Site-specific phosphorylation of yeast RNA polymerase I

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Ich erkläre hiermit, dass ich diese Dissertation selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Diese Arbeit war bisher noch nicht Bestandteil eines Prüfungsverfahrens; andere Promotionsversuche wurden nicht unternommen.

Regensburg, 24.04.2008

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1 Introduction

1.1 Cell growth and ribosome biogenesis

A growing cell needs to synthesize numerous biomolecules to gain size and to divide. Especially a large number of proteins must be produced, as they are either directly or indirectly involved in almost every cellular function. Prerequisite to this task is the provision of a sufficiently large quantity of ribosomes (Rudra and Warner, 2004). The cells in a logarithmically growing culture of the budding yeast *Saccharomyces cerevisiae* contain about 200.000 ribosomes and accordingly need to produce up to 2000 new ribosomes per minute during each round of the cell cycle (Warner, 1999).
Ribosome biogenesis is a very complex and consumptive process (Figure 1) (Tschochner and Hurt, 2003). The mature yeast ribosome consists of four different ribosomal RNAs (rRNA) plus 79 ribosomal proteins (RP), arranged in two separate subunits (Wilson and Nierhaus, 2003; Link *et al*., 1999; Gerbasi *et al*., 2004). The production of these components involves a considerable portion of the capacities of the cellular transcription, translation and transport machineries. For instance the transcription of rRNA by RNA polymerase I (Pol I) accounts for 60% of the total transcription of a yeast cell (Warner, 1999). 50 % of all RNA polymerase II (Pol II) transcription initiation events give rise to a messenger RNA (mRNA) for one of the ribosomal proteins, which in turn need to be translated and the products transferred into the nucleus. The successive assembly of the pre-ribosomes, their maturation and intracellular transport involves about 150 ribosome biogenesis factors and 100 snoRNPs (Tschochner and Hurt, 2003; Kressler *et al*., 1999).

Thus, while a high rate of ribosome production is required for a fast growth rate, the associated consumption of a high percentage of the cells resources necessitates a very tight regulation in dependence of the growth conditions, like the availability of nutrients (Warner, 1999). The status of these determinants for cell growth is forwarded to key regulatory steps of the ribosome biogenesis machinery via signal transduction pathways, including the TOR (*T*arget *O*f *R*apamycin) and PKA (*P*rotein *K*inase *A*) pathways (Warner, 1999; Rudra and Warner, 2004). In the resulting regulatory network different positive or negative signals are integrated to precisely adjust the activities of the target proteins. Intracellular signal transduction is commonly accomplished via reversible phosphorylation through protein kinase cascades. Phosphorylation at specific sites modulates the functions of the enzymes in the pathway and finally of the target proteins, *e.g.* by changing the activities, facilitating specific interactions or initiating transports to other cellular compartments (Cohen, 2002; Hunter, 2000; Pawson and Scott, 2005).

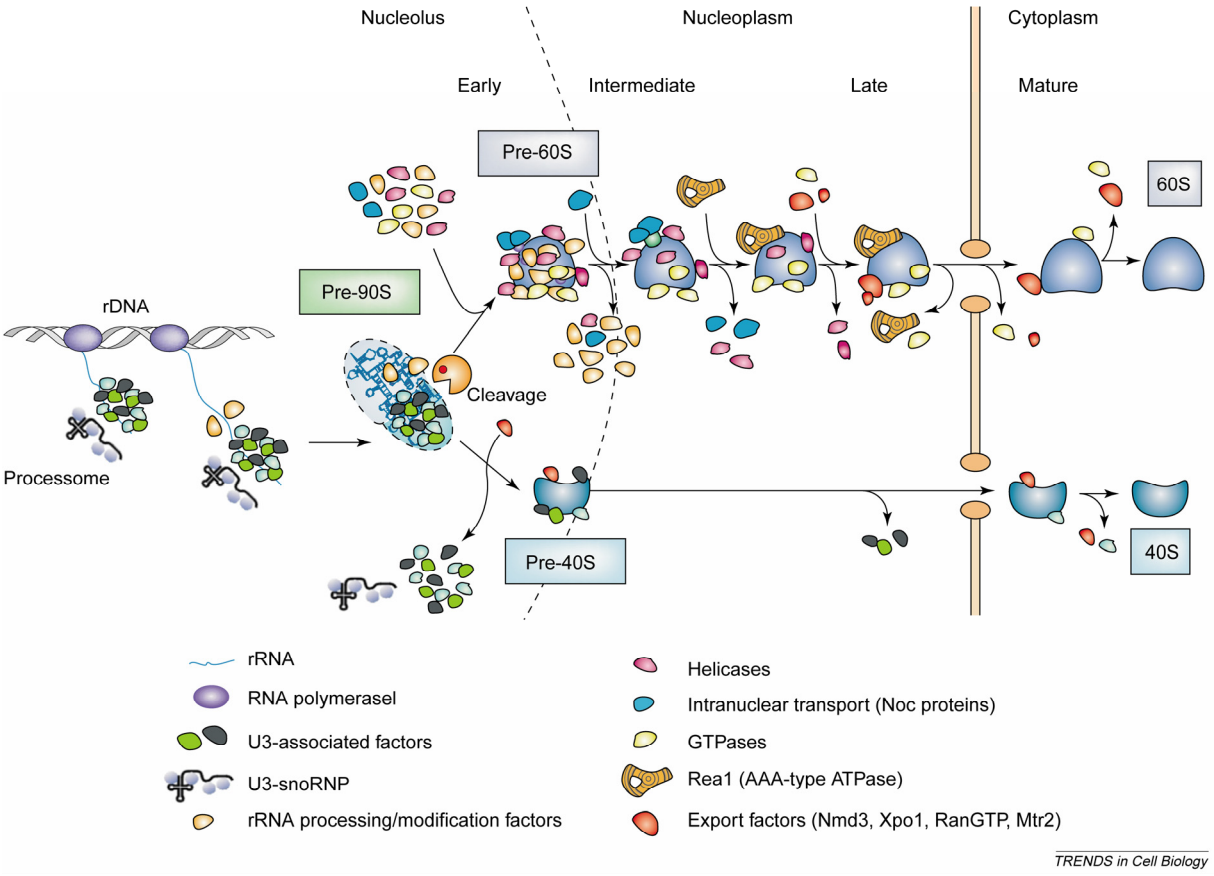

Figure 1. Overview of ribosome biogenesis. The rRNA is transcribed as one large polycistronic precursor by RNA polymerase I and subsequently cleaved during several maturation steps. About 150 ribosome biogenesis factors associate dynamically to the intermediate complexes and about 100 snoRNPs guide the modifications of the rRNA. Furthermore the 79 ribosomal subunits are assembled during the maturation steps to yield the large 60S and the small 40S ribosomal subunits. [from Tschochner and Hurt, 2003]

About 30 years ago, *in vivo* phosphorylated subunits have been discovered in the yeast Pol I complex (Bell *et al*., 1976; Bell *et al*., 1977a; Buhler *et al*., 1976b) (see 1.2.5). As one of the first steps of ribosome biogenesis, the transcription of the large rRNA-precursor by Pol I is also one of the main targets for regulation, and a number of experiments have shown that Pol I activity is linked to its phosphorylation state (Fath *et al*., 2001; Fath *et al*., 2004). However, little is known about the positions and functions of these post-translational modifications.

1.2 The RNA Polymerase I transcription system of *Saccharomyces cerevisiae*

1.2.1 Cellular localization, template and promoter of Pol I

1.2.1.1 The nucleolus

The major site of ribosome biogenesis in eukaryotic cells is the nucleolus, a specialized sub-compartment of the nucleus. This highly dynamic subnuclear organelle assembles around the cluster of tandemly repeated ribosomal DNA (rDNA) genes upon the presence of rRNA precursor transcripts (Trumtel *et al*., 2000; Dousset *et al*., 2000) and is large enough to be visible in conventional light microscopy. Under the electron microscope (EM), three morphological different regions of the nucleolus can be distinguished (Léger-Silvestre *et al*., 1999) (Figure 2). Early to late steps of ribosome biogenesis have been assigned to these structures according to protein localizations, in-situ hybridizations or aberrant morphologies in conditional mutants (Léger-Silvestre *et al*., 1999, Oakes *et al*., 1998; Trumtel *et al*., 2000). The rDNA is localized in the fibrillar center (FC). Pol I is concentrated at the boundary between the FC and the surrounding dense fibrillar component (DFC), and apparently this is also the site of rDNA transcription. The nascent pre-rRNA seems to spread into the DFC, where the first processing steps occur. Further ribosome maturation occurs in the granular component (GC), before the assembled ribosomal subunit precursors leave the nucleolus.

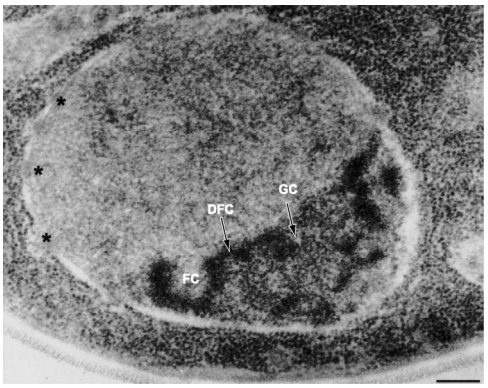

Figure 2. The nucleolus of *Saccharomyces cerevisiae*. The electron micrograph shows the nucleus surrounded by the nuclear envelope, including nuclear pores (asterisks). The nucleolus can be seen as a crescent-shaped density inside the nucleus. Three morphological different regions can be distinguished in the nucleolus: the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC). The bar represents 200 nm. [from Léger-Silvestre *et al*., 1999]

Interestingly, the nucleolus is not exclusively dedicated to the production of ribosomes (Pederson, 1998; Boisvert *et al*., 2007). Throughout the last years, additional functions of this organelle have been reported, linking it to the regulation of mitosis and cell-cycle progression, stress-response or the maturation of other ribonucleoprotein particles like snRNPs (Visintin and Amon, 2000; Boisvert *et al*., 2007; Gerbi *et al*., 2003).

1.2.1.2 The ribosomal DNA genes

Multiple copies of the rDNA genes exist in all eukaryotic organisms studied so far. In *S. cerevisiae* a cluster of approximately 150 rDNA repeats is localized on chromosome XII (Schweizer *et al*., 1969; Petes 1979). Each of the head-to-tail arranged 9.1 kb rDNA units contains a gene for the 35S pre-rRNA (Bell *et al*., 1977b; Nath and Bollon, 1977) (Figure 3), but only approximately 50 % are actively transcribed by Pol I at a time (Dammann *et al*., 1993). The active genes seem to be randomly distributed in the 150 repeats and can be distinguished from the inactive genes by their chromatin structure (Dammann *et al*., 1993; Dammann *et al*., 1995).

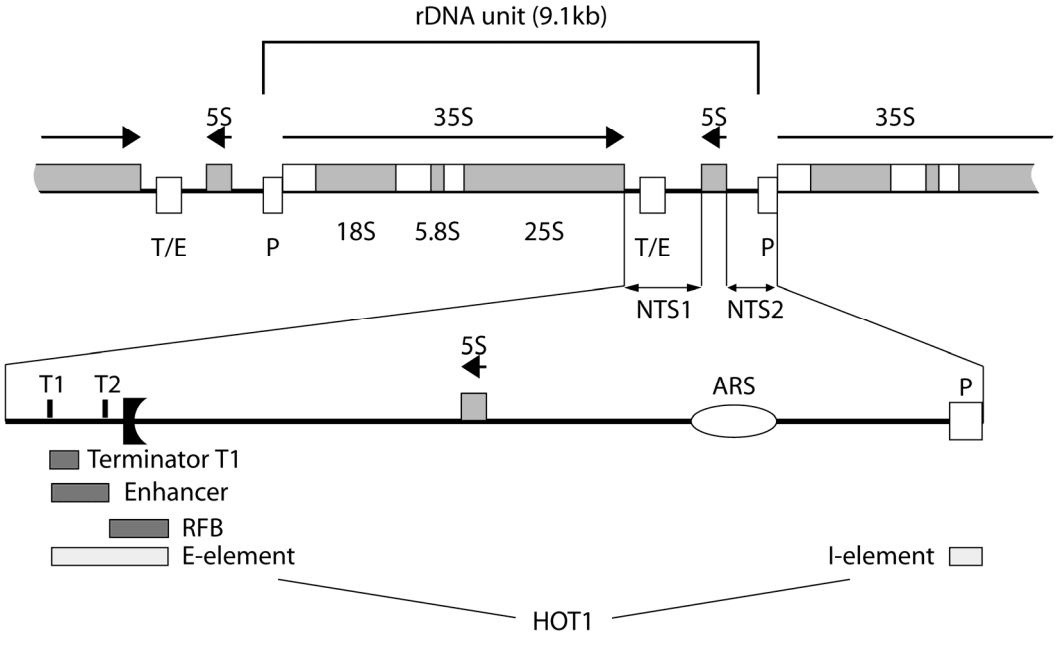

Figure 3. Scheme of a yeast rDNA unit. The 18S, 5.8S and 25S rRNA are transcribed as a 35S polycistronic precursor rRNA by Pol I. The gene for the 5S rRNA transcribed by Pol III is located between two 35S rRNA genes and coded on the opposite strand. A number of *cis*-acting elements were identified in the non-transcribed spacers (NTS): Pol I promoter (P); Pol I terminator / enhancer (T/E); autonomous replication sequence (ARS); replication fork barrier (RFB); recombination hot-spot 1 (HOT1). [from Nomura *et al*., 2004 (modified)]

The 35S pre-rRNA includes three of the four rRNA species found in mature ribosomes, transcribed as one large polycistronic precursor. In 5'→ 3' direction, the 18S rRNA (the RNA component of the small ribosomal subunit) is followed by the 5.8S and the 25S rRNAs (both RNA components of the large ribosomal subunit, along with the 5S rRNA) (Udem and Warner, 1972; Bell *et al*., 1977b; Nath and Bollon, 1977). They are separated on the primary transcript by the two internal transcribed spacer sequences ITS1 and ITS2 and framed by the 5' and 3' external transcribed spacers (5'- and 3'-ETS, respectively). As an exception to most other eukaryotic organisms, in yeast the 5S rRNA gene which is transcribed by RNA polymerase III (Pol III) is also included in each rDNA unit (Rubin and Sulston, 1973). It is located within the intergenic spacer (IGS) between the 35S rRNA genes, coded on the opposite DNA-strand and accordingly transcribed into the other direction (Kramer *et al*., 1978; Philippsen *et al*., 1978). Despite the spatial proximity of the two rDNA genes, there is no apparent direct coupling of their transcription, *e.g.* via *cis*-acting elements (Paule, 1998; Neigeborn and Warner, 1990).

A number of *cis*-elements were identified in the IGS region and have been extensively studied (Figure 3). They include an autonomous replication sequence (ARS) in each rDNA unit and a replication fork barrier (RFB), allowing DNA replication only in the same direction as Pol I transcription (Brewer and Fangman, 1988; Brewer *et al*., 1992). The RFB-element was also shown to be part of a recombination hot spot termed HOT1 (Voelkel-Meiman *et al*., 1987; Ward *et al*., 2000) along with an adjacent element originally termed Pol I enhancer (Elion and Warner, 1986; Wai *et al*., 2001) and the Pol I promoter (Musters *et al*., 1989). Pol I transcription terminator elements can be found at the 3' end of the 35S rDNA gene, but also directly upstream of the Pol I promoter (Lang and Reeder, 1993; Lang and Reeder, 1995; Reeder *et al*., 1999; Kulkens *et al*., 1989; Morrow *et al*., 1989). The function of the upstream terminator does not seem to be related to transcription termination, because unlike described for metazoan rDNA repeats, no additional functional Pol I promoters in the IGS (termed spacer promoters) were found in yeast (Paule, 1998; Moss and Birnstiel, 1979; Murtif and Rae, 1985; Cassidy *et al*., 1987). Moreover the 'ribomotor' model has been proposed describing a direct interaction between factors bound to the 5'- and 3'-terminator elements, resulting in consecutive loops of the 35S rRNA gene and the IGS including the 5S rRNA gene (Kempers-Veenstra *et al*., 1986; Kulkens *et al*., 1992; Planta, 1997). As a consequence Pol I molecules terminating at the end of the transcript would be in close proximity to the promoter for re-initiation, thus facilitating the high processivity of the Pol I transcription system.

1.2.1.3 The rDNA promoter and Pol I transcription factors

The Pol I promoter in the rDNA of *S. cerevisiae* has been mapped to the region from - 155 to + 24 base pairs (bp) relative to the transcription start site of the 35S rRNA (Musters *et al*., 1989). It consists of two DNA elements (Figure 4), namely the core element (- 28 to + 8 bp), an essential element spanning the transcription start site, and the upstream element (- 146 to - 50 bp), which is dispensable for transcription initiation *in vitro*, but required to achieve high levels of transcription (Musters *et al*., 1989; Kulkens *et al*., 1991; Keys *et al*., 1996; Kenner *et al*., 1998). The correct spacing between the elements was demonstrated to be crucial for efficient transcription initiation (Musters *et al*., 1989; Choe *et al*., 1992). Most of the transcription factors binding to the promoter elements are specific for the Pol I transcription system (Nogi *et al*., 1991b), but also more common proteins like the histones H3 and H4 or the TATA-binding protein (TBP) are involved (Keener *et al*., 1997; Cormack and Struhl, 1992; Schultz *et al*., 1992; Hernandez, 1993).

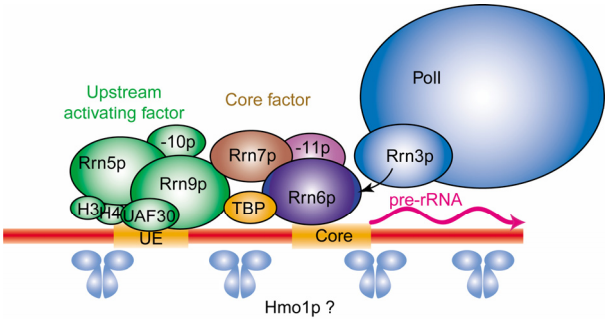

Figure 4. Pol I initiation complex at the yeast rDNA promoter. The multiprotein factors UAF (upstream activating factor) and CF (core factor) bind to the UE (upstream element) and core element, respectively, connected by TBP (TATA binding protein). The initiation-competent Pol I-Rrn3p-complex binds to the Rrn6p-subunit of the core factor to initiate transcription. Hmo1p can be found throughout the complete rDNA gene; its role in transcription initiation at the rDNA promoter is unclear. [from Moss, 2004]

The upstream element is recognized by the multiprotein complex 'upstream activation factor' (UAF) (Keys *et al*., 1996). It consists of six subunits: Rrn5p, Rrn9p, Rrn10p, Uaf30p and the histones H3 and H4 (Keys *et al*., 1996; Siddiqi *et al*., 2001; Keener *et al*., 1997). Besides its stimulatory function, UAF has been shown to be crucial to maintain an epigenetic state, which allows only Pol I transcription from this promoter. In yeast strains carrying the deletion of a gene for any of the Pol I specific components of UAF, a polymerase switch phenotype can be observed, *i.e.* the rRNA is transcribed from the chromosomal locus by Pol II, albeit with an altered transcription start site (Vu *et al*., 1999; Oakes *et al*., 1999; Siddiqi *et al*., 2001).

The ‘core factor’ (CF) is another Pol I specific multiprotein transcription factor. It binds to the essential core element and is sufficient for a basal transcription level *in vitro* (Kulkens *et al*., 1991; Keener *et al*., 1998). The three components of the core factor are Rrn6p, Rrn7p and Rrn11p (Keys *et al*., 1994; Lalo *et al*., 1996; Lin *et al*., 1996). The TATA-binding protein (TBP) was also described to complex with CF, but it is not required for the basal *in vitro* transcription (Keener *et al*., 1998). Moreover it was shown to interact with components of both, UAF and CF, thus helping to recruit CF to the promoter after the initial binding of UAF (Steffan *et al*., 1996; Steffan *et al*., 1998).

