerase cleft and hence the stability of early elongation complexes having short transcripts (e.g. 5-mer) [167]. It remains to be tested whether Spt4-Spt5 can 'remotely' modulate RNAPII conformation at interiors of the cleft (e.g. the Switches) or even the active site.

These structural models suggest that Spt4-Spt5/NusG proteins bind polymerase at sites that are also bound by transcription initiation factors. In bacteria, sigma factor binds to the CC domain in the Clamp [168, 169], and RfaH functionally competes with sigma factor [147, 170]. In eukaryotes, initiation factor TFIIE binds to the Clamp of RNAPII [162] at a site that appears to overlap the NGN binding site. This conflict suggests that TFIIE and Spt4-Spt5 may compete for access to the Clamp. Werner and coworkers used single-molecule FRET to directly address this problem in archaea [171]. Their results confirm (i) an overlap (*e.g.* the shared CC motif) between the RNA polymerase binding sites of the archaeal TFIIE and Spt4-Spt5, and (ii) the predicted binding competition between these factors [171]. More interestingly, their transcription activity assay reveals that the relative abilities of Spt4-Spt5 and TFE to compete for polymerase depends upon the functional state of the polymerase complex: TFE out-competes the inhibitory effect of Spt4-Spt5 on pre-initiation complex assembly; whereas Spt4-Spt5 displaces TFE from the elongation complex, and hence may exert a positive effect on transcription by facilitating the factor handover process during the transition from initiation to elongation [41, 172].

Factor competition during the initiation-to-elongation transition may also be coordinated with progression of the early elongation complex, particularly in terms of accumulated length of the transcript. Cheng and Price have shown that DSIF enjoys an increased affinity toward complexes with longer RNAs (>35 nucleotides) [119], consistent with the RNA gel-shift data mentioned earlier, RNA-Spt5 crosslinking within an elongation complex [120], and the proposal that Spt4-Spt5 directly interacts with the nascent transcript emerging from the RNA exit pore. If so, one may speculate that Spt4-Spt5 bridges the Rpb1 Clamp and the 5′ RNA to form a structural relay that could coordinate the status of 5′ end of the transcript with events occurring inside the central cleft or even at the 3′ end of the transcript. It might also be possible that such bridging could serve as a ratchet to help prevent the 5′ RNA from retrograde movement and hence reduce pausing. A similar binding organization on RNAPII has been demonstrated for the RNA splicing factor U2AF65 as mentioned earlier. Progressive change of RNAPII conformation along the transcription coordinate (from initiation to promoter clearance to elongation) might be another parameter that influences Spt4-Spt5 interactions [66]. This mechanism might be especially significant when RNAPII traverses from ~20 to ~30 nucleotides during which range the polymerase displays a change of behaviors in terms of its tendency to backtrack [173] and slip over repetitive sequences [174].

Several additional difficulties may be anticipated for efforts to solve the complete structure of eukaryotic Spt4-Spt5 complexes. First, these Spt5 proteins contain five to six KOW domains separated by linker sequences that are predicted to be disordered (Fu, unpublished). It is reasonable to expect that some of these domains directly participate in coupling RNAPII elongation with various other nuclear pathways including nucleosome modification, RNA processing and DNA modifications. However, it is not known if the different KOW domains serve distinct or overlapping functions. Furthermore, if two or more of the KOW domains can interact with the same surface on RNAPII, it may be difficult to obtain a uniform population of complexes for structural studies of Spt4-Spt5/RNAPII interactions. In addition to problems presented by the KOW domains of eukaryotic Spt5, protein disorder prediction algorithms indicate that both the N-terminal acidic and the CTR regions do not adopt a stable fold (Fu, unpublished). Thus, as is the case for the RNAPII CTD [175], Spt5's N- and C-termini are likely not amenable to crystallographic analysis.