The resulting assembly is capable to recruit an initiation-competent form of Pol I, *i.e.* Pol I in complex with the specific transcription factor Rrn3p (Yamamoto *et al*., 1996; Milkereit and Tschochner, 1998). Rrn3p is required for Pol I transcription initiation (Keener *et al*., 1998), and the template-independent pre-formation of the Pol I-Rrn3p-complex (Yamamoto *et al*., 1996; Schneider and Nomura, 2004) seems to be a crucial step in the regulation of Pol I transcription (Milkereit and Tschochner, 1998; Peyroche *et al*., 2000; Claypool *et al*., 2004; Laferté *et al*., 2006). Rrn3p bridges Pol I to the Rrn6p subunit of CF, thus bringing the polymerase into the correct position for transcription initiation (Peyroche *et al*., 2000).

Additionally, Hmo1 can be found at the rDNA promoter, as well as throughout the complete rDNA gene (Hall *et al*., 2006). It has a stimulating effect on Pol I transcription and is synthetic lethal with some non-essential Pol I subunits (see 1.2.3.5 and 1.2.3.6) (Gadal *et al*., 2002; Berger *et al*., 2007), but its function is not known yet. Hmo1 is also present at the promoters of many genes coding for ribosomal proteins (RPs) (Hall *et al*., 2006), and was suggested to be involved in the coordination of rDNA transcription and the expression of RPs (Berger *et al*., 2007).

1.2.2 Pol I is one of three conserved nuclear multisubunit RNA polymerases

1.2.2.1 The nuclear RNA polymerases and their functions in the cellular transcription apparatus

Pol I is one of three nuclear RNA polymerases present in all eukaryotic cells (Roeder and Rutter, 1969; Roeder and Rutter, 1970; Ponta *et al*., 1971; Adman *et al*., 1972). After the initial characterizations of a *bona fide* single eukaryotic RNA polymerase (Weiss and Gladstone, 1959; Weiss, 1960; Furth and Loh, 1963; Frederick *et al*., 1969), the transcription activity in crude nuclear extracts was found to be further separable on DEAE anion exchange chromatography columns (Roeder and Rutter, 1969). The order of their elution gave also rise

to the nomenclature of RNA polymerase I, II and III (or RNA polymerase A, B and C, respectively).
Recently the ongoing genome sequencing projects of various organisms have revealed a fourth subclass of nuclear RNA polymerases in plants, referred to as RNA polymerase IV (Pol IV) (Arabidopsis Genome Initiative, 2000; Vaughn and Martienssen, 2005). However, the corresponding genes are absent in all fungi, animals and protozoa sequenced so far. According to phylogenetic analyses, Pol IV is most likely a variant of Pol II, which evolved from the duplication of the genes for the two largest subunits (Luo and Hall, 2007).

Each nuclear RNA polymerase synthesizes specific classes of RNAs. As already mentioned, RNA polymerase I transcribes the large polycistronic precursor for three of four ribosomal RNAs (Tocchini-Valentini and Crippa, 1970; Zylber and Penman, 1971; Reeder and Roeder, 1972; Nogi *et al*., 1991b). Apparently this is the only essential role of Pol I as demonstrated by the possibility to rescue deletion strains of essential Pol I subunits. Functional 35S rRNA can be produced from cloned rDNA genes under the control of a Pol II promoter to complement the loss of Pol I function (Nogi *et al*., 1991a; Nogi *et al*., 1991b).
The primary but not exclusive role of RNA polymerase II is the transcription of mRNAs from protein-coding genes (Suzuki and Giza 1976; Detke *et al*., 1978; Lee and Young, 2000). Furthermore Pol II is involved in the production of many non-coding RNAs including snRNAs, snoRNAs, siRNAs and microRNAs (Kiss, 2004; Costa, 2005; Mattick and Makunin, 2006).
Pol III has two main functions in the cellular transcription apparatus, namely the production of the tRNAs and of the small 5S rRNA (Weinmann and Roeder 1974; Weil and Blatti 1976). Additionally the U6 snRNA is synthesized by this RNA polymerase (Kiss, 2004).
The plant-specific Pol IV appears to be dedicated to the production of siRNAs (Zhang *et al*., 2007).

One characteristic feature often used to distinguish the three common nuclear RNA polymerases in experiments is their different sensitivity to the inhibitor α-amanitin, a toxic cyclic octapeptide from the death cap mushroom *Amanita phalloides* (Wieland, 1968). While Pol II is completely inhibited at low concentrations, Pol III can resist higher inhibitor concentrations, and Pol I is insensitive to α-amanitin (Lindell *et al*., 1970; Kedinger *et al*., 1970; Bushnell *et al*., 2002). In yeast however, Pol I was found to be even more α-amanitin-sensitive than Pol III (Schultz *et al*., 1976; Valenzuela *et al*., 1976b).

1.2.2.2 Composition of yeast Pol I

The gene-specificity of the RNA polymerases is accomplished through interactions with other proteins of the different transcription machineries, including the corresponding transcription factors which recognize specific promoter elements. The enzymatic transcription activity is identical for all RNA polymerases. This is reflected by the high level of conservation between the cellular RNA polymerases (eukaryotic nuclear RNA polymerases, as well as the RNA polymerases of eubacteria and archaea) (Allison *et al*., 1985; Berghöfer *et al*., 1988; Langer *et al*., 1995). They are all multiprotein complexes, consisting of two large subunits, which harbor the active center, and a number of smaller subunits, required for enzyme assembly, regulation and protein interactions (Cramer, 2002).

Table 1. The subunits of yeast RNA polymerase I, their counterparts in other RNA polymerases and the genes coding for the Pol I subunits.

	Protein							Gene					
Subunit	MW (kDa, calculated)	amino acids	pI	postransl. modifications	homolog. bRNAP	homolog. Pol II	metal	gene locus	alias	systematic name	chromosome	bp	knockout
A190	186,430	1664	7,05	phos; sumo	β'	Rpb1	Mg; Zn	RPA190	RRN1	YOR341W	XV (960982 - 965976)	4995	lethal
A135	135,741	1203	7,61		β	Rpb2	Mg; Zn	RPA135	RRN2	YPR010C	XVI (581193 - 577582)	3612	lethal
A49	46,650	415	10,36			-		RPA49		YNL248C	XIV (182607 - 181360)	1248	conditional
A43	36,224	326	4,63	phos		Rpb7		RPA43		YOR340C	XV (960177 - 959197)	981	lethal
AC40	37,686	335	5,25		α	Rpb3		RPC40	RPC5	YPR110C	XVI (746833 - 745826)	1008	lethal
A34.5	26,875	233	9,47	phos		-		RPA34		YJL148W	X (140355 - 141056)	702	viable
ABC27	25,079	215	9,82			Rpb5		RPB5		YBR154C	II (549003 - 548356)	648	lethal
ABC23	17,910	155	4,36	phos	ω	Rpb6		RPO26	RPB6	YPR187W	XVI (911253 - 911796)*	544 (468)	lethal
AC19	16,151	142	4,14	phos	α	Rpb11		RPC19		YNL113W	XIV (412771 - 413199)	429	lethal
ABC14.5	16,511	146	4,36			Rpb8		RPB8		YOR224C	XV (761265 - 760825)	441	lethal
A14	14,585	137	4,93			Rpb4		RPA14		YDR156W	IV (769520 - 769933)	414	viable
A12.2	13,660	125	7,79			Rpb9	Zn	RPA12	RRN4	YJR063W	X (555109 - 555486)	378	conditional
ABC10β	8,278	70	7,77			Rpb10	Zn	RPB10		YOR210W	XV (738320 - 738532)	213	lethal
ABC10α	7,716	70	10,5			Rpb12	Zn	RPC10	RPB12	YHR143W-A	VIII (387235 - 387447)	213	lethal

*RPO26 contains an intron (bp 21-96; 911273-911348).

Yeast RNA polymerase I consists of 14 subunits (Table 1; Carles *et al*., 1991; Paule, 1999), as revealed by the analyses of Pol I-complexes purified by diverse methods and to different extents of completeness and purity (Ponta *et al*., 1972; Buhler *et al*., 1974; Van Keulen *et al*., 1975; Valenzuela *et al*., 1976a; Hager *et al*., 1977; Keener *et al*., 1998). However, the detailed composition of yeast Pol I remained unclear for a long time due to the nearly identical sizes of some small subunits (Carles *et al*., 1991). The high degree of conservation especially between the three classes of eukaryotic nuclear RNA polymerases is further underlined by the existence of a number of shared subunits. Two subunits are identical in Pol I and Pol III (Valenzuela *et al*., 1976d; Mann *et al*., 1987; Dequard-Chablat *et al*., 1991), and another five subunits are common to all three RNA polymerases (Buhler *et al*., 1976a; Valenzuela *et al*., 1976b; Valenzuela *et al*., 1976d; Woychik *et al*., 1990a; Carles *et al*., 1991). In the common nomenclature for Pol I subunits each protein is named by a combination of the letters A, B

and/or C according to its appearance in Pol I, Pol II and/or Pol III, respectively, and the molecular weight in kDa as determined by SDS-PAGE (Table 1).

All but four of the Pol I subunits are essential proteins (Mémet *et al*., 1988; Yano and Nomura, 1991; Thuriaux *et al*., 1995; Mann *et al*., 1987; Woychik *et al*., 1990a; Dequard-Chablat *et al*., 1991; Treich *et al*., 1992; Woychik and Young, 1990b). Mutant yeast strains lacking the gene coding for A49 are viable, but exhibit a slow growth phenotype (Liljelund *et al*., 1992), and strains with a deleted gene coding for A12.2 are unable to grow at elevated temperatures (Nogi *et al*., 1993). The deletion of the gene coding for A14 results in a yeast strain which grows slower at high temperatures (Smid *et al*., 1995), and only the deletion of the gene coding for A34.5 does not result in a growth defect phenotype (Gadal *et al*., 1997). Triple mutants lacking A49, A34.5 and A12.2 are viable, but deletion of one of these subunits in combination with a deletion of A14 is lethal (Gadal *et al*., 1997).

1.2.2.3 The structure of the yeast Pol I complex resembles the general architecture of multisubunit RNA polymerases

The structure of yeast Pol I has been intensively investigated by EM, immuno-EM and cryo-EM analyses (Schultz *et al*., 1993; Klinger *et al*., 1996; Bischler *et al*., 2002; De Carlo *et al*., 2003; Kuhn *et al*., 2007). As suggested by the high level of conservation between the polymerase subunits, it resembles the general architecture of multisubunit RNA polymerases (Cramer *et al*., 2002; Bischler *et al*., 2002; Kuhn *et al*., 2007). The overall structure is globular, notched by a central cleft, one side of which is formed by a mobile clamp (Figure 5). At the front end of the cleft jaws are apparent, while the backside is closed by a wall. A stalk-like structure protrudes on the clamp side of the polymerase and at the bottom a funnel shaped cavity is visible which is connected to the cleft through a pore.

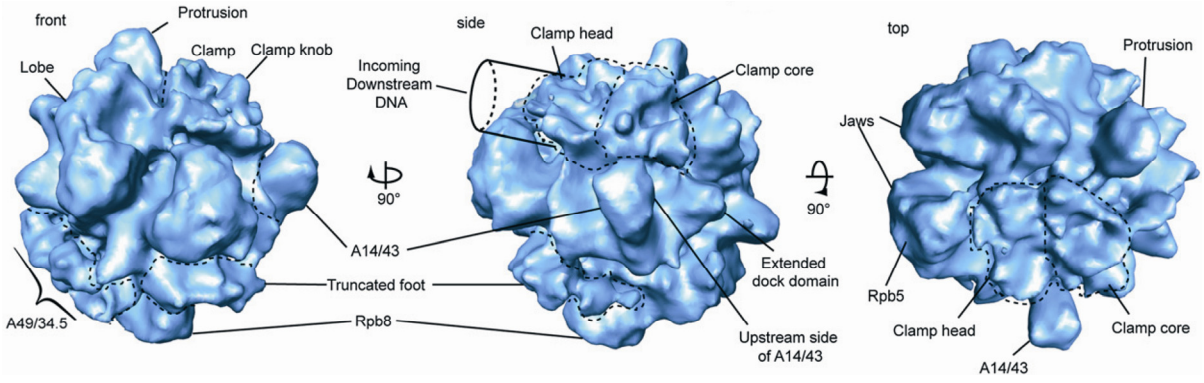

Figure 5. Cryo-EM structure of yeast RNA polymerase I at 12 Å. Front, side and top views are presented (from left to right). The positions of the Pol I subunits A43/A14, A49/A34.5, ABC27 (= Rpb5) and ABC14.5 (= Rpb8), as well as of the downstream rDNA and several domains are indicated. [from Kuhn *et al*., 2007]

Molecular details of multisubunit RNA polymerases have been revealed by the crystal structures of the bacterial RNA polymerase from *Thermus aquaticus* (Zhang *et al*., 1999; Vassylyev *et al*., 2002) and of the eukaryotic RNA polymerase II from *Saccharomyces cerevisiae* (Fu *et al*., 1999; Cramer *et al*., 2000; Cramer *et al*., 2001; Bushnell and Kornberg, 2003; Armache *et al*., 2003; Armache *et al*., 2005; Kornberg, 2007) (Figure 6). Furthermore structures of elongating yeast Pol II (Gnatt *et al*., 2001; Westover *et al*., 2004; Kettenberger *et al*., 2004; Wang *et al*., 2006) and of Pol II molecules in complex with associated factors or inhibitors have been obtained, including the transcription factor TFIIB (Bushnell *et al*., 2004), the elongation factor TFIIS (Kettenberger *et al*., 2003) or the inhibitor α-amanitin (Bushnell *et al*., 2002).

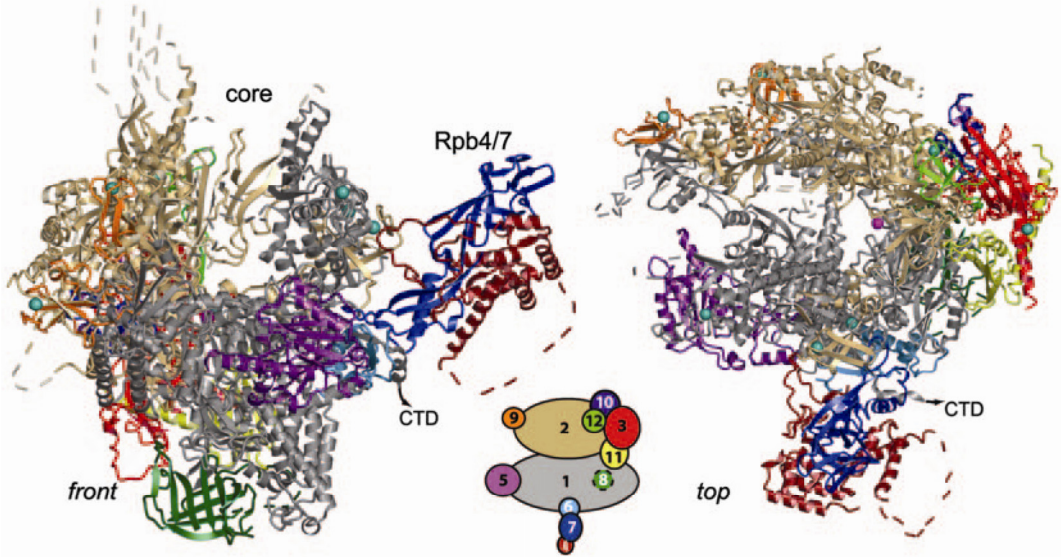

Figure 6. X-ray structure of yeast RNA polymerase II at 3.8 Å. Front and top views of the complete 12 subunit form including the Rpb4/7 heterodimer are shown. The color key for the single subunits is given in the scheme representing the top view. [from Armache *et al*., 2005]

Many molecular details of the transcription mechanism were derived from these analyses. The downstream DNA enters the RNA polymerase through the jaws and binds to the bottom of the cleft, while the clamp closes over the template (Gnatt *et al*., 2001; Westover *et al*., 2004; Kettenberger *et al*., 2004). Upon encountering the wall at the backside, the DNA makes a sharp bend upwards and exits the polymerase at the top. The active center can be found directly at this sharp bend, where the DNA is melted to form the transcription bubble (Cramer *et al*., 2000; Gnatt *et al*., 2001; Westover *et al*., 2004; Kettenberger *et al*., 2004). NTPs apparently enter the polymerase through the funnel / pore beneath the active site (Cramer, 2000). Incorporation of the nucleotides into the nascent RNA chain and translocation involves the movement of two important mobile structures named ‘bridge helix’ and ‘trigger loop’

(Gnatt *et al*., 2001; Wang *et al*., 2006). The RNA apparently exits the polymerase through an opening above the stalk-structure (Cramer *et al*., 2001; Gnatt *et al*., 2001; Cramer, 2002).

The Pol II crystal structure could be fitted into the Pol I cryo-EM maps (Bischler *et al*., 2002; Kuhn *et al*., 2007). The five common subunits occupied the equivalent positions and matched perfectly into the map. Most homologous regions also fitted very well, while some strong deviations were apparent in other parts (Kuhn *et al*., 2007). Based on the structural similarities, sequence alignments and a new crystal structure of the Pol I subunits A43 and A14, it was possible to construct a 12 subunit Pol I homology model (Kuhn *et al*., 2007). Due to the absence of A49 and A34.5 counterparts in Pol II, these subunits are missing in the model. However, their position in the complex could be derived from the difference between cryo-EM maps of the complete Pol I and of a variant lacking the A34.5/A49 heterodimer (Kuhn *et al*., 2007; Huet *et al*., 1975; see 1.2.3.6).

Similar cryo-EM analyses have recently been applied to elucidate the structures of yeast Pol III (Jasiak *et al*., 2006; Fernández-Tornero *et al*., 2007) and of the archael RNA polymerase from *Pyrococcus furiosus* (Kusser *et al*., 2008).

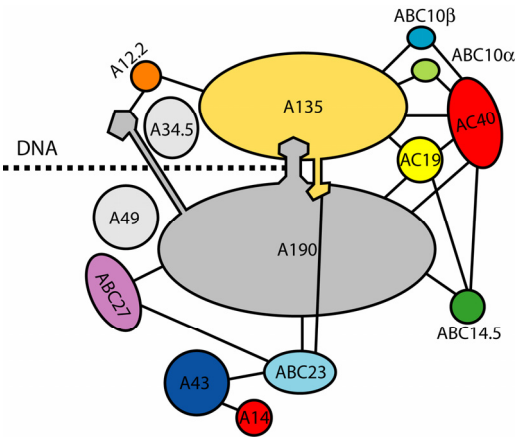

Figure 7. Interaction diagram for the Pol I subunits. The scheme was derived from interaction diagrams of the 10 subunit Pol II (Cramer *et al*., 2000; Cramer *et al*., 2001) and represents the top view. The colors of the subunits correspond to the respective Pol II counterparts shown in Figure 6. [from Nomura *et al*., 2004 (modified)]

1.2.3 Pol I subunits

1.2.3.1 A190 and A135 – the two largest subunits

A190 and A135 are the two largest Pol I subunits; together they make up more than 50 % of the 600 kDa holoenzyme. They form the central mass of the polymerase complex and are localized on the opposite sites of the cleft (Cramer *et al*., 2000; Bischler *et al*., 2002) (Figure 7) (see 1.2.2.3). According to their homology to the largest subunits of the bacterial RNA polymerase (bRNAP), A190 and A135 are also referred to as β'- and β-like subunits, respectively (Sweetser *et al*., 1987; Mémet *et al*., 1988; Yano and Nomura, 1991; Cramer, 2002). In the primary sequences of β'- and β-like RNA polymerase subunits, eight and ten conserved domains (designated a - h and A - J, respectively) are present (Figure 8) (Jokerst *et al*., 1989; Sweetser *et al*., 1987; Cramer *et al*., 2001). Domain d of the largest (β'-like) RNA polymerase subunits contains a cluster of three invariant aspartate residues, which coordinate the active site metal A, *i.e.* an Mg^{2+}-ion, required for the enzymatic mechanism (Cramer *et al*., 2000; Cramer *et al*., 2001). The other half of the active center and binding site for the metal B is constituted by domain F of the second largest (β-like) subunits (Cramer *et al*., 2001). Consistent with the presence of the catalytic center in these Pol I subunits, A190 and A135 were found to interact with both DNA and NTPs (Valenzuela *et al*., 1978).

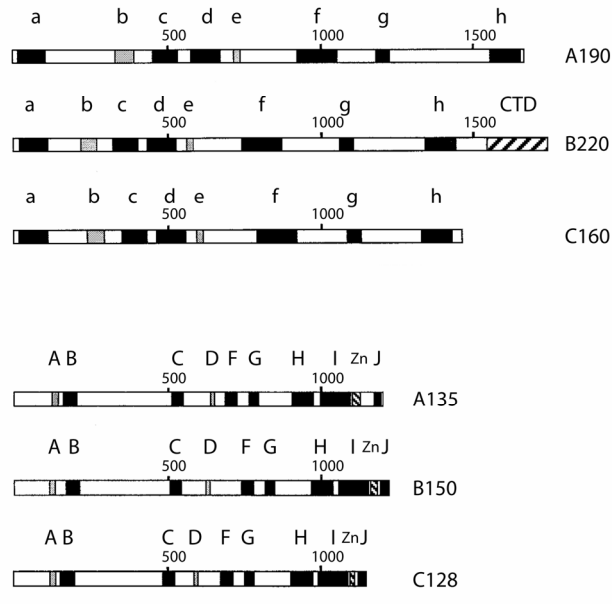

Figure 8. Conserved regions a-h of the β'-like (largest) subunits and regions A-J of the β-like (second largest) subunits of the three nuclear RNA polymerases from *S. cerevisiae*. Black boxes represent highly conserved regions; more divergent regions are depicted in gray. Additionally the zinc-binding regions of the β-like subunits are shown, as well as the CTD of the largest Pol II subunit which has no counterpart in the corresponding β'-like subunits of Pol I and Pol III. [from Flores *et al*., 1999 (modified)]

Furthermore A190 and A135 are two of five Pol I subunits with zinc binding domains (Treich *et al*., 1991). The function of the zinc ions in the polymerase activity remains unclear, but mutations in the N-terminal zinc binding domain of A190, as well as analogous mutations in the largest Pol II subunit, result in temperature-sensitive phenotypes (Wittekind *et al*., 1988; Himmelfarb *et al*., 1987). The corresponding Zn-binding domain of the *E.coli* RNA polymerase was implicated to function either in downstream DNA binding or intrinsic transcription termination (Nudler *et al*., 1998; King *et al*., 2004). Replacement of the second of four metal-coordinating cysteine residues in the C-terminal zinc binding domain of A135 by an alanine is lethal for the yeast cell, while the mutation of all four cysteines retains viability (Naryshkina *et al*., 2003).
Additionally to the post-transcriptional modification by phosphorylation (see 1.2.5; Bell *et al*., 1976; Bell *et al*., 1977a; Buhler *et al*., 1976b), A190 was shown to be a substrate for sumoylation in yeast (Panse *et al*., 2004).

1.2.3.2 AC40 and AC19 – the α-like subunits shared by Pol I and Pol III

AC40 and AC19, the two subunits shared by Pol I and Pol III, are the α-like subunits of these complexes. Although AC40 and AC19 are two different proteins, as opposed to the two identical α-subunits of the bacterial enzyme (subunit composition $\alpha_2\beta\beta'\omega$), 'α-motifs' can be found in both proteins and in the homologous Pol II subunits Rpb3 and Rpb11 (Martindale, 1990; Dequard-Chablat *et al*., 1991; Cramer, 2002). The homodimerization of the bacterial α-subunits is the first step of the assembly of the RNA polymerase in *E. coli*, followed by the subsequent binding of the β- and β'-subunit (in this order) (Ishihama, 1981). A similar mechanism for eukaryotic RNA polymerases was suggested by the isolation of a stable Pol II subassembly of subunits Rpb3, Rpb11 and Rpb2 from *Schizosaccharomyces pombe*, resembling the intermediate $\alpha_2\beta$-complex (Kimura *et al*., 1997; see also Shpakovski and Shematorova, 1999a), and by the analysis of Pol II assembly mutants in *S. cerevisiae* (Kolodziej *et al*., 1991). Finally the heterodimerization of AC40 and AC19 has been demonstrated *in vivo* (Lalo *et al*., 1993; Flores *et al*., 1999), and a subcomplex of the homologous Rpb3 and Rpb11 along with Rpb10 (= ABC10β) and Rpb12 (= ABC10α) is evident in the yeast Pol II crystal structure (Cramer *et al*., 2000).
Besides their role in the assembly of the multiprotein complex, the bacterial α-subunits were shown to be involved in promoter DNA binding and in interactions with activator proteins (Ebright and Busby, 1995). However, the C-terminal domain required for these interactions is absent in the archaeal and eukaryotic α-like polymerase subunits, and similar functions have not been observed to date.
AC19 is phosphorylated in Pol I and in Pol III (Bell *et al*., 1977a); whether the same site is modified in both enzymes remains to be investigated.

1.2.3.3 ABC27, ABC23, ABC14.5, ABC10α and ABC10β – the common subunits

The five common subunits ABC27, ABC23, ABC14.5, ABC10α and ABC10β together account for approximately 15% of the total mass of the Pol I complex (Carles *et al*., 1991). ABC23 (= Rpb6) is the eukaryotic counterpart of the bacterial RNAP ω-subunit (Minakhin *et al*., 2001), which is also the only phosphorylated common subunit (Bell *et al*., 1977; Kolodziej *et al*., 1990). Its function seems to be related to the assembly of the enzyme (Nouraini *et al*., 1996; Minakhin *et al*., 2001). In the proposed mechanism ABC23 latches the C-terminus of the largest polymerase subunit to a more N-terminal region of the protein, thus inducing a conformational change, which promotes the binding to the $\alpha_2\beta$-like intermediate complex (Minakhin *et al*., 2001; see also Lanzendörfer *et al*., 1997; Ghosh *et al*., 2001). ABC23 features a highly conserved C-terminal half, required for the interactions with the largest polymerase subunits, and a variable acidic N-terminal half (McKune *et al*., 1994). The evolutionary conservation is especially evident by the full exchangeability for its mammalian counterparts (McKune *et al*., 1994). Similarly ABC27, ABC14.5, ABC10α and ABC10β can be substituted by the corresponding human proteins, but the exchange of ABC27 results in a thermosensitive phenotype (Shpakovski *et al*., 1995; McKune *et al*., 1995).

The structure of the five common polymerase subunits has been solved within the crystal structure of yeast Pol II lacking two subunits (Cramer *et al*., 2000; see also Krapp *et al*., 1998; Todone *et al*., 2000). The structure of the complete twelve subunit Pol II complex (Armache *et al*., 2003) visualized another important feature of ABC23, which was anticipated from preceding experiments. It forms the main interaction interphase between the core polymerase and two heterodimerized subunits important for interactions with the promoter, *i.e.* subunits Rpb7 and Rpb4 in Pol II or the homologous A43 and A14 in Pol I, respectively (Peyroche *et al*., 2002) (see 1.2.3.4). A connection between ABC23, A43 and A14 was first established by their co-dissociation from the polymerase under stringent purification conditions in a ΔA14 deletion strain (Smid *et al*., 1995; Lanzendörfer *et al*., 1997) or upon lowering of the pH to 4 (Bull *et al*., 1981). A direct interaction of recombinant ABC23 and A43 was shown by co-immunoprecipitation from crude extracts of an *E. coli* culture expressing both subunits (Peyroche *et al*., 2002; see also Woychik *et al*., 1990a). Finally the docking of the atomic model of Pol II lacking Rpb4 and Rpb7 (Cramer *et al*., 2000) into a 25 Å cryo-EM electron density map of Pol I revealed the position of ABC23 near a protruding structure termed 'stalk', which contains A43 and A14 (Bischler *et al*., 2002, Peyroche *et al*., 2002; Lanzendörfer *et al*., 1997).

The dissociation of ABC23 was in each case associated with a complete inactivation of Pol I, when tested in an unspecific, promoter-independent transcription assay (Valenzuela *et al*., 1976c; Bull *et al*., 1981; Lanzendörfer *et al*., 1997). Pol I ΔABC23 was still able to bind DNA in a gel-shift retardation assay, but unable to catalyze the formation of phosphodiester bonds (Lanzendörfer *et al*., 1997; see also Valenzuela *et al*., 1978). Activity could be partially

restored by the addition of recombinant ABC23 to the polymerase preparation from the ΔA14 deletion strain (Lanzendörfer *et al*., 1997).

ABC23 is the only common subunit with a homolog in the bacterial RNA polymerase. However, there is no evidence that the five eukaryotic proteins resembling the $\alpha_2\beta\beta'\omega$ complex are sufficient to form a functional polymerase. Moreover ABC10α (= Rpb12) and ABC10β (= Rpb10), two zinc-binding subunits (Treich *et al*., 1991; Carles *et al*., 1991), also seem to be crucial for the assembly of the eukaryotic enzyme (Rubbi *et al*., 1999; Gadal *et al*., 1999). As already mentioned, they form a subcomplex with the α-like polymerase subunits, and the zinc-binding domains seem to be involved in interactions with the large subunits (Cramer *et al*., 2000). Interestingly mutants of ABC10α and ABC10β were isolated, which are specifically defective in the assembly of Pol III (Rubbi *et al*., 1999) or Pol I (Gadal *et al*., 1999), respectively. Mutations in a conserved eukaryote-specific motif (HVDLIEK) in ABC10β result in a strong temperature-sensitive phenotype, which can be fully rescued by transcribing the 35S rRNA from a Pol II promoter (Gadal *et al*., 1999). Besides its function in enzyme assembly, interactions between ABC10α and the Pol III-specific transcription factor TFIIIC have been reported, linking this subunit to the formation of a stable pre-initiation complex of RNA polymerase III (Dumay *et al*., 1999; Lefebvre *et al*., 1994; Rubbi *et al*., 1999). In a similar manner interactions between the common polymerase subunit ABC27 (= Rpb5) and the Pol II-specific transcription factor TFIIF were found (Miyao *et al*., 1998; Wei *et al*., 2001). Its location in the polymerase complex is near the entry site for the downstream DNA, in a structure termed 'jaws', and a contact between this subunit and the DNA has been proposed (Cramer *et al*., 2000). Furthermore synthetic lethal defects between *rpb5* mutants and the deletion of A12.2 or its homolog Rpb9, which are located on the opposite 'jaw' of the respective polymerase complex, have been reported (Zaros *et al*., 2007).

Less functional information is available for the common subunit ABC14.5 (= Rpb8). It is the only eukaryote specific Pol I subunit, *i.e.* without any known homologous subunit in the archaeal RNA polymerase (Cramer, 2002). The structure of ABC14.5 contains an OB-fold (Krapp *et al*., 1998; Cramer *et al*., 2000), a protein folding motif capable of binding oligonucleotides or oligosaccharides (Murzin, 1993), but its function remains unclear (see also Voustina *et al*., 1999; Briand *et al*., 2001).

1.2.3.4 A43 and A14 – the 'stalk' subunits

As described above, the Pol I-specific subunits A43 and A14 form a heterodimer, which makes up the protruding 'stalk', apparent in EM analyses (Bischler *et al*., 2002, Peyroche *et al*., 2002; Lanzendörfer *et al*., 1997) (see 1.2.2.3 and 1.2.3.3). Despite the weak sequence homology, which is largely limited to the N-terminal part, A43 was shown to be the Pol I counterpart of Rpb7 in Pol II, C25 in Pol III and subunit E in the archaeal RNA polymerase,

respectively (Peyroche *et al*., 2002; Meka *et al*., 2003; Zaros and Thuriaux, 2005; Shpakovskiĭ and Shematorova, 1999b; see also Sadhale and Woychik, 1994). Accordingly the A43/A14 heterodimer was considered to belong to the family of Rpb4/Rpb7 (Pol II), C25/C17 (Pol III) and the archaeal polymerase subunits E/F (termed the 'RpoE/RpoF-family' or 'Rpb4/7-family'). Although there is no obvious sequence homology between A14 and any of the corresponding proteins in this family, a distant relatedness based on structural similarity was proposed. Finally the comparison of the recently solved crystal structure of a recombinant A43/A14 heterodimer with the structures of the other RpoE/RpoF family members confirmed this relationships on a structural level (Kuhn *et al*., 2007; Armache *et al*., 2005; Jasiak *et al*., 2006; Todone *et al*., 2001) (Figure 9). The structures of A43, Rpb7, C25 and RpoE can be separated into two distinct domains: the N-terminal part, involved in the binding to ABC23 (called 'tip domain'), and the C-terminal half, forming the most outer part of the stalk (called 'OB-domain'). The latter is named for the OB-fold (Murzin, 1993) found in each of these proteins. Due to this protein fold, each of the heterodimers is capable to bind single stranded RNA *in vitro* (Meka *et al*., 2003; Orlicky *et al*., 2001; Jasiak *et al*., 2006), but the significance of this feature *in vivo* remains unclear. A14 consists of a 'tip-associated domain', wrapped around the 'tip-domain'of A43, but the more C-terminal 'HRDC-domain' present in the other members of this protein family seems to be lacking (Kuhn *et al*., 2007; see also Meka *et al*., 2003).

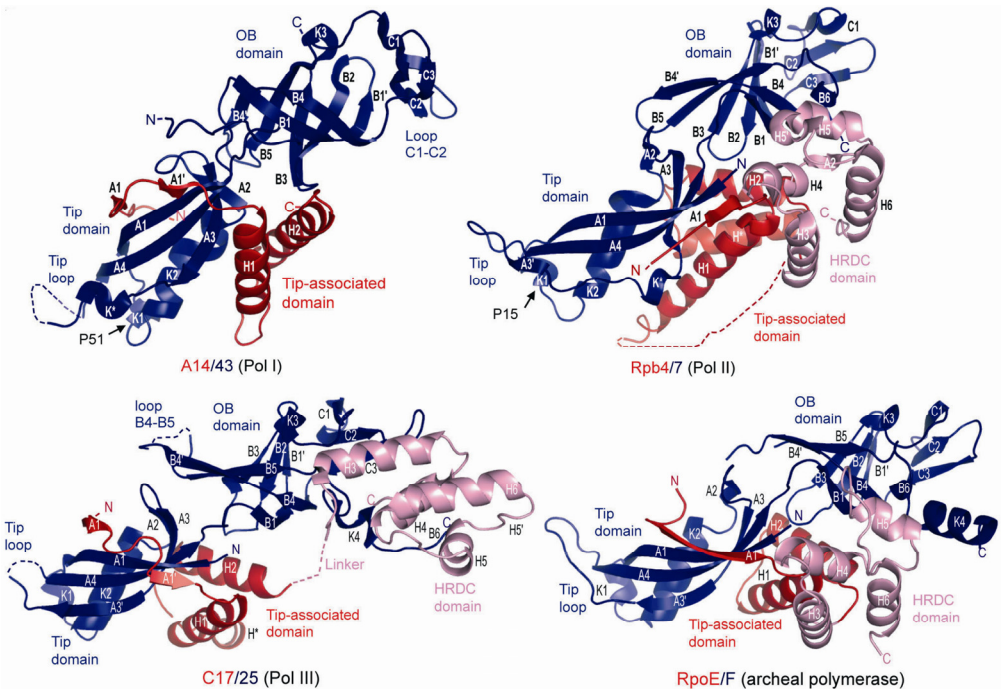

Figure 9. Structural comparison of the yeast A43/A14 heterodimer and its counterparts in the other nuclear RNA polymerases and the archaeal RNA polymerase. A43, Rpb7, C25 and RpoE are shown in blue; A14, Rpb4, C17 and RpoF in red. The counterparts of A14 possess an additional HRDC domain (light red) which is absent in A14. [from Kuhn *et al*., 2007]

The most important common feature of the RpoE/RpoF-like heterodimers is their function in transcription initiation through contacts with the respective transcription factors (Peyroche *et al*., 2000; Choder, 2004; Ferri *et al*., 2000; Zaros and Thuriaux, 2005; Ouhammouch *et al*., 2004). A43 was found to be the interaction partner for the Pol I specific transcription factor Rrn3p (Peyroche *et al*., 2000) (see 1.2.1.3). Pol I in complex with Rrn3p forms the initiation-competent form of Pol I, required for the recruitment of Pol I to the rDNA promoter (Milkereit and Tschochner, 1998; Yamamoto *et al*., 1996). The Pol I-Rrn3p complex represents only about 2 % of the total cellular Pol I content, and its formation is apparently the key switch in the regulation of rDNA transcription (Milkereit and Tschochner 1998; Claypool *et al*., 2004; Laferté *et al*., 2006). Both A43 and Rrn3p are phosphoproteins *in vivo* (Bell *et al*., 1976; Bell *et al*., 1977a; Buhler *et al*., 1976b; Fath *et al*., 2001).
The exact role of A14 is unclear. It is one of the non-essential Pol I subunits and seems to stabilize A43 and ABC23 in the polymerase complex (Smid *et al*., 1995; Lanzendörfer *et al*., 1997). Furthermore the A14 ortholog in *S. pombe* was described to stabilize the A43-Rrn3p interactions (Imazawa *et al*., 2005). However, recombinant, co-expressed A43 and Rrn3p of *S. cerevisiae* are able to interact in the absence of A14 (Peyroche *et al*., 2000).

While being absolutely indispensable for promoter-specific transcription initiation, the A43/A14 heterodimer is not required for promoter-independent, non-specific *in vitro* transcription (Lanzendörfer *et al*., 1997; Peyroche *et al*., 2000), *i.e.* the basal transcription mechanism is independent of these two subunits. The same is true for the other RpoE/RpoF-like heterodimers (Edwards *et al*., 1991; Sadhale *et al*., 1994; Werner and Weinzierl, 2002; Naji *et al*., 2007).

1.2.3.5 A12.2 – a TFIIS-like subunit

A12.2 is the smallest Pol I-specific subunit. It is non-essential under normal growth conditions, but required for growth at elevated temperatures (Nogi *et al*., 1993). This is consistent with its Pol II homolog Rpb9 (Woychik *et al*., 1991), while the homologous Pol III subunit C11 appears to be an essential protein (Chédin *et al*., 1998). The members of this protein family, which also includes the archaeal TFS (Langer *et al*., 1995; Hausner *et al*., 2000), represent another example for the high degree of conservation between the multisubunit RNA polymerase systems. They share a tripartite composition, with the N- and C-terminal domains each containing a zinc binding domain (Treich *et al*., 1991; Van Mullem *et al*., 2002; Woychik *et al*., 1991; Cramer *et al*., 2000; Chédin *et al*., 1998; Hausner *et al*., 2000). The short intermediate linker-domain is involved in contacts to the largest polymerase subunit, *i.e.* it adds a β-strand to a sheet in Rpb1 in the Pol II structure (Cramer *et al*., 2000). The C-terminal domain contains a highly conserved motif (Q.RSADE..T.F; only Rpb9

contains variations), which is also present in the Pol II elongation factor TFIIS. Thus, A12.2, Rpb9, C11 and TFS are sometimes referred to as TFIIS-like RNA polymerase subunits. However, this homology is limited to the C-terminal domain of A12.2 and the C-terminal zinc binding domain of the much larger TFIIS (Van Mullem *et al*., 2002; Chédin *et al*., 1998). TFIIS induces the hydrolytic cleavage of the nascent RNA chain from the 3' end of a backtracked Pol II after encountering a transcriptional block (Fish and Kane, 2002). The two acidic residues (DE) in the conserved motif were shown to be crucial for the mechanism of TFIIS-induced RNA cleavage (Jeon *et al*., 1994; Kettenberger *et al*., 2003), and mutation of the same residues in C11 is lethal (Chédin *et al*., 1998). Both Pol I and Pol III exhibit an intrinsic RNA cleavage activity, capable of a similar shortening of the RNA from the 3' end in an artificial or stalled ternary complex *in vitro* (Kuhn *et al*., 2007; Whitehall *et al*., 1994). This cleavage activity was shown to be dependent on the C-terminal domain of A12.2 or on C11, respectively (Kuhn *et al*., 2007; Chédin *et al*., 1998). Pol II on the other hand required the addition of TFIIS for efficient cleavage under the same conditions (Kuhn *et al*., 2007), possibly due to the altered TFIIS-like motif in Rpb9. A previously described dissociable Pol I RNA cleavage factor (Tschochner, 1996; Labhart, 1997) might be required for the backtracking of the enzyme, thus facilitating the 3' trimming of the RNA chain by the intrinsic cleavage activity (Kuhn *et al*., 2007). Finally, similar to TFIIS deletion mutants, yeast strains lacking the gene coding for A12.2 are sensitive to the NTP-pool depleting drug 6-azauracil (6AU), a phenotype often associated with defects in transcription elongation (Van Mullem *et al*., 2002; Exinger and Lacroute, 1992; Archambault *et al*., 1992; Hampsey, 1997). But despite the partial homology, including a conserved motif, and the functional and phenotypic similarities, the location of TFIIS and the TFIIS-like proteins in the RNA polymerase complex contradicts a common RNA cleavage mechanism. While a loop of TFIIS (which includes the conserved residues) enters through the polymerase 'pore' underneath the active center, A12.2, Rpb9 and C11 are located on top of the 'jaw' domain of the largest polymerase subunit (Kettenberger *et al*., 2003; Cramer *et al*., 2000; Kuhn *et al*., 2007; Fernández-Tornero *et al*., 2007).

The TFIIS-like polymerase subunits are also involved in the transcription termination processes (Prescott *et al*., 2004; Chédin *et al*., 1998; Landrieux *et al*., 2006). Yeast strains lacking RPA12 (the gene encoding A12.2) were shown to accumulate Pol I molecules in the IGS regions of the rDNA locus, but transcripts of this DNA element were not detectable, probably due to degradation (Prescott *et al*., 2004). In the case of Pol III, C11 was identified as a termination factor, but the associated intrinsic RNA cleavage activity does not seem to be necessary (Landrieux *et al*., 2006). The same may be true for Pol I, consistent with the non-essential character of A12.2.
Rpb9 and C11 were additionally shown to function in proofreading and transcriptional fidelity of the respective RNA polymerases (Nesser *et al*., 2006; Koyama *et al*., 2007; Alic *et al*., 2007), but this hasn't been tested for A12.2 yet.

However, in spite of all the described processes involving A12.2, its C-terminal domain, which includes the highly conserved motif, can be deleted without any effects on cell growth. The analysis of deletion mutants lacking either the N-terminal (A12.2 ΔN) or C-terminal half of the protein (A12.2 ΔC) revealed, that the ΔN-mutant is not able to bind to the polymerase, and thus was phenotypically indistinguishable from a strain with a full Δrpa12 knock-out (Van Mullem *et al*., 2002). In contrast yeast strains expressing the A12.2 ΔC mutant do not show the temperature sensitive (ts) phenotype and grow like a wild-type yeast strain. Most probably the binding of the N-terminal half of A12.2 is required to assure the correct conformation of A190. This is consistent with the observation, that the ts-phenotype of a ΔA12.2 strain can be suppressed by overexpression of A190, and that binding of A12.2 promotes the assembly of the largest subunit and associated to this, increases its stability (Nogi *et al*., 1993).
This additional consequence of the deletion of A12.2 hampers the interpretation of the results obtained with Δrpa12 strains, *i.e.* to distinguish between effects due to the lacking activity of A12.2 or an incorrect folding of A190. For instance the 6AU-sensitivity mentioned above could also be suppressed by the overexpression of A190, more arguing for the latter case (Van Mullem *et al*., 2002). The instability of A190 is probably also the cause for an observed synthetic lethal defect of ΔA12.2 with ΔA14 (Gadal *et al*., 1997) or Δhmo1 (Berger *et al*., 2007).

1.2.3.6 A49 and A34.5 – Pol I specific subunits without counterparts in other RNA polymerases

A34.5 and A49 are the only two Pol I-specific subunits without any known counterpart in the other RNA polymerases (Gadal *et al*., 1997; Liljelund *et al*., 1992). A34.5 is phosphorylated *in vivo* (Bell *et al*., 1976; Bell *et al*., 1977a; Buhler *et al*., 1976b). Both subunits are non-essential proteins; together they form a heterodimer (Kuhn *et al*., 2007). Consistent with this, Pol I purified from a ΔA34.5 deletion strain also lacks A49 (Gadal *et al*., 1997), and both subunits are susceptible to be lost together from the wild-type complex during purification procedures (Valenzuela *et al*., 1976a) and upon lowering of the pH (Bull *et al*., 1981). They can also be dissociated in a coordinated manner by urea treatment, resulting in an active 12 subunit Pol I preparation referred to as Pol I* or Pol A* (Huet *et al*., 1975). This Pol I variant lacking A34.5 and A49 can transcribe an artificial $d(A\text{-}T)_n$ template with a comparable efficiency to the wild-type enzyme, but is less active on native calf thymus DNA. Genetic analyses of the deletion strains for each of the two subunits revealed synthetic lethal effects with deletions of A14. Furthermore synthetic growth defects of ΔA34.5 with the deletion of DNA Topoisomerase I (Top1) and Hmo1 (Gadal *et al*., 1997; Berger *et al*., 2007) and of ΔA49 with deletions of DNA Topoisomerase III (Top3) and Hmo1 were found (Gadal *et al*.,

2002; Berger *et al*., 2007). Together these data indicate a function of the A34.5/A49 heterodimer in Pol I elongation. This is supported by a recent *in vitro* RNA extension assay (Kuhn *et al*., 2007). An artificial transcription bubble could only be efficiently elongated to the end of the template by Pol I* after adding back the two dissociated subunits. Furthermore a ΔA34.5 yeast strain was found to be sensitive to 6AU. Bioinformatic analyses suggested a weak homology of A34.5/A49 with two subunits of the Pol II-associated factor TFIIF (Kuhn *et al*., 2007).
A34.5 and A49 were, along with A190 and A135, found to be polymerase subunits involved in contacts with the DNA (Valenzuela *et al*., 1978). However, this was not confirmed in photocross-linking experiments with the mammalian Pol I at the rDNA promoter (Bric *et al*., 2004). Possibly these A34.5/A49-DNA-interactions exist only transiently during the elongation phase, as they are not strictly required for polymerase activity (Valenzuela *et al*., 1978).

1.2.4 The life and death of Pol I

1.2.4.1 Expression of the Pol I subunits

DNA Sequence analyses revealed a common element in the promoter regions of the genes for all Pol I and Pol III specific and shared subunits, which is absent in the promoters for the Pol II subunits, including the five common ones. It was therefore termed the 'PAC box' (for RNA *P*olymerase *A* and *C* box) (Dequart-Chablat *et al*., 1991). This promoter element was later on found to be often associated with another motif called 'RRPE' (for *R*ibosomal *R*NA *P*rocessing *E*lement) (Hughes *et al*., 2000) in the promoters of about 200 genes, which form together the RRB regulon (*rR*NA *B*iosynthesis regulon) (Wade *et al*., 2001; Wade *et al*., 2006). The genes included in the RRB regulon code for proteins with various functions in ribosome biogenesis, ranging from the Pol I and Pol III subunits to ribosome biogenesis factors (*e.g.* RNA-modifying enzymes), but no ribosomal proteins. Transcription factors, which bind to the PAC and RRPE boxes, were not identified yet, but the protein Sfp1 was found to be a factor involved in the regulation of these promoters (Jorgensen *et al*., 2002; Fingerman *et al*., 2003). Although no ribosomal proteins (RPs) are included in the RRB regulon, a coordination of their expression with the expression of Pol I subunits might be facilitated through a 'RPG box' (for *R*ibosomal *P*rotein *G*ene) (Leer *et al*., 1985). This motif is present in most RP gene promoters and was also found in the upstream regions of the genes for A190, A43 and AC40 (RPA190, RPA43 and RPC40, respectively) (Mémet *et al*., 1988; Mann *et al*., 1987). Interestingly RPA190 and RPA43 (the genes coding for A190 and A43,

respectively) are transcribed divergently from a shared promoter region (Thuriaux *et al*., 1995; Goffeau *et al*., 1996).

The amount of Pol I in a yeast cell was quantified to a number of about 15200 copies per cell, averaged for three different Pol I specific subunits (A190, A135 and A43) (Bier *et al*., 2004). Taken into consideration, that Pol I transcription accounts for about 60 % of the total cellular transcription (Warner, 1999), it is remarkable, that the copy number of Pol I subunits is only half the amount of Pol II molecules (*i.e.* 30000 copies per cell) (Borggrefe *et al*., 2001). Probably these numbers reflect the fact, that Pol I is highly concentrated in the nucleolus and, according to the ribomotor-model (Kempers-Veenstra *et al*., 1986; Planta, 1997) (see 1.2.1.2), uses an efficient re-initiation mechanism. The copy numbers for Pol I and Pol II subunits obtained through a systematic large scale quantification of the yeast proteome (Ghaemmaghami *et al*., 2003) do not seem to be very accurate, as the amounts calculated for the single components of the stoichiometric polymerase complex vary over a large range.

1.2.4.2 Assembly and nuclear import of the complex

The assembly of single subunits into a RNA polymerase complex is best studied for the five subunit RNA polymerase of *E. coli* (RNAP), and assembly of the polymerase core of the eukaryotic enzymes seems to follow the same mechanism (see 1.2.3.2). The first step of *E. coli* RNAP assembly is the dimerization of the α-subunits ($\rightarrow \alpha_2$), followed by the binding of the β-subunit ($\rightarrow \alpha_2\beta$) (Ishihama, 1981). Finally the largest subunit β' joins this intermediate complex, assisted by the ω subunit ($\rightarrow \alpha_2\beta\beta'\omega$) (Ghosh *et al*., 2001). The details for the α-like (AC40 and AC19; see 1.2.3.2), β- and β'-like (A135 and A190, respectively; see 1.2.3.1) and ω-like (ABC23; see 1.2.3.3) subunits of Pol I are described above. Furthermore the subunits ABC10α, ABC10β were found to be involved in the correct assembly of Pol I (see 1.2.3.3). One single copy of each subunit is present in the completed Pol I molecule (Hager *et al*., 1977; Paule, 1998). ABC27 was originally described to be present in a stoichiometric ratio of 2 relative to the other subunits (Hager *et al*., 1977), but this common subunit is apparently also present as a single copy (Kuhn *et al*., 2007), like in the Pol II and Pol III complexes (Cramer *et al*., 2000; Lorenzen *et al*., 2007; see also Kolodziej *et al*., 1990).

It is unclear, in which cellular compartment the assembly of Pol I takes place, *i.e.* the subunits could be imported into the nucleus independently or assembled into intermediate subcomplexes or in form of the complete polymerase. One study analyzed the nuclear import of Pol III subunits in wild-type yeast compared to an N-terminal deletion mutant of the second largest Pol III subunit (RPC128ΔN), which shows a mislocalization of this subunit to the cytoplasm, but no obvious growth defect (Hardeland and Hurt, 2006). The authors report

the coordinated nuclear import of some, but not all Pol III subunits. However, the current model of RNA polymerase assembly ($\alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'\omega$; see above) contradicts the formation of a subcomplex from the subunits described to be co-imported. Thus, these results point more towards a coordinated import mechanism for the single subunits.

Once assembled, Pol I stays intact without subunit exchange through multiple rounds of the transcription cycle (Schneider and Nomura, 2004). This includes the pool of Pol I molecules not actively engaged in transcription. These initiation-incompetent complexes were found to be able to dimerize under the conditions optimal for *in vitro* transcription (Milkereit *et al*., 1997; Bischler *et al*., 2002; see also Bull *et al*., 1981), but no extra peak corresponding to a Pol I dimer is obvious after glycerol gradient centrifugation of yeast whole cell extracts (Schneider and Nomura, 2004).

1.2.4.3 The Pol I transcription cycle

In order to initiate transcription at the rDNA promoter, Pol I needs to be in an initiation-competent form, *i.e.* in a complex with transcription factor Rrn3p (Milkereit and Tschochner, 1998) (see 1.2.1.3). This complex is formed independent from interactions with the promoter (Yamamoto *et al*., 1996; Schneider and Nomura 2004) and involves the Pol I subunits A43 and possibly A190 (Peyroche *et al*., 1998; Kuhn *et al*., 2007) (see 1.2.3.4). At the promoter the Pol I-bound Rrn3p interacts with CF (*i.e.* with the CF-subunit Rrn6p) (Peyroche *et al*., 1998), which itself seems to be dependent on UAF and TBP to be recruited to the promoter (Aprikian *et al*., 2001; Bordi *et al*., 2001) (see 1.2.1.3). Being correctly positioned for transcription initiation, Pol I escapes from the promoter and switches to elongation mode, including the dissociation of Rrn3p from the complex (Milkereit and Tschochner 1998; Aprikian *et al*., 2001; Bier *et al*., 2004). During this post-initiation phase CF and TBP apparently leave the promoter again, while UAF remains stably associated to the upstream element for the next initiation event (Aprikian *et al*., 2001; Bordi *et al*., 2001).

Directly after the promoter-escape of one Pol I molecule, the next pre-initiation complex can start to form. This results in multiple polymerases transcribing the same rDNA repeat at the same time. A spreading technique ('Miller spreading') enabled the electron microscopic visualization of these active rDNA genes (Miller and Beatty, 1969; Miller, 1981; Rattner *et al*., 1982). The pictures resemble a 'Christmas tree', with the DNA forming the 'trunk', highly packed with Pol I molecules, and each one giving rise to a 'branch' of nascent rRNA transcripts with growing length along the gene (Figure 10). Using this method, the polymerase loading of the active rDNA repeats in the cells of an exponentially growing yeast culture was counted to be 51 Pol I molecules per gene in average (French *et al*., 2003). This

equals a density of one polymerase every 132 nucleotides of rDNA, and the initiation interval at one promoter was calculated to be about 2.2 seconds.

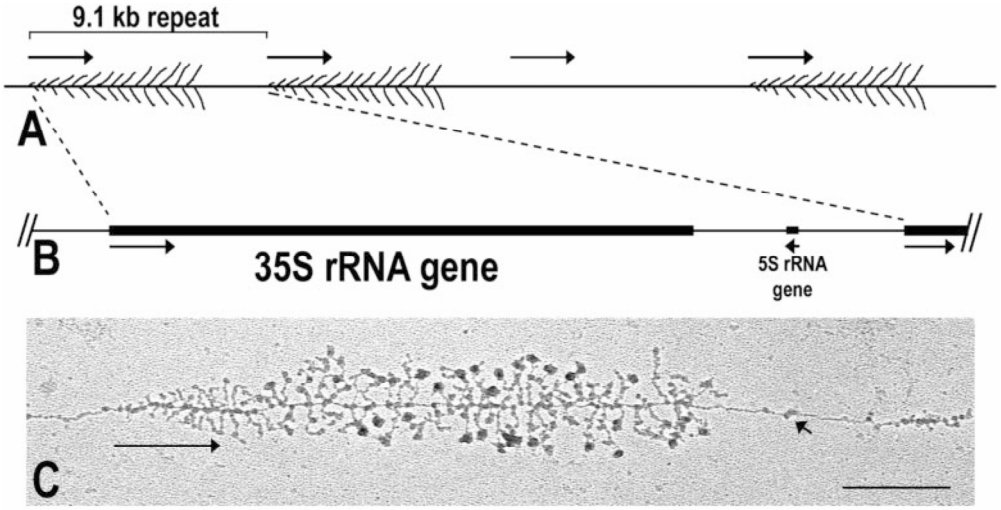

Figure 10. Transcribed rDNA units resemble 'Christmas trees'. (A) Schematic representation of active (with rRNA 'branches') and inactive (without rRNA) rDNA units of yeast. **(B)** One rDNA unit contains the 35S rDNA and the intergenic spacer which includes the 5S rDNA. Only the 35S rDNA forms the 'Christmas tree'. **(C)** Electron micrograph of a transcribed rDNA unit after Miller spreading (aligned with the map in (B)). A Pol I molecule can be found at the origin of each nascent rRNA chain. The bar represents 400 nm. [from French *et al*.,2003]

During the transcription of the 35S pre-rRNA, ribosome biogenesis factors start to assemble on the nascent transcripts, visible as characteristic terminal 'balls' in the miller spreads (Mougey *et al*., 1993; Dragon *et al*., 2002; Tschochner and Hurt, 2003; Osheim *et al*., 2004). Furthermore the correct function of these co-transcriptional processes seem to depend on the accurate production of the rRNA precursor, as suggested by the rRNA processing and ribosome assembly defects in a Pol I mutant, defective in transcription elongation (A135 D784G) (Schneider *et al*., 2007). Similar effects were also found in a deletion strain for the Pol II elongation factor Spt4p which was described to additionally function in the Pol I transcription system in a complex with Spt5p (Schneider *et al*., 2006). No other Pol I elongation factors are known so far, but the idea of subunits A34.5 and A49 as 'built-in' Pol I elongation factors exists (Kuhn *et al*., 2007) (see 1.2.3.6). Furthermore the function of the Pol I subunit A12.2 is linked to transcription elongation (Van Mullem *et al*., 2002) (see 1.2.3.5).

Under optimal growth conditions, Pol I needs approximately two minutes to transcribe the 35S rRNA precursor from the transcription start site to the terminator, *i.e.* the calculated elongation rate of Pol I is about 60 nucleotides per second at 30° C (French *et al*., 2003; see also Ryals *et al*.,1982).

Termination of the Pol I transcript predominantly appears at a T-rich DNA element 93 bp downstream of the 25S rRNA sequence, 17 bp upstream of a binding site for the termination factor Reb1p (Lang and Reeder, 1993; Reeder *et al*., 1999). The Reb1 site causes Pol I to pause elongation, thus facilitating transcript release at the T-rich element (Lang and Reeder, 1995; Jeong *et al*., 1995; Reeder and Lang, 1997). About 10 % of all transcripts were found to be further extended to a second 'fail safe' terminator about 250 bp downstream (Reeder *et al*., 1999). Two other proteins were identified as Pol I termination factors: Rnt1p, the yeast RNase III, which was found to cleave the primary transcript near the 3' end of the 25S rRNA sequence co-transcriptionally (Kufel *et al*., 1999; Chanfreau *et al*., 2000; Henras *et al*., 2004; Prescott *et al*., 2004) and the Pol I subunit A12.2 (Prescott *et al*., 2004) (see 1.2.3.5).

Following termination, the free Pol I needs to be converted into a form which is competent for complex-formation with Rrn3p again (Milkereit and Tschochner, 1998), rendering it capable of running another round of the transcription cycle.

1.2.4.4 Degradation

The degradation of RNA polymerase subunits is best analyzed for the largest Pol II subunit Rpb1, which was found to be a substrate for the E3 ubiquitin-protein ligase Rsp5 (Huibregtse *et al*., 1997; Crews, 2003). Interestingly, Rpb1 of arrested Pol II elongation complexes is the preferential substrate for ubiquitination (Somesh *et al*., 2005). Apparently these polymerases are degraded by the 26S proteasome in order to avoid a lethal blockage of essential genes (Crews, 2003). The mapped ubiquitination-sites are conserved in A190 (Somesh *et al*., 2007; Kuhn *et al*., 2007), but as Rpb1 ubiquitination seems to depend on the CTD-domain (Huibregtse *et al*., 1997; Somesh *et al*., 2005) it is unclear whether a similar mechanism exists for Pol I.

1.2.5 Pol I phosphorylation

1.2.5.1 Pol I is a phosphoprotein complex

The presence of phosphorylated proteins in the Pol I complex of *S. cerevisiae* was first described about 30 years ago (Bell *et al*., 1976; Buhler *et al*., 1976b). Starting with the discovery of protein phosphorylation / dephosphorylation as the mechanism modulating the activity of glycogen phosphorylase in 1955 (Fischer and Krebs, 1955; Krebs and Fischer,

1956; Cohen, 2002), the importance of this reversible posttranslational modification as a common regulatory concept was emerging through the early 1970s (Holzer and Duntze, 1971; Rubin and Rosen, 1975). In 1976 two independent research groups reported the analysis of Pol I preparations from ^{32}P-labeled, exponentially growing yeast cultures, identifying the five subunits A190, A43, A34.5, ABC23 and AC19 as *in vivo* phosphorylated proteins (Bell *et al*., 1976; Buhler *et al*., 1976b; Bell *et al*., 1977) (Figure 11). Additionally the *in vivo* phosphorylation of Pol II (Buhler *et al*., 1976b; Bell *et al*., 1977) and Pol III (Bell *et al*., 1977) was investigated, establishing that all three RNA polymerases are phosphoprotein complexes. In these analyses the shared subunits ABC23 and AC19 were found to be phosphorylated in each case. Another seven years later the amount of phosphate groups present in the *in vivo* ^{32}P-labeled Pol I preparations was quantified to be in the range of 15 ± 3 phosphates per enzyme (Bréant *et al*., 1983). The measurement of the specific radioactivity incorporated into the single subunits resulted in the following average distribution of phosphate groups per molecule: A190 (6), A43 (4), A34.5 (2), ABC23 (1-2) and AC19 (1-2).

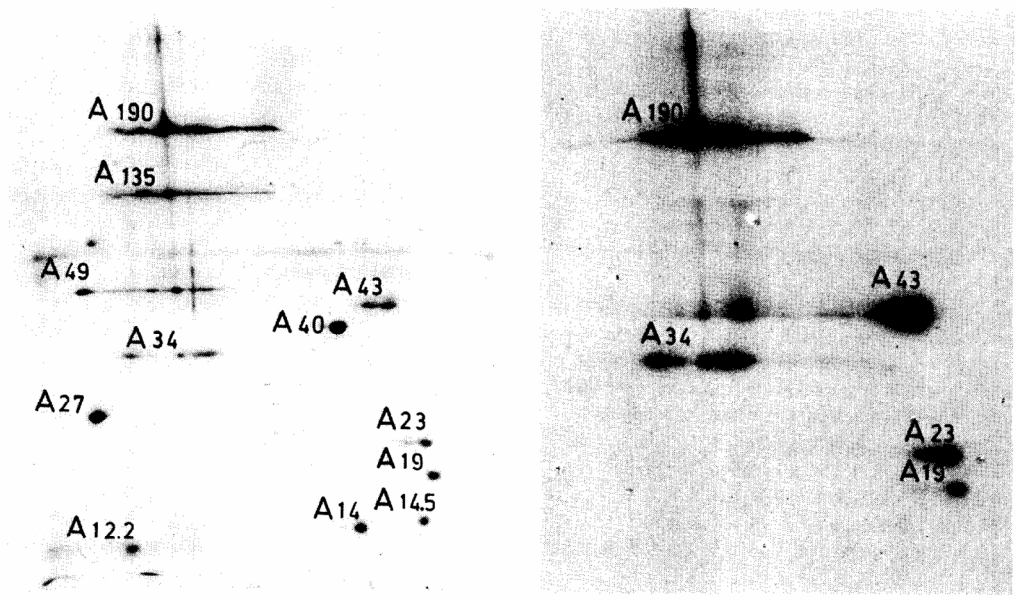

Figure 11. *In vivo* phosphorylated subunits of yeast RNA polymerase I. Pol I was purified from ^{32}P-labeled logarithmically growing yeast cells and separated by 2D-gelelectrophoresis. The gel was stained with coomassie blue (left) and subjected to autoradiography (right). [from Buhler *et al*., 1976b]

Several experimental data point towards a regulatory role of Pol I phosphorylation. Initiation-competent Pol I (see 1.2.4.3) differs from total Pol I in its phosphorylation pattern (Fath *et al*., 2001) (Figure 12). Dephosphorylation of a fraction enriched in the Pol I-Rrn3p complex results in a strong reduction of initiation competence in a reconstituted promoter-specific transcription assay and in the destabilization of the otherwise highly salt-resistant complex.

Furthermore, *in vitro* dephosphorylated Pol I fails to form a stable complex with recombinant Rrn3p, as opposed to a preparation of native Pol I (Fath *et al*., 2001). Finally, Pol I becomes inactivated in a non-specific transcription assay upon dephosphorylation by alkaline phosphatase (Fath *et al*., 2004). Interestingly the kinetics of this assay indicate that the removal of the first (*i.e.* the most easily accessible) phosphate groups leads to an increased activity. This suggests that only part of the modifications is required to activate the enzyme, while phosphorylation at other sites has an inhibitory role.

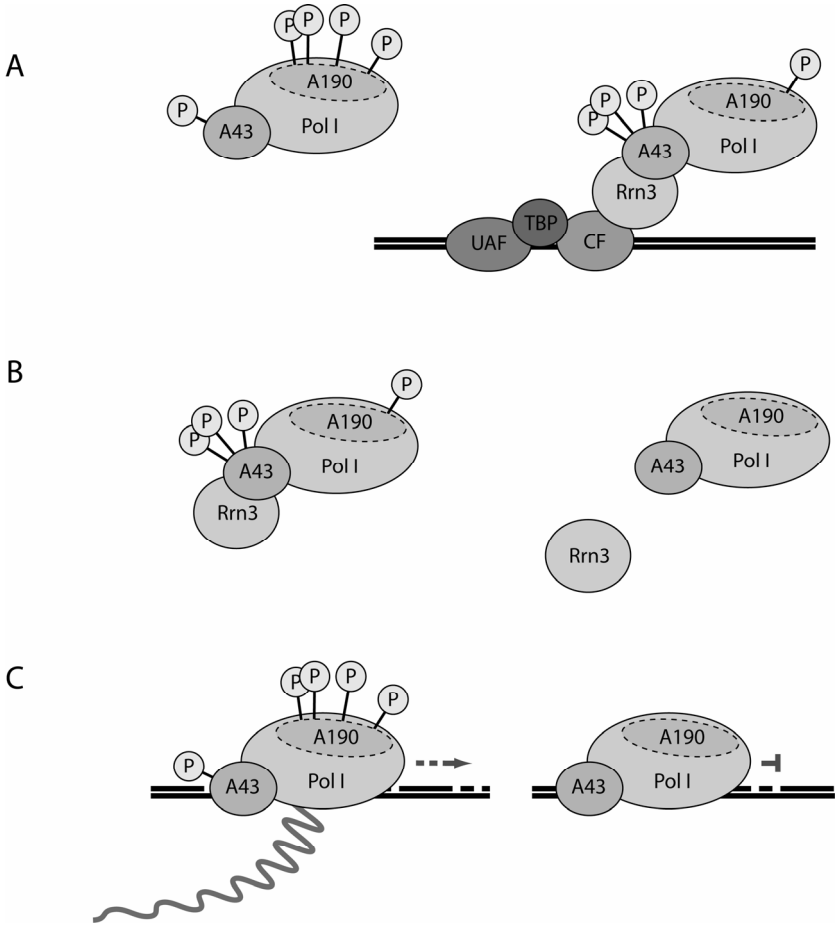

Figure 12. The activity of Pol I is linked to its phosphorylation state. (A) Initiation-competent Pol I (*i.e.* complexed with Rrn3p) differs from total Pol I in its phosphorylation pattern. (Fath *et al*., 2001) **(B)** Dephosphorylated Pol I fails to form a stable complex with recombinant Rrn3p (Fath *et al*., 2001). **(C)** Dephosphorylated Pol I is inactive in promoter-independent transcription (Fath *et al*., 2004).

The only information about Pol I phosphorylation sites came from large scale phosphorylation analyses of the yeast proteome. The approach by Ficarro and colleagues (2002) identified 216 phosphopeptides, including one peptide of A190 with the three clustered phosphosites S1413, S1415 and S1417 (Ficarro *et al*., 2002). Gruhler *et al*. investigated in a quantitative analysis the changes in protein phosphorylation associated with the yeast pheromone signaling

pathway (Gruhler *et al*., 2005). Among the 585 phosphopeptides without alterations in relative abundance (of 729 phosphopeptides in total), one A43 peptide can be found, which includes the phosphosite S285. The largest dataset was recently published by Li and co-workers, who applied large scale phosphorylation analysis to α-factor arrested yeast cells (Li *et al*., 2007). They managed to identify a total of 2288 phosphosites, included in 5985 redundant phosphopeptides. With this analysis they confirmed the A43 phosphorylation site (S285) of the Gruhler dataset. In addition they published a new A190 phosphosite (S1636), a cluster of three phosphates in A34.5 (S10, S12 and S14) and one phosphorylated threonine in AC19 (T33). However, the A190 site is among the 766 phosphorylation sites with an ambiguous localization on the identified phosphopeptide, and accordingly should be assigned S1636 or S1638.
A fourth large scale phosphorylation analysis reported a phosphopeptide of A14 (S121) (Chi *et al*., 2007), but as this subunit is not phosphorylated *in vivo* (see Figure 11), this result is rather questionable.

Up to date, detailed analyses of phosphorylation sites and their function in eukaryotic RNA polymerases have been largely limited to the carboxy-terminal domain (CTD) of the largest Pol II subunit, which was subject to many investigations due to its importance for Pol II transcription (Phatnani and Greenleaf, 2006). The highly conserved CTD consists of 25 to 52 repeats (depending on the organism) of the heptapeptide sequence YSPTSPS, and the pattern of phosphorylation on serines 2 and/or 5 is linked to its regulatory function. However, no analogous domain exists in A190.

1.2.5.2 Kinases and phosphatases

A number of kinases and phosphatases were described to be involved in the Pol I transcription system. However, information about Pol I subunits as their possible targets is missing.

The first potential kinase found to have a function in the Pol I system was TFIIH (Iben *et al*., 2002). This multiprotein factor was originally identified as a component of the Pol II basal transcription machinery. It contains a cyclin-dependent CTD kinase, an ATP-dependent DNA helicase and several subunits involved in nucleotide excision repair (Zurita and Merino, 2003). A strong decrease of 35S rRNA production was observed in two different TFIIH ts-mutants (*i.e.* mutants of the kinase Kin28 and TFB1, a part of the DNA-repair system) with similar kinetics to an A43 ts-mutant, but the ability of TFIIH to activate Pol I transcription in the presence of a nonhydrolyzable ATP analog in a reconstituted mammalian Pol I transcription system contradicts the involvement of the kinase activity of TFIIH (Iben *et al*., 2002).

CTD kinase I (CTDK-I), *i.e.* its catalytic subunit Ctk1, is another kinase of the Pol II transcription machinery implicated in Pol I transcription (Bouchoux *et al*., 2004). Its described function is the phosphorylation of serine 2 of the CTD, which is associated with the elongation phase of Pol II (Komarnitsky *et al*., 2000; Sims *et al*., 2004). Ctk1 was found to co-immunoprecipitate with Pol I, and the nucleolar fraction PA600 purified from a Δctk1 deletion strain appeared to be inactive in an *in vitro* transcription assay (Bouchoux *et al*., 2004).

The only phosphatase, described to play a role in Pol I transcription so far, is Fcp1 (Fath *et al*., 2004). In the Pol II machinery, it helps to overcome transcriptional pauses during elongation, and due to its ability to dephosphorylate both serines 2 and 5 of the CTD, it is involved in the recycling of the polymerase after termination (Sims *et al*., 2004). The analysis of ts-mutants suggested another role of Fcp1 in the production of 35S rRNA. It was also found to increase the efficiency of Pol I transcription in unspecific or promoter dependent *in vitro* assays, but it is not required for the formation of the Pol I-Rrn3p-complex and transcription initiation. Rather, the Pol I function of Fcp1 was suggested to be associated to elongation (Fath *et al*., 2004).

The kinase Tor1, which is a component of the TORC1-complex (*T*arget *O*f *R*apamycin *C*omplex 1) (Loewith *et al*., 2002; De Virgilio and Loewith, 2006), was recently described to be associated with the rDNA promoter under normal growth conditions and excluded from the nucleus upon nutrient depletion or rapamycin treatment (Li *et al*., 2006). One consequence of the inhibition of TORC1 by rapamycin is the reduction of Pol I-Rrn3p-complexes and the Rrn3 mediated recruitment of Pol I to the promoter (Claypool *et al*., 2004, Laferté *et al*., 2006), which is probably the cause for the previous reported strong decrease of 35S rRNA production (Zaragoza *et al*., 1998; Powers and Walter, 1999). Whether this is a direct effect or includes a signaling cascade downstream of TORC1 was not elucidated yet. At the rDNA promoter Tor1 might directly phosphorylate Pol I and/or Rrn3p to facilitate the complex formation. Although this event is not dependent on a DNA template (Yamamoto *et al*., 1996; Schneider *et al*., 2004), the ribomotor model (see 1.2.1.2) would suggest complex formation in close proximity to the promoter to allow efficient re-initiation of the close-by terminated polymerases (see 1.2.4.3). Analysis of a mutant expressing an A43-Rrn3-fusionprotein (named CARA for *C*onstitutive *A*ssociation of *R*rn3 and *A*43) pointed towards Rrn3 as the target for modification (Laferté *et al*., 2006). This would be consistent with the rapamycin induced changes in the phosphorylation pattern of the mammalian homolog TIF-IA, which has been studied in detail (Grummt, 2003; Mayer and Grummt, 2006). However, the sites of modification are not conserved and the analyses of TIF-IA suggest that a more complex regulatory mechanism for the mammalian system evolved, certainly associated with the requirements to precisely control the growth of different cell types in a multicellular

organism. Furthermore, yeast Rrn3p was suggested to interact with Pol I in its nonphosphorylated or hypophosphorylated state, based on the ability of recombinant Rrn3 to mediate initiation *in vitro* (even in the presence of an nonhydrolyzable ATP analog) and on the comparison of ^{32}P-labeling of Pol I-bound Rrn3p to the total population (Fath *et al*., 2001). Thus yeast TORC1 might activate a phosphatase, which in turn renders Rrn3p capable of complex formation, or as mentioned above, Tor1 could directly modify Pol I.

1.3 Identification of phosphorylation sites using mass spectrometry

To further elucidate the details of the presumed regulation of Pol I via phosphorylation, it is crucial to identify the sites of modification. The advances in mass spectrometry (MS) facilitated the development of methods which employ this powerful technique to investigate posttranslational modifications of proteins.

Mass spectrometry allows to directly detect protein phosphorylation as an additional mass of + 79.9663 Da (Figure 13). First, the peptide-mixtures resulting from proteolytic digestion of the proteins (usually digested with Trypsin, due to its high specificity) are analyzed to select for putative phosphopeptides showing this specific mass shift compared to the theoretical mass of the unmodified version. Further fragmentation of the candidate peptides in a subsequent tandem MS (or MS/MS) analysis provides sequence information, allowing to verify the identity of the phosphopeptide and to pin down the site of modification to a certain amino acid (for detailed information on the mass spectrometric analysis of proteins and peptides please refer to Baldwin, 2005; Wells and McLucky, 2005; Medzihradszky, 2005).

Unfortunately straightforward MS analyses of phosphoproteins are hampered by a number of unfavorable properties of phosphorylated peptides. Some are associated to the type of mass spectrometer used (*e.g.* MALDI or ESI ion source; TOF, quadrupole or ion trap mass analyzer etc.), each of which has its advantages and disadvantages (Smith, 2002), but this introduction will focus on the MALDI-TOF/TOF (*M*atrix *A*ssisted *L*aser *D*esorption/*I*onization – *T*ime-*O*f-*F*light/ *T*ime-*O*f-*F*light) mass spectrometer (Karas and Hillenkamp, 1988; Medzihradszky *et al*., 2000; Vestal and Campbell, 2005) used throughout the study.
For most phosphoproteins, only a minor fraction of the total quantity is actually phosphorylated at a time. Accordingly the phosphopeptides are underrepresented in the protein digests, which anyway comprise a complex peptide-mixture. In the mass spectrometer all peptides need to be ionized in order to obtain information about their masses. Related to this ionization process, low abundant peptides are often poorly detected in the presence of a large excess of other analytes, a well known phenomenon referred to as ‘suppression effect’ (Kratzer *et al*., 1998; Schlosser *et al*., 2005). This applies especially for phosphopeptides

(Areces *et al*., 2004) due to their lower ionization efficiency compared to the non-phosphorylated analogs (Janek *et al*., 2001).

Furthermore phosphopeptides are susceptible to decomposition in the mass spectrometer, *i.e.* to the loss of H_3PO_4 ('neutral loss') (Annan and Carr, 1996; Areces *et al*., 2004). While this can be used as a phosphopeptide-marker in some applications (Annan and Carr, 1996; see also Lehmann *et al*., 2007), it also results in a further splitting of the low abundant phosphopeptides into fractions with and without the phosphate group (which is still different from the non-phosphorylated peptide because the dehydro amino acids resulting from neutral loss of H_3PO_4 lack the OH-group of the original serines or threonines).

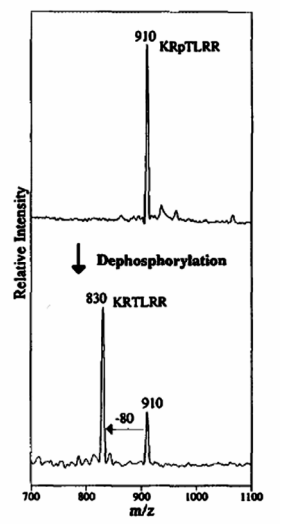

Figure 13. MALDI mass spectra of a phosphopeptide before (top) and after dephosphorylation (bottom). In mass spectra the x-axis corresponds to the mass to charge (m/z) ratio of the detected ions, while the y-axis shows their relative abundance. The highest peak is set to 100 % (referred to as base peak). In MALDI-MS the charge is typically +1 (caused by the transfer of one proton during the ionization process), thus the m/z ratio equals the molecular mass +1 Da (MH^+). Phosphorylation can be detected as an additional mass of +79.9663 Da compared to the non-phosphorylated counterpart. [from Liao *et al*., 1994]

There are a number of approaches to deal with these problems, but no 'standard method' for the mass spectrometric analysis of protein phosphorylation has evolved yet, as reflected by the number of reports describing new or improved methods still published every month (*e.g.* about 12 in November 2007).

Most of these methods rely on an enrichment of the phosphorylated peptides to facilitate their detection. This is achieved by binding of negatively charged phosphopeptides to Fe^{3+}- or Ga^{3+}-IMAC (*I*mmobilized *M*etal *A*ffinity *C*hromatography) columns (Andersson and Porath, 1986; Posewitz and Tempst; 1999; Lund and Ardö, 2004), pH-dependent adsorption of the phosphate groups to TiO_2- or ZrO_2-beads (Pinkse *et al*., 2004; Kweon and Håkansson, 2006) or the highly specific binding to phosphotyrosine antibodies (Kalo and Pasquale, 1999; De Corte *et al*., 1999) (for an overview please refer to Witze *et al*., 2007; Reinders and Sickmann, 2005; Areces *et al*., 2004). In some cases additionally a pre-enrichment of negatively charged peptides on a strong cation-exchanger (SCX) was used (Nühse *et al*., 2003; Gruhler *et al*., 2005). Furthermore the fractionation of the peptides via C_{18} reversed phase chromatography, to further lower the complexity of the sample and thus minimize

suppression effects, was found to be advantageous (Areces *et al*., 2004). The HPLC is coupled directly to the mass spectrometer (referred to as LC-MS); for MALDI-ion sources this is facilitated by fraction collection on the MALDI sample plate (LC-MALDI) (Wall *et al*., 2002; Zhen *et al*., 2004).

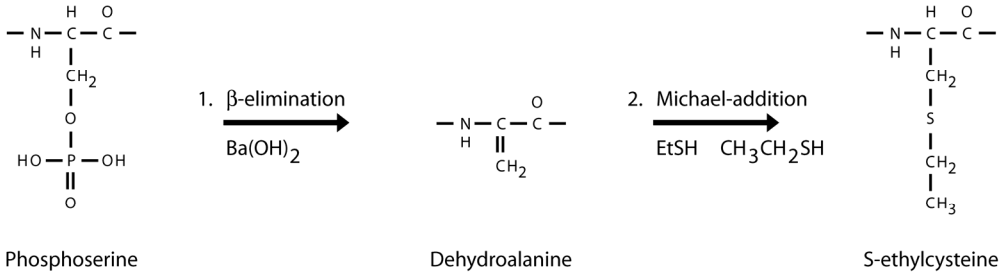

Figure 14. Reaction scheme of the chemical derivatization of phosphopeptides. The derivatization of a phosphoserine with ethanethiol (EtSH) is shown as an example. The phosphate group is β-eliminated in the presence of $Ba(OH)_2$. The resulting dehydroamino acid is susceptible to Michael-addition of an S- or N-nucleophile.

In this study an alternative approach based on chemical derivatization of the phosphopeptides to increase their stability and raise the ionization efficiency was used. The phosphate-group is removed in a β-elimination reaction at high pH in the presence of Ba^{2+}-ions and replaced by an S- or N-nucleophile (*e.g.* ethanethiol) in a Michael-addition reaction (Byford *et al*., 1991; Molloy and Andrews, 2001; Klemm *et al*., 2004) (Figure 14). This technique was originally developed to facilitate the detection of phosphoresidues in Edman sequencing (Annan *et al*., 1982; Byford *et al*., 1991; Meyer *et al*., 1991) and was later on found to be also very useful in MALDI-MS applications (Molloy and Andrews, 2001; Jaffe *et al*., 2001). The derivatization results in a unique mass shift which allows the identification of the originally phosphorylated residues.

1.4 Objective

RNA polymerase I catalyzes the transcription of the 35S rRNA precursor, which is one of the first steps of ribosome biogenesis. It is crucial for a cell to control this process in dependence of the growth conditions. The regulatory mechanism apparently involves signal transduction via reversible phosphorylation through cascades of protein kinases and phosphatases to several key regulatory steps of the ribosome biogenesis system.

About 30 years ago, two independent research groups identified five *in vivo* phosphorylated subunits (A190, A43, A34.5, ABC23 and A34.5) among the fourteen subunits of the Pol I

complex from the yeast *Saccharomyces cerevisiae* by ^{32}P-labeling (Bell *et al*., 1976; Buhler *et al*., 1976b; Bell *et al*., 1977). The phosphate content was calculated to be about 15 ± 3 phosphate groups per enzyme (Bréant *et al*., 1983) and a connection between the phosphorylation state of Pol I and its activity was established *in vitro* (Fath *et al*., 2001; Fath *et al*., 2004). However, little information is available about the positions and functions of the sites of modification.

The main objective of this thesis was the identification of phosphorylation sites of yeast RNA polymerase I. Prerequisite was the development of a rapid procedure to purify Pol I from logarithmically growing yeast cells while maintaining its phosphorylation status. An additional intended purpose of this purification procedure was to obtain enzyme preparations for structural analyses in cooperation with the research group of Patrick Cramer. Thus, besides trying to obtain the highest purity possible, it was required to enable easy upscaling of the procedure. Another prerequisite for the identification of Pol I phosphorylation sites was the establishment of a method to analyze protein phosphorylation using the available MALDI-TOF/TOF mass spectrometer. Upon successful identification, the phosphorylation sites should be characterized by site-directed mutagenesis and growth analyses of the resulting mutant yeast strains to gain information about the functions of the reversible phosphorylation of Pol I.

2 Results

2.1 Pol I purification

Prerequisite for the identification of Pol I phosphorylation sites was the purification of the multisubunit enzyme from yeast whole cell extracts (WCE). Furthermore the purified Pol I complex should be used for structure determination in cooperation with the laboratory of Patrick Cramer. Thus, besides trying to obtain the highest purity possible, the purification procedure would need to be upscaleable afterwards.

The yeast strain used for Pol I purification was GPY2 (Fath *et al*., 2000), which carries the gene coding for an N-terminal His_6 / HA-tagged variant of subunit A43 on a low-copy plasmid, while the chromosomal locus was knocked out with the LEU2-marker gene. The growth behavior of this strain is similar to a wild-type yeast strain, and the binding of the His_6 / HA-tagged A43 to Ni-NTA resin was already tested before (Fath *et al*., 2000; Hauger, 2002).
Yeast cultures were grown in YPD-media to the logarithmic growth phase (OD_{600} = 1-2), in which the ribosome biogenesis machinery is fully active (Warner, 1999). To ensure the integrity of the polymerase in the starting material, the buffer conditions and procedure for cell breakage were taken from the protocol for the preparation of the transcriptional active nucleolar fractions PA600 and B600 / B2000 (Tschochner, 1996; Milkereit and Tschochner, 1998). This protocol includes an initial ultracentrifugation step (100.000 g for 90 minutes), which separates the crude cell lysate into three phases. The clear middle phase, *i.e.* the soluble fraction of the lysate, was collected while avoiding the turbid insoluble matter in the lower phase and the top layer of lipids, and designated WCE.

The first step of the new Pol I purification protocol is the dialysis of the high salt WCE (400 mM $(NH_4)_2SO_4$) against a low salt buffer (50 mM KAc) (Figure 15A). As a consequence, a relative small fraction of proteins precipitates from the cell extract, including Pol I (Figure 15B). The principle of this low-salt precipitation (Englard and Seifter, 1990) was previously used to enrich Pol I from eluates of a DEAE column (a weak anion exchanger) in the fractionation scheme mentioned above (Tschochner, 1996; Milkereit and Tschochner, 1998). Western blot analyses revealed, that Pol I can be quantitatively precipitated from the whole cell extract upon complete dialysis (Figure 15B). Pol II and Pol III co-precipitate to some extent, but in both cases the major amount remains in the soluble fraction.

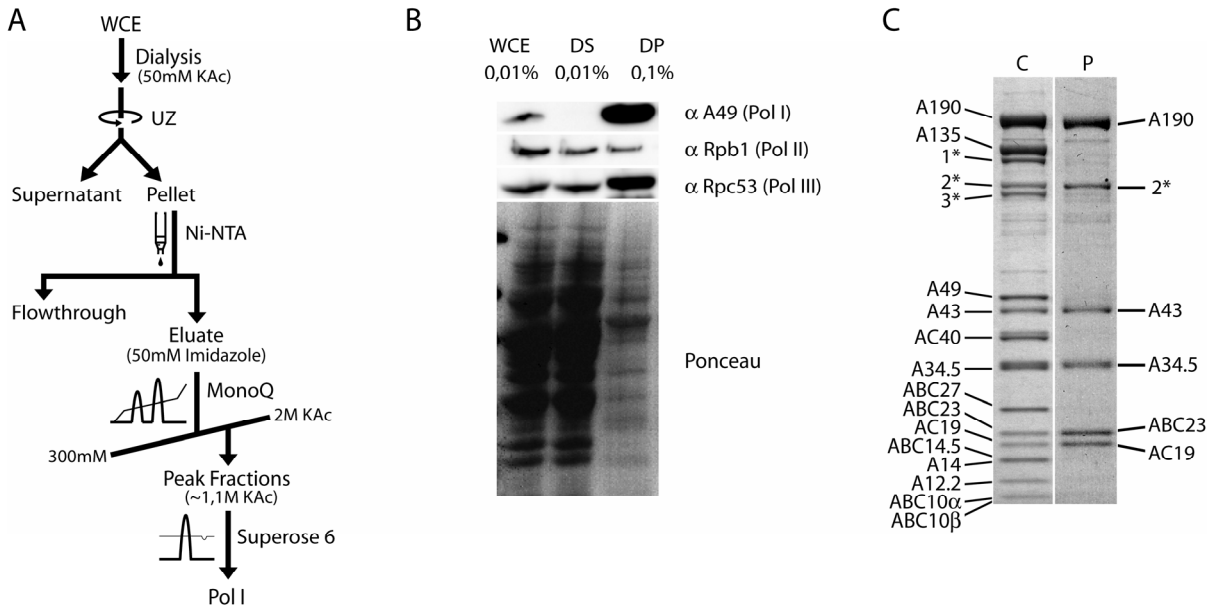

Figure 15. Yeast RNA polymerase I purification. (A) Pol I purification scheme. **(B)** Initial low-salt precipitation step. Aliquots of the whole cell extract [WCE] and of the dialysate after centrifugation (dialysate supernatant [DS]; dialysate pellet [DP]) were analyzed by western blotting. The membrane was stained with Ponceau-S for total protein content before immunodetection of Pol I (A49), Pol II (Rpb1) and Pol III (Rpc53) subunits. Note that due to the much lower volume and total protein content 0.1 % by volume of the dialysate pellet was loaded on the gel instead of the 0.01 % of the other fractions. **(C)** Coomassie staining of purified Pol I [C] and phosphoprotein staining of the same gel [P] using phosphostain ProQ Diamond (Invitrogen). Three proteins of an eIF3 subcomplex co-purified with Pol I, namely Rpg1p (1*), Nip1p (2*) and Prt1p (3*).

Following centrifugation of the dialysate, the precipitated proteins in the pellet can be resolubilized in another buffer. The major advantages of this precipitation at the beginning of the purification procedure are the removal of bulk impurities (*i.e.* they remain in the supernatant, which is discarded) and the concentration of the proteins in a small volume, facilitating the subsequent column steps. Furthermore sample handling was minimized by using the binding buffer of the following nickel-affinity chromatography for resolubilization (Figure 15A). The yeast Pol I complex is stable at salt concentrations up to 2 M KAc, as demonstrated by the functional polymerase in the B2000 fraction (Tschochner, 1996; Milkereit and Tschochner, 1998). Thus, it was possible to apply very stringent conditions (1.5 M KAc) with the binding buffer. In the last wash steps and the elution steps of the Ni-NTA column, the salt concentration was lowered to 300 mM KAc, to adjust the binding conditions for the subsequent MonoQ anion exchange chromatography (Figure 15A), from which Pol I elutes at approximately 1.1 M KAc. The final polishing step is a gelfiltration chromatography using a Superose 6 column to obtain a homogeneous Pol I preparation.

A coomassie stained polyacrylamide gel of the purified Pol I is shown in (Figure 15C). The pattern of the stained protein bands resembled the expected pattern for the complete Pol I complex (see Paule, 1998) and the identity of the subunits was verified by MALDI-TOF/TOF

mass spectrometry. The three co-purified additional protein bands were identified as the components of an eIF3-subcomplex, *i.e.* Rpg1p (= Tif32p), Nip1p and Prt1p (Phan *et al*., 2001). However, these are rather contaminating proteins than specific interaction partners.

Starting from 10 l yeast culture (OD_{600} = 1-2) this purification procedure yielded approximately 200 µg Pol I. The upscaling, including some optimizations with respect to the crystallization approach (*e.g.* omitting the detergents from the buffers), was developed by Claus Kuhn in the Cramer laboratory. An additional MonoS cation exchange chromatography subsequent to the MonoQ column and prior to the gelfiltration removed the last contaminating proteins. In this large-scale version the yield was about 5 mg Pol I from 200 l yeast culture.
The purity of the established Pol I purification method was suitable for structure determination by cryo-EM to a resolution of 12 Å (Kuhn *et al*., 2007) (see 1.2.2.3, Figure 5). The purified Pol I was also suitable for crystallization, but the interpretation of the x-ray diffraction pattern is still in progress.

The enzyme preparation was tested to be active in a promoter-independent unspecific transcription assay, indicating the preservation of the native protein conformation and also of the posttranslational modifications throughout the purification procedure, since the dephosphorylation of Pol I by alkaline phosphatase results in the loss of transcriptional activity (Fath *et al*., 2004). Interestingly, in early versions of the purification, the activity was completely lost during the Ni-NTA affinity chromatography, when using $(NH_4)_2SO_4$ instead of KAc.
To analyze the phosphorylation status of the purified Pol I, a sample of the preparation was separated by SDS-PAGE and the gel stained with the phosphoprotein stain Pro-Q Diamond (Invitrogen). The five Pol I subunits, which are phosphorylated *in vivo*, were also stained in the purified Pol I (Figure 15C). The only other band visible in this staining was the co-purified Nip1p.

2.2 Pol I phosphorylation sites

2.2.1 Chemical derivatization of phosphopeptides – establishing the method

Prior to the analysis of the Pol I phosphorylation sites, synthetic phosphopeptides and a model phosphoprotein (*i.e.* α-casein from bovine milk) were used to establish a method facilitating the detection of phosphorylated peptides on the available MALDI-TOF/TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer). Figure 16A shows an ion-spectrum of the phosphopeptide EAIpSAAPFAK (peptide mass MH^+ 1083.524 Da) in the

normal positive ion reflector mode, using α-cyano-4-hydroxycinnamic acid (CHCA, 2 mg/ml) as matrix. Next to the peak of the expected mass to charge ratio (m/z) a second major peak is visible (MH^+ 988.6377 Da), which corresponds to the same peptide after the neutral loss of H_3PO_4 (see 1.3).

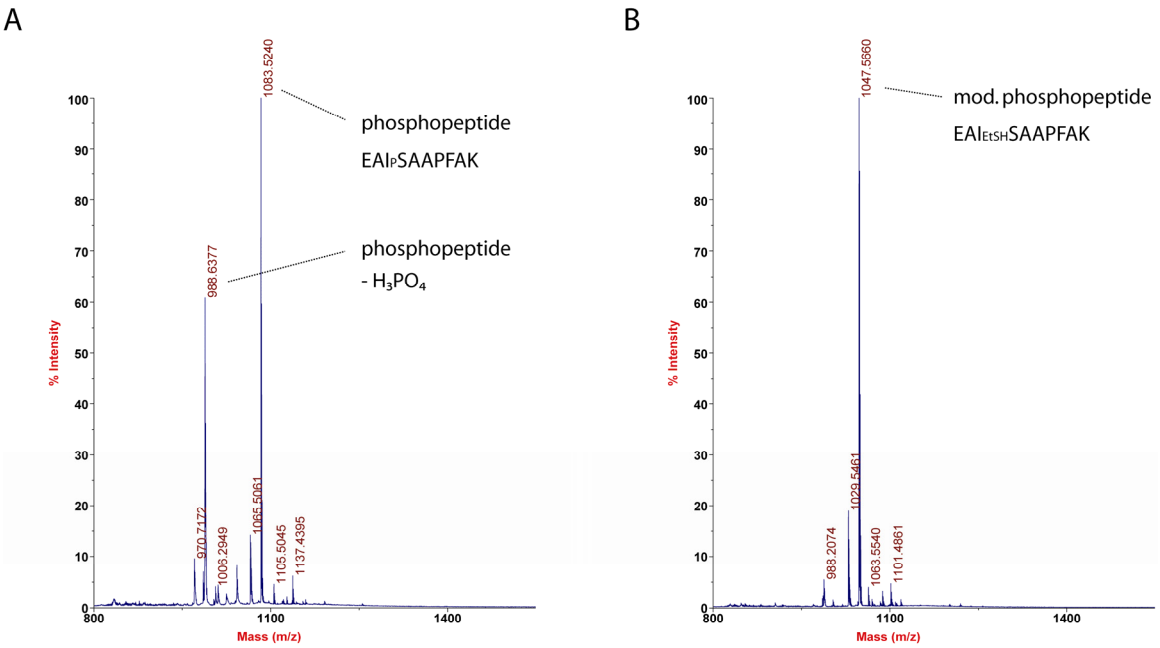

Figure 16. MALDI-MS of phosphopeptides and their derivates. Mass spectrum of the synthetic phosphopeptide EAIpSAAPFAK before **(A)** and after **(B)** β-elimination of the phosphate group and Michael-addition of ethanethiol (EtSH). Please see text for details.

Note that in MALDI-MS normally only singly charged ions are detected (Karas *et al.*, 2000), thus the mass to charge ratio (m/z) given in the spectra equals the molecular mass in Da (= M) plus one dalton from the proton added during the ionization process (= MH^+). However, the $(MH\text{-}H_3PO_4)^+$ -ion is not displayed at the exact mass in the spectrum recorded in reflector mode (Annan and Carr, 1996; Areces *et al.*, 2004), because the neutral loss of H_3PO_4 from the metastable phosphopeptides occurs in the flight tubes of the mass analyzer. The phosphopeptide is accelerated in the ion source with one mass (MH^+) but reaches the ion mirror with a lower mass [$(MH\text{-}H_3PO_4)^+$] (*i.e.* with a lower kinetic energy), which finally confuses the measurement (see Annan and Carr, 1996 for details). Neutral loss of H_3PO_4 results in a mass shift of -97.9769 Da and thus the actual mass of the $(MH\text{-}H_3PO_4)^+$ -ion would be 985.5471 Da. The other small peaks apparent in the spectrum result from additional fragmentation events (*e.g.* the loss of H_2O (MH^+ -18.0106 Da)) or the formation of adducts (*e.g.* Na^+-adducts (MH^+ +21.9819 Da)), but these are not linked to the presence of a

phosphoresidue in the peptide (Ballard and Gaskell, 1993; Paizs and Suhai, 2005; Leite *et al*., 2004).

In order to stabilize the phosphopeptides, a chemical derivatization procedure was tested. The phosphate group is removed by a β-elimination reaction upon exposure to $Ba(OH)_2$. The resulting dehydroamino acids are susceptible to nucleophilic derivatization with alkanethiols (Michael-addition) (see 1.3, Figure 14). However, in each study employing this method different reaction conditions are described. Thus, a protocol based on the procedure described by Molloy and Andrews (2001) was used and several parameters tested according to the protocols by Byford (1991), Jaffe *et al*. (2001) and Klemm *et al*. (2004). These included the concentrations of $Ba(OH)_2$ and of the alkanethiol in the reaction mixture, the solvent used and the temperature and length of the incubation. Optimization of the protocol was monitored by analyzing the degree of modification of the synthetic phosphopeptide by MALDI-MS.
Briefly, the final protocol included the following steps: Cysteine residues were first blocked with iodoacetic acid to prevent their conversion to dehydroalanine (Byford, 1991) and their subsequent modification by the alkanethiol (due to sequence information this could still be discriminated from the modified phosphosites, but it would result in additional variables for the data evaluation). The β-elimination and Michael-addition were then conducted simultaneously in one reaction batch with ethanethiol (EtSH) or pentanethiol (PeSH) as nucleophile. Incubation was performed at 37 °C for 90 minutes in the presence of approximately 65 mM $Ba(OH)_2$ (taken from a freshly prepared saturated solution) and 30 % 1-propanol as solvent. Note that in some of the later experiments the incubation temperature and solvent were changed to 50 °C and 30 % acetonitrile (ACN), respectively. After the incubation, the Ba^{2+}-ions were precipitated as insoluble $BaCO_3$ upon addition of NH_4HCO_3, and residual alkanethiols, solvent and ammonium carbonate were removed by lyophilization. The resulting modified, lyophilized peptides could then be further processed by standard sample preparation methods (*e.g.* desalting with ZipTip C_{18} pipette tips (Millipore)) and analyzed by MALDI-TOF/TOF mass spectrometry.

Figure 16B shows the MS spectrum of the phosphopeptide EAIpSAAPFAK after β-elimination and Michael-addition of ethanethiol. The peak of the original phosphopeptide (MH^+ 1083.524 Da) is no longer visible. Instead a single major peak at m/z 1047.566 (MH^+ 1047.566 Da) appeared which corresponds to the derivatized phosphopeptide as confirmed by MS/MS analysis (Figure 17). In this tandem MS mode a selected peptide is fragmented by collision with gas molecules (CID, *C*ollision *I*nduced *D*issociation) and a spectrum of the resulting fragments is recorded which provides information on the sequence of the peptide. Besides confirming the identity of the peptide, the originally phosphorylated serine was thus clearly identified as the site of modification.

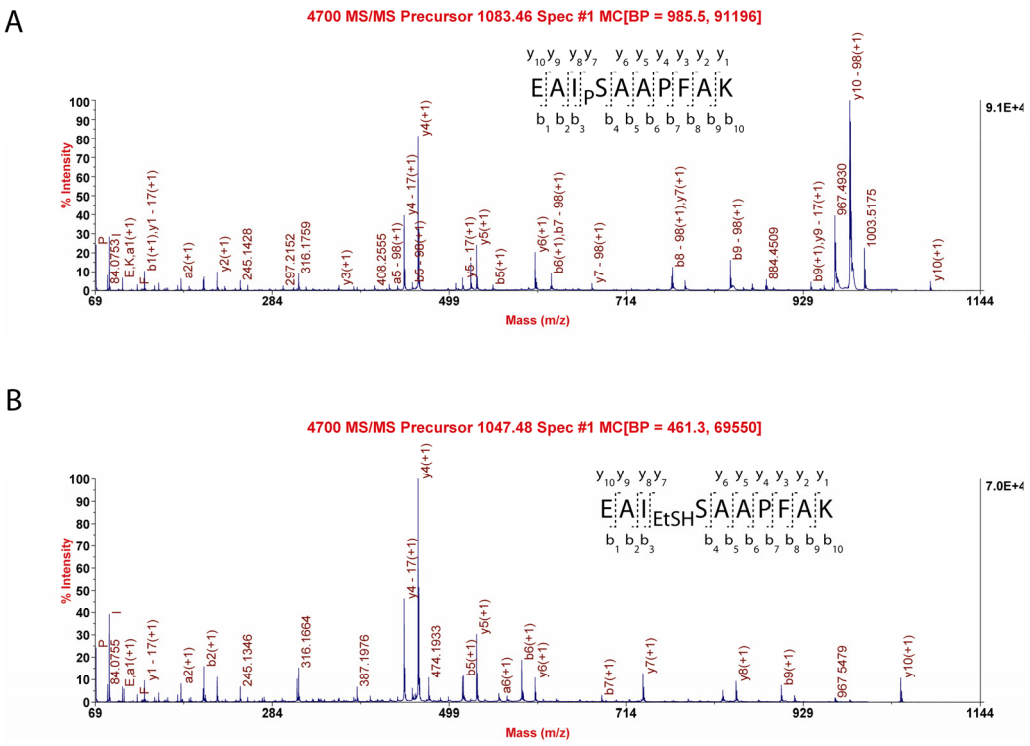

Figure 17. MS/MS analyses of phosphopeptides and their derivates. The synthetic phosphopeptide EAIpSAAPFAK **(A)** before (precursor mass 1083.524 Da) and **(B)** after chemical derivatization with ethanethiol (precursor mass 1047.566 Da) (see Figure 16 for the respective MS spectra) were subjected to MS/MS analyses. In this mode selected peptides are fragmented along the peptide backbone by *C*ollision *I*nduced *D*issociation (CID). The analysis of the resulting peptide fragments of different lengths provides sequence information facilitating the confirmation of the identity of the peptide and the identification of the site of modification (please refer to Medzihradszky, 2005; Wells and McLuckey 2005; Paizs and Suhai, 2005 for detailed information). Due to the preferential protonation at the C-terminal lysine or arginine of tryptic peptides in the MALDI ion source and the fragmentation conditions in the collision cell, mainly the y-fragment ion series are visible in the spectra. The specific mass shifts starting from the y_7-ion (*i.e.* y_7-y_{10}) identify the serine at this position as the modified amino acid. The -98 Da shifts in the MS/MS spectrum of the underivatized phosphopeptide result from the neutral loss of H_3PO_4. Both spectra were recorded with the 'optimized precursor'-option of the 4000 Series Explorer software (Applied Biosystems).

The next strongest peak in the MS-spectrum resulted from the loss of H_2O, apparently from the glutamate in the N-terminal position of the peptide (MH^+ 1029.546 Da). A peak which probably corresponds to the loss of ethanethiol in the mass spectrometer (MH^+ 988.2 Da) is present only at a very low relative intensity, further arguing for the stabilization of the modified peptide in MALDI-MS as compared to the non-derivatized phosphopeptide.
Some other nucleophiles different from alkanethiols were also tested (see Klemm *et al.*, 2004), but with none of them a complete derivatization comparable to ethanethiol could be achieved.

Next, the β-elimination / Michael-addition method was tested with a tryptic digest of the model-phosphoprotein α-casein. This protein is quantitatively phosphorylated at eight different sites, which are normally distributed to four tryptic peptides (Kjellström and Jensen,

2004). In the MALDI-spectrum only two of these were apparent, *i.e.* the singly phosphorylated peptides. After the chemical derivatization with ethanethiol, peaks at the expected masses for all four originally phosphorylated peptides could be detected.

Because protein identification by mass spectrometry relies on comparison of experimentally determined masses with theoretical masses in a database, any modification must be included as variable in the search parameters. Thus, at this stage the masses of serine and threonine derivatized with ethanethiol (monoisotopic mass 131.040489 Da (S) and 145.056139 Da (T)) or pentanethiol (monoisotopic mass 173.08744 Da (S) and 187.10309 Da (T)) were added to the modifications file (…/mascot/config/mod_file) of the Mascot search engine (Perkins *et al*., 1999) which is implemented in the GPS explorer software (Applied Biosystems) used for data evaluation.
Allowing these variables, the derivatized α-casein phosphopeptides were unambiguously identified in the peptide mass fingerprint (PMF). MS/MS analyses confirmed their identity; even of the five-time phosphorylated 2.7 kDa peptide, which is normally hard to detect in peptide mixtures. No apparent modification of any originally non-phosphorylated peptide could be detected after the β-elimination / Michael-addition procedure.

2.2.2 Identification of Pol I phosphorylation sites

The established β-elimination / Michael-addition procedure for the detection of phosphopeptides in MALDI-MS was applied to yeast RNA polymerase I.
A scheme of the complete strategy is shown in (Figure 18). Although the polymerase is highly purified, the few phosphopeptides would still represent only a minor fraction of the peptide-mixture derived from the whole 600 kDa complex. Thus, a gel-based strategy was preferred to an in-solution digestion strategy, to start from the single purified Pol I subunits. The analyses were restricted to the five subunits which were described to be phosphorylated *in vivo* (see 1.2.5).

Briefly, Pol I subunits were separated by SDS-PAGE, the gel was stained with coomassie blue and the bands of the phosphosubunits were excised. Following standard in-gel digestion with trypsin (Shevchenko *et al*., 2007) the resulting peptides were eluted and modified by the β-elimination / Michael-addition procedure described above (2.2.1), using either ethanethiol or pentanethiol as nucleophiles replacing the phosphoryl-group. The modified peptides were then either desalted with ZipTip C_{18} reversed phase microtips (Millipore) and spotted manually on the MALDI target using the dried-droplet method (Cañas *et al*., 2007) or separated on a nanoHPLC C_{18} reversed phase column, with the fractions directly collected on the MALDI sample plate using a Dionex Probot system (LC-MALDI). The identification of

the unknown phosphopeptides in the samples is based on the specific mass shift after chemical derivatization compared to the expected mass of the non-phosphorylated form (Figure 19 and Figure 20).

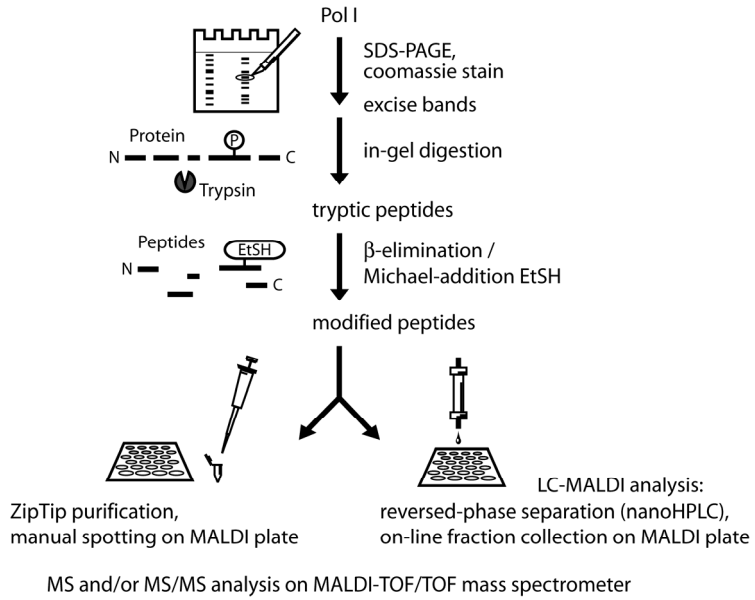

Figure 18. Identification of phosphorylation sites from purified Pol I. Scheme of the strategy. Either ethanethiol (EtSH) or pentanethiol (PeSH) were used for chemical derivatization. Modified peptides were alternatively analyzed after ZipTip purification and manual spotting on the target plate or via the LC-MALDI approach.

Since the objective of these analyses was the identification of post-translational modification sites on known proteins and not the identification of unknown proteins in the samples, a small 'yeast Pol I sequence database' was created for the data evaluation. It includes the 14 subunits as well as all co-purifying proteins, trypsin and known interaction partners which possibly co-purify in small amounts (*i.e.* Rrn3p). The advantage of this customized database is the possibility to use more variable modifications in the search parameters without creating false positive hits (Johnson *et al*., 2005). As mentioned above the identification is based on the comparison of experimental and theoretical masses. Any possible modification (post-translational or artificial; *e.g.* deamidation of asparagines or glutamines caused by the β-elimination reaction conditions (Karty and Reilly, 2005)) would change the experimental mass, thus prevent a match. Variable modifications in the search parameters can compensate for this, but with the number of variables (especially for frequently occurring amino acids) and of the number of proteins in the database, the chance for false assignments increases, which finally hamper straightforward analyses.

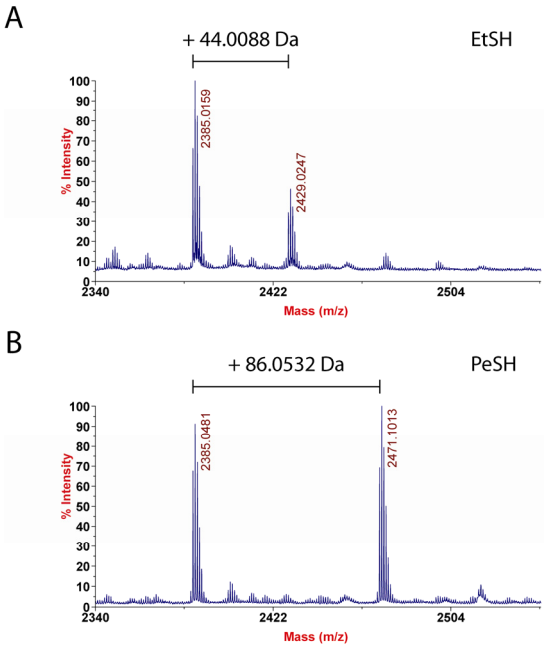

Figure 19. Identification of originally phosphorylated peptides via specific mass shifts upon derivatization. The example shows part of a mass spectrum containing the tryptic peptide A43 242-264 in the non-phosphorylated and in the phosphorylated form after β-elimination and Michael-addition of **(A)** ethanethiol or **(B)** pentanethiol. A large excess of the non-phosphorylated form is present in most cases but is not required for identification. The actual identification of the modified peptides in the spectra is performed during the computer-based evaluation by defining the respective specific mass shifts as variable modification.

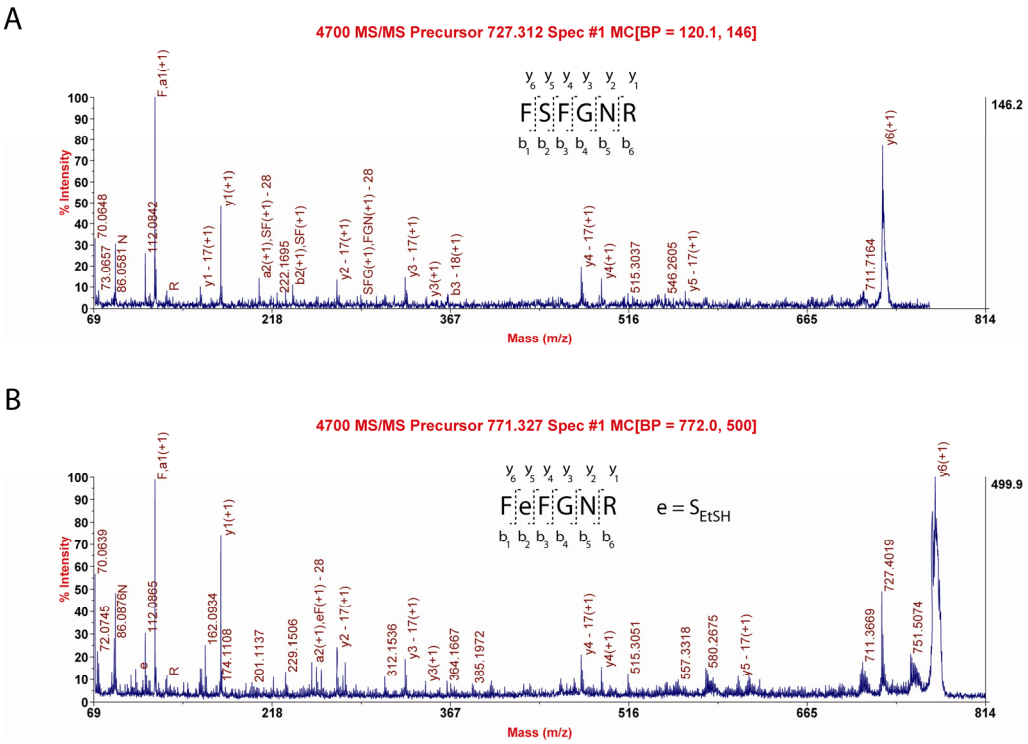

Figure 20. Identification of the modified amino acid in the phosphopeptide. The MS/MS mass spectra of peptide A43 207-212 in **(A)** the non-phosphorylated and **(B)** the phosphorylated form after chemical modification with ethanethiol (EtSH) are shown as example. The precursor mass of (B) corresponds to the precursor mass of (A) plus the specific additional mass of + 44.0085 Da resulting from the derivatization of the phosphorylated form. The appearance of the additional + 44.0085 Da starting from the y_5-ion of the derivatized phosphopeptide (i.e. the y_5 and y_6-ions) identifies this amino acid as the site of modification. Additionally the b_2-ion of the corresponding mass is visible in the spectrum. The -17 Da mass shift of the y-ions starting from y_2 results from the loss of ammonia from the asparagine residue.

Table 2. Identified Pol I phosphopeptides and –sites and corresponding amino acids in the Pol I homology model.

Subunit	Phosphopeptide			Amino acid in homology model [3]
	Position	*Sequence*[1]	*Phosphosite*	
A190	352-366	AD**S**FFMDVLVVPPTR	S354	Rpb1 W234
	684-689	D**S**FFTR	S685	Rpb1 T539
	935-955	G***S***NVNV***S***QIMCLLGQQALEGR	S936 or S941[2]	Rpb1 S754 / A759
A43	207-212	F**S**FGNR	S208	N/D
	213-228	SLGHWVD**S**NGEPIDGK	S220	A43 S220
	242-264	VVSVDGTLISDADEEGNGYN***SS***R	S262 or S263[2]	N/D
	278-289	IVFDDEV**S**IENK	S285	N/D
A34.5	7-31	DYV**S**D**S**D**S**DDEVISNEFSIPDGFKK	S10 / S12 / S14	N/D
ABC23	98-119	ALQI**S**MNAPVFVDLEGETDPLR	S102	ABC23 S102
AC19	21-46	HIQEEEEQDVDM**T**GDEEQEEEPDREK	T33	Rpb11 A3
	49-77	LL***T***QA***TS***EDGTSASFQIVEEDHTLGNA LR	T51 or T54 or S55[2]	Rpb11 I21 / D24 / T25

[1] Phosphoserines /-threonines are presented as bold, underlined characters.

[2] Three phosphopeptides could be identified due to the specific mass shift after chemical derivatization and a partial MS/MS spectrum, but the phosphorylation site could not be unambiguously assigned. The remaining possible serines and threonines are shown in bold, i letters in the peptide sequence.

[3] Some phosphosites could not be localized in the Pol I homology model due to weak homology of the respective protein regions to Pol II, missing homologous subunits or deletions in the A43/14 crystal structure.

In total 11 Pol I phosphopeptides including 13 phosphorylation sites were identified (Table 2): three sites on the A190 subunit (S354, S685 and S936/S941), four on A43 (S208, S220, S262/S263 and S285), a cluster of three phosphorylation sites on one phosphopeptide of A34.5 (S10, S12 S14), one site on the common subunit ABC23 (S102) and two on AC19 (T33 and T51/T54/S55) which is shared by Pol I and Pol III.

Three of the sites could not be unambiguously assigned on the corresponding phosphopeptides (peptides A190 935-955, A43 242-264 and AC19 49-77). The peptides were identified due to the specific mass shift after derivatization with ethanethiol and pentanethiol and their identity confirmed by a partial MS/MS spectrum. While some of the serines and threonines on these peptides could be excluded as the site of modification, some candidate residues were left due to missing peaks in the fragment-ion series.

The phosphosites A43 S285, A34.5 S10/S12/S14 and AC19 T33 were confirmed by the proteome-wide large scale phosphorylation analyses reported by Gruhler *et al*. (2005) and Li *et al*. (2007) (see 1.2.5.1). Note that the methods applied in these analyses were completely different from the chemical derivatization approach used in this study. Both relied on the enrichment of phosphopeptides from the whole cell extracts using immobilized metal affinity

chromatography (IMAC), one in combination with a pre-fractionation on strong cation exchange (SCX) columns, the other after separation by preparative SDS-PAGE.
Furthermore the additional A190 phosphosites identified in the proteome-wide approaches by Ficarro *et al*. (2002) (three clustered sites S1413, S1415 and S1417) and Li *et al*. (2007) (S1636) should be mentioned.

2.2.3 Localization of the phosphorylation sites in the Pol I complex

Figure 21 shows the positions of the identified Pol I phosphorylation sites in the primary structures of the five phosphosubunits with respect to the protein's domain organizations and in the context of previously published mutations.

A Pol I homology model facilitated the three-dimensional localization of the identified phosphorylation sites in the Pol I complex (Figure 22). The homology model was constructed by Claus Kuhn and Patrick Cramer based on the new Pol I cryo-EM structure (see 2.1), the 10 subunit Pol II crystal structure, sequence-alignments of the Pol I and Pol II subunits and the new crystal structure of the A43/A14 heterodimer (Kuhn *et al*., 2007). The corresponding amino acids of the Pol I phosphosites in the homology model are listed in Table 2. Seven of the thirteen identified phosphosites could be located in the model. The others could not be assigned due to missing homologous subunits in Pol II (A34.5 S10, S12, S14) or deleted regions in the A43/14 crystal structure (A43 S208, S262/263, S285). The A190 triple cluster (A190 S1413, S1415 and S1417) is in a region of weak homology to Rpb1 and thus also not included in the model. Most of the sites can be found at the surface of the enzyme, consistent with the accessibility for modifying enzymes (*i.e.* kinases and the phosphatases) (Pang *et al*., 2007) (Figure 22).