How could Spt4/5's association with RNAPII be regulated by other cellular factors? Gaynor and coworkers have identified consensus arginine motifs between human KOW domains 3 and 4 that are recognized by protein arginine methyltransferase (PRMT) 1 and 5 [176]. Inhibition of methylation, either by mutating the targeted residues or by using a methylation inhibitor, enhanced Spt4-Spt5 activity in terms of both DRB-induced inhibition and Tat-induced activation. Further, they have found that methylation inhibition is associated with increased association of Spt4-Spt5 with the IL-8 promoter as assayed by ChIP and elevated association with RNAPII as measured by immunoprecipitation and Western blotting. The broader significance of these findings has yet to be tested in a genome-wide study. However, it is conceivable that PRMT-mediated methylation of Spt5 may modulate the stability of elongation complexes and promote their turnover during termination; facilitated removal of Spt4-Spt5 may allow RNAPII to disengage from the DNA template and be prepared for reinitiation by leaving an un-obstructed nucleic acid cleft. An understanding of how methylation of Spt5 modulates its association with RNAPII will require structural information as to where KOW 3 and 4 are placed on the surface of RNAPII.

12. Perspectives

Tremendous progress has been made in elucidation of the structure and function of Spt4-Spt5 complexes. We now have a clear view of the deep structural and functional conservation shared among members of the Spt5/NusG family of proteins. However, a comprehensive structural framework is not yet available for understanding how Spt4-Spt5 engages and modulates the properties of elongating RNAPII nor for understanding how other nuclear functions are temporally and mechanistically linked to transcription elongation. Future challenges include: obtaining more complete structures for Spt4-Spt5/RNA polymerase complexes; determining how Spt4-Spt5 impacts the enzymatic mechanisms of RNAPII and its translocation down a DNA template; understanding how Spt4-Spt5 may participate in regulatory transitions from initiation to elongation and elongation to termination; and more deeply examining Spt4-Spt5's roles in RNA processing and chromatin remodeling.

Acknowledgments

This work was supported in part by grants to GAH and JF from the NIH.

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Highlights

- Spt4-Spt5 is a positive and negative regulator of transcription elongation.
- Spt5/NusG proteins are found in all organisms and are essential for life.
- Spt4-Spt5 links transcription elongation to chromatin remodeling and RNA processing.
- Structural studies are beginning to provide insights into Spt4-Spt5 function.