The A190 phosphosite S354 can be found on top of the mobile clamp, which encloses the DNA template during transcription elongation (Gnatt *et al*., 2001; Cramer, 2002). It is located directly at the Clamp head – Clamp core transition (see Cramer *et al*., 2001; Kuhn *et al*., 2007). Furthermore the phosphorylation site A190 S1636 is positioned in a small region at the extreme C-terminus of A190 which additionally contributes to the Clamp core.

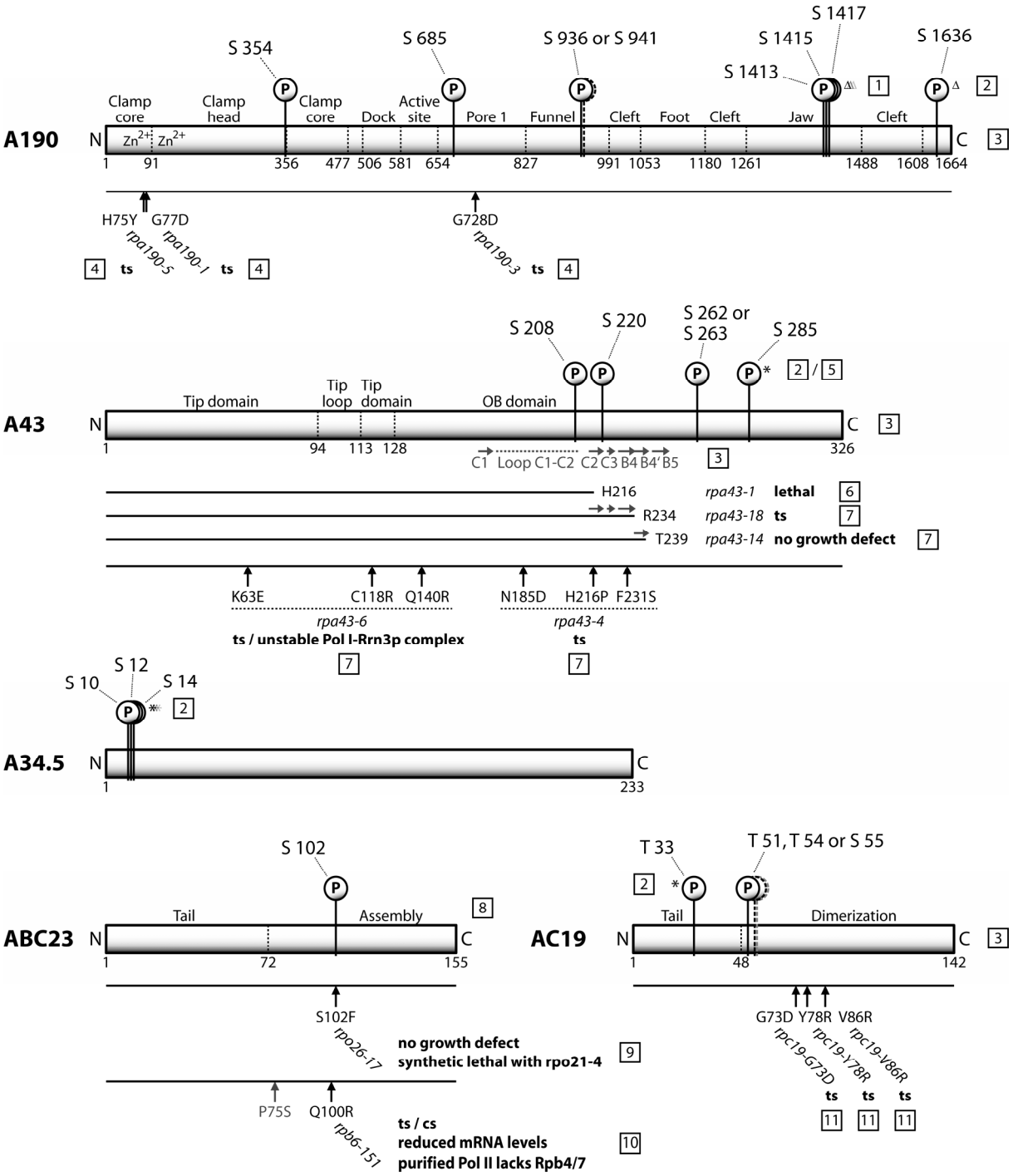

Figure 21. Phosphorylation sites of Pol I. Positions of the identified Pol I phosphorylation sites in the primary structure of the five phosphorylated subunits. Phosphorylation sites marked with an asterisk were confirmed by proteome wide, large-scale phosphorylation analyses (see text). Sites identified exclusively by proteome wide analyses are marked by a triangle. The domain organization of the subunits and the positions of amino acid exchanges of known Pol I mutants are shown, as well as a part of the secondary structure of A43. References: [1] Ficarro *et al*., 2002; [2] Li *et al*., 2007; [3] Kuhn *et al*., 2007; [4] Wittekind *et al*., 1988; [5] Gruhler *et al*., 2005; [6] Thuriaux *et al*., 1995; [7] Peyroche *et al*., 2000; [8] Cramer *et al*., 2001; [9] Nouraini *et al*., 1996; [10] Tan *et al*., 2003; [11] Lalo *et al*., 1993.

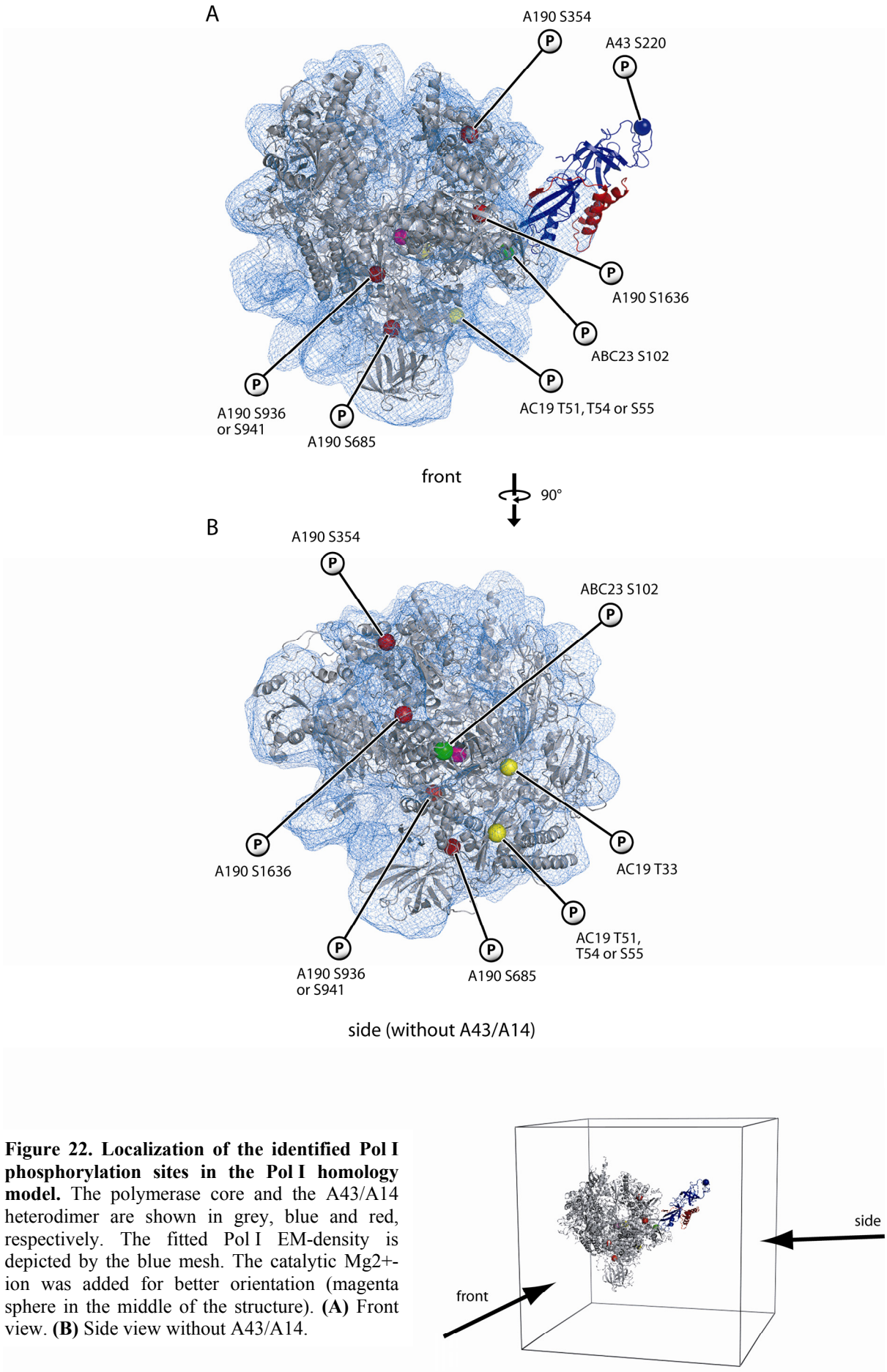

Figure 22. Localization of the identified Pol I phosphorylation sites in the Pol I homology model. The polymerase core and the A43/A14 heterodimer are shown in grey, blue and red, respectively. The fitted Pol I EM-density is depicted by the blue mesh. The catalytic Mg2+-ion was added for better orientation (magenta sphere in the middle of the structure). **(A)** Front view. **(B)** Side view without A43/A14.

A190 S685 and S936/941 are located in the Pore 1- and Funnel-region, through which NTPs enter the active site during transcription and from which the 3' end of the RNA is presumed to exit upon backtracking of the enzyme (Cramer *et al*., 2000; Batada *et al*., 2004).

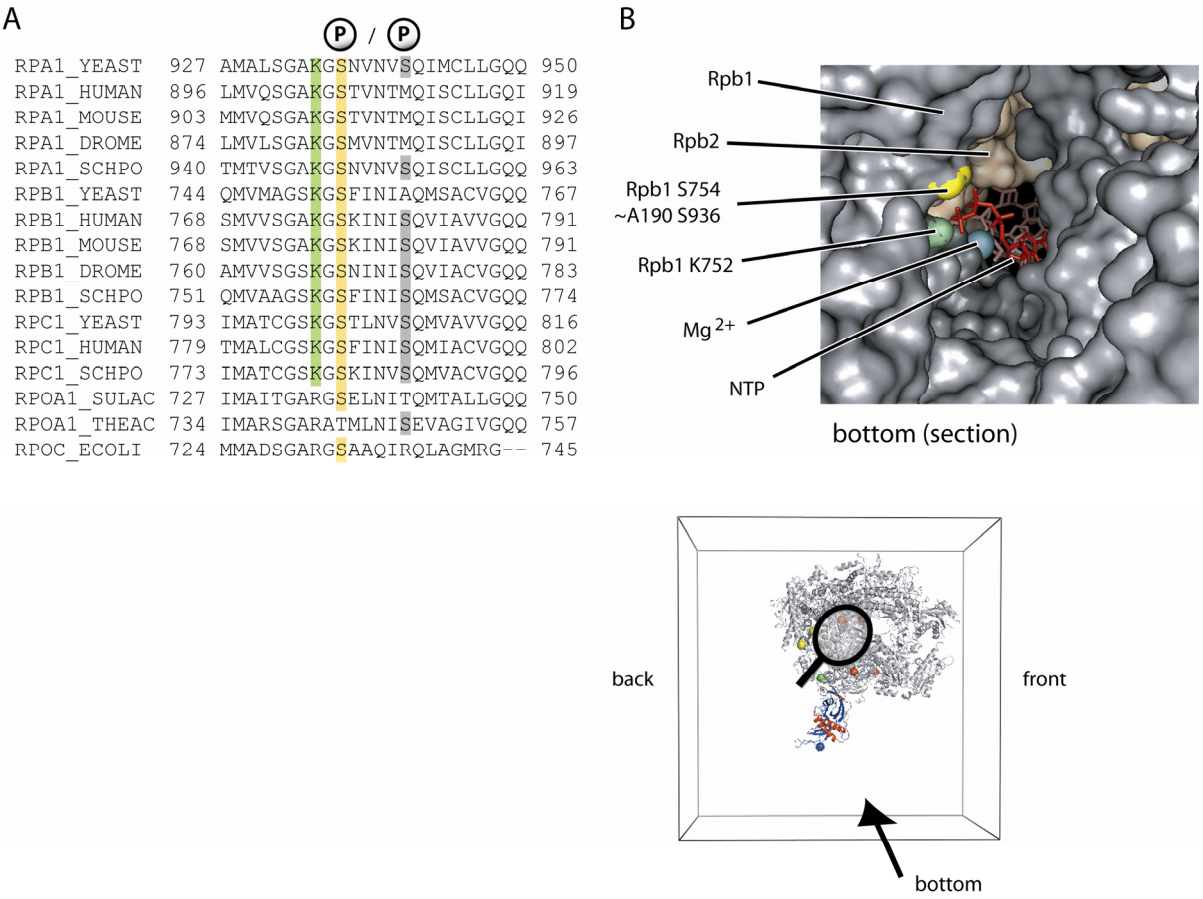

Figure 23. Localization of A190 S936/941 in the polymerase funnel. (A) Sequence alignment of the largest subunits of several eukaryotic RNA polymerases, as well as the homologous subunits from two Archaea species and E. coli. Highlighted amino acids are shown in the structure of the pore/funnel-region **(B)**. A section of the bottom view of a Pol II elongation complex (PDB 1Y77) is presented with the downstream DNA on the right hand side. Rpb1 A759 is not located directly on the surface and thus not shown.

The most striking position is that of A190 S936/941 inside the funnel (Figure 23). This protein region is highly conserved among the nuclear RNA polymerases as well as among different species (Figure 23). Right next to the homologous amino acids in the largest Pol II subunit (*i.e.* Rpb1 S754 / A759) is the conserved K752, which was described to be involved in NTP-binding in the A-site (Kettenberger *et al*., 2004; Westover *et al*., 2004; Wang *et al*., 2006). In the structure of an elongating Pol II (PDB 1Y77; Kettenberger *et al*., 2004) the serine (Rpb1 S754) corresponding to A190 S936 is slightly more outside. Rpb1 A759 on the other hand is not located on the protein surface in this structure.

In contrast to S936/941, the other phosphorylation site of the pore/funnel region A190 S685 is not located inside the pore but on the part of the pore 1-domain which contributes to the backside of the polymerase (Figure 24A). There, it is located in the vicinity of ABC14.5, the AC40/AC19 heterodimer and a conserved loop of the hybrid binding-domain of A135 (see Cramer *et al*., 2001). Interestingly, the amino acids corresponding to A190 S685 and G728, the mutation of which leads to a temperature-sensitive phenotype (G728D, rpa190-3), are in close proximity in the Pol I homology model, while the intermediate protein region is looped out (Figure 24B).

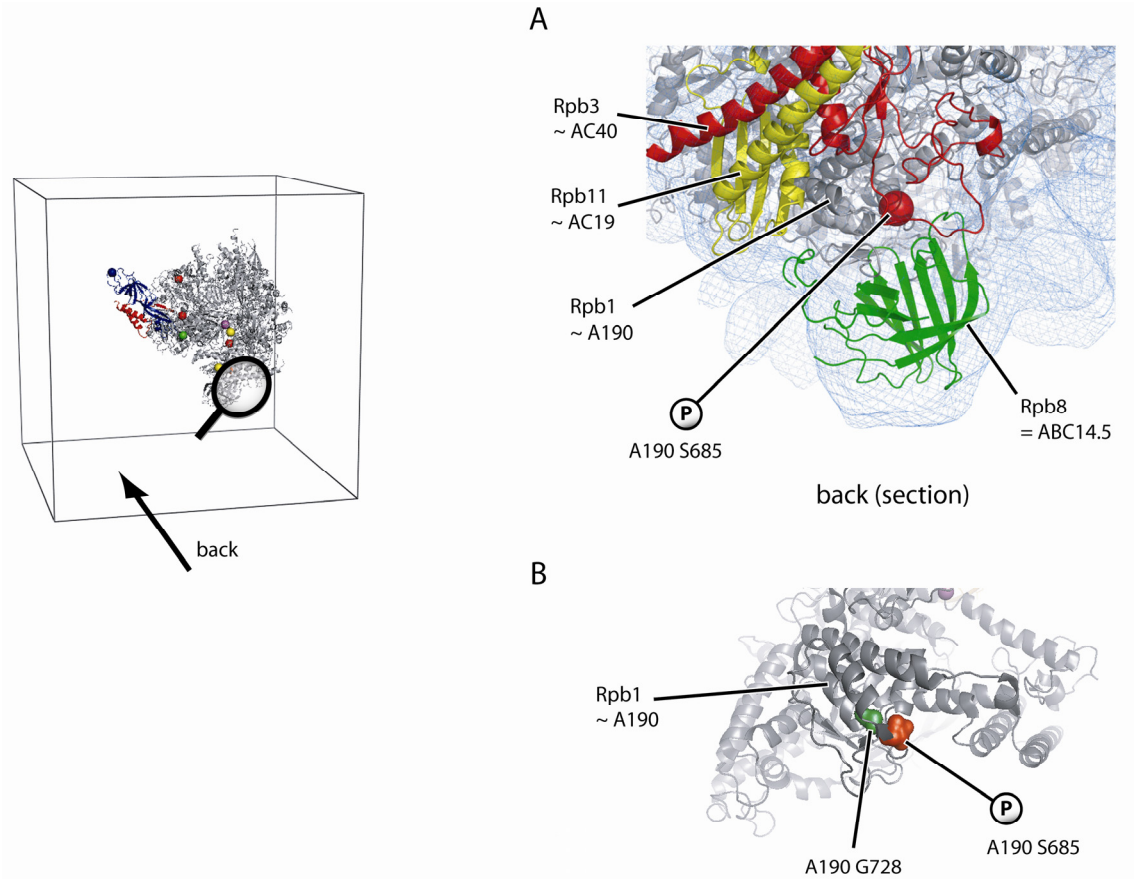

Figure 24. Localization of A190 S685 on the backside of the polymerase. (A) A magnification of the lower part of the homology model is shown. **(B)** Phosphorylation site A190 685 is in close spatial proximity to A190 G728 which is mutated to aspartate in the ts-mutant rpa190-3 (Wittekind *et al*., 1988).

The three clustered phosphosites on A190 (A190 S1413 / S1415 / S1417) are placed in the Jaw-domain in a region of weak homology and, thus, not included in the homology model.

All A43 phosphosites can be found in the C-terminal OB-domain, with two sites (S262/263 and S285) being located in a non-essential part of the protein (Peyroche *et al*., 2000). On the other hand, the region including the other two A43 phosphosites (S208 and S220) is indispensable for cell viability as demonstrated by a C-terminally truncated mutant [rpa43-1

(H216stop)] (Thuriaux *et al*., 1995). The 18 amino acids longer mutant rpa43-18 (R234stop) (Peyroche *et al*., 2000), which still contains the two sites, is viable but exhibits a ts-phenotype. Interestingly, the different truncations of these mutants correspond well to subsequent truncations N-terminally of the β-strands B4', B4 and C2 (Figure 21) which were recently revealed by the A43/A14 crystal-structure (Kuhn *et al*., 2007). Furthermore the mutations in another ts-mutant called rpa43-4 are within the same protein region as these two phosphosites.
The new A43 crystal structure in the homology model contains only one of the four A43 phosphorylation sites (S220). The parts with the other three sites (S208, S262/263 and S285) needed to be deleted in order to facilitate the crystallization of the recombinant protein (A43 Δ173-209 Δ252-326). S208 is positioned in an unordered loop between the β-strands C1 and C2, and the complete C-terminus