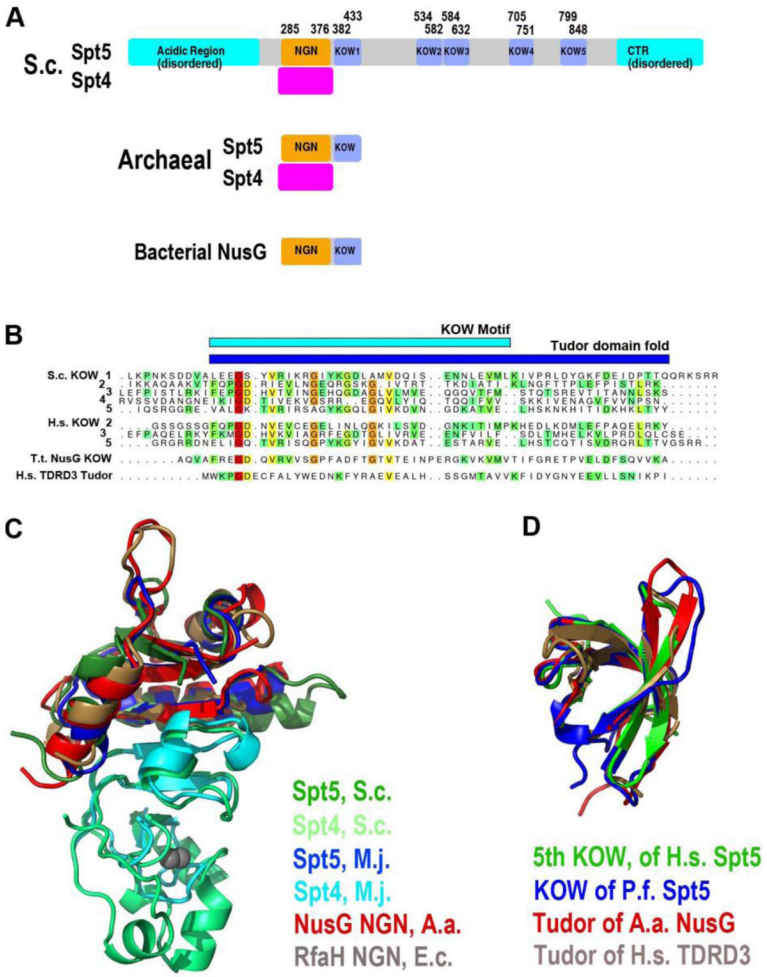

Figure 1.

(A) Domain organization of Spt4 and Spt5 proteins. Domain boundaries are drawn to proportions according to their polypeptide lengths. (B) Alignment of sequence segments from Spt5 and NusG proteins that encompass the KOW motif. Note a Tudor domain sequence of TDRD (Tudor domain-containing protein 3) is also included. Color variation (from red to green) reflects degree of conservation (invariant to similar). (C) Structural superposition of homologous Spt4 and Spt5 proteins. Protein IDs are indicated with the different colors. (D) Structural superposition of KOW domains of Spt5 and NusG together the Tudor domain of TDRD3. Abbreviations: S.c. - *Saccharomyces_cerevisiae*; M.j. - *Methanococcus jannaschii*; A.a. - *Aquifex aeolicus*; P.f. - *Pyrococcus furiosus*; T.t. - *Thermus thermophilus*; E.c. - *Escherichia coli*; H.s. - *Homo sapiens*; and TDRD3 - Tudor domain-containing protein 3.

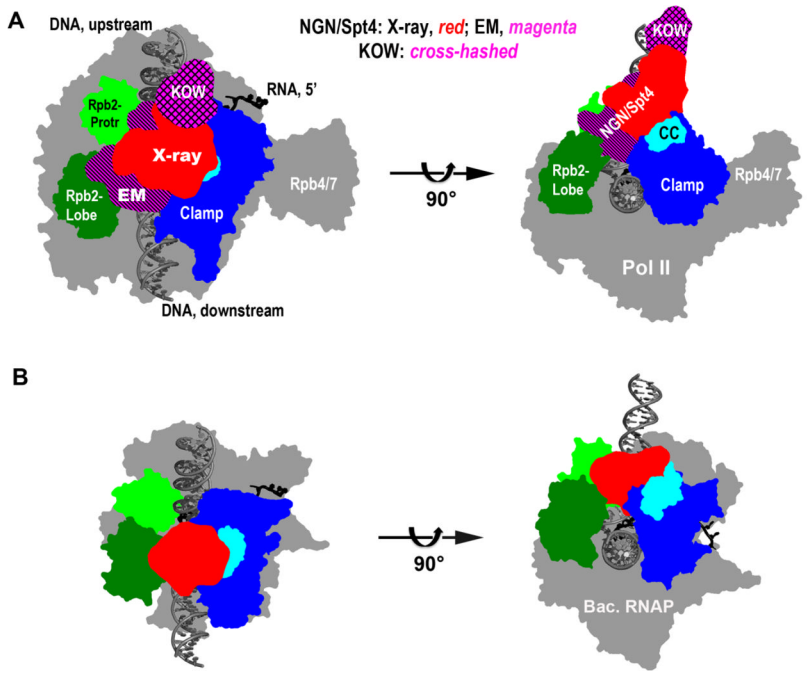

Figure 2.

Quaternary organization of (A) Spt4-Spt5-RNA polymerase elongation complex of archaea and eukaryotes and (B) bacterial NusG-polymerase elongation complex. Left hand images present a top view of the complexes and right hand images a front view. Significant structural domains are marked and colored differently. Two locations of the Spt5 NGN domain are included in *A*, with the X-ray and EM results rendered in *red* and *magenta*, respectively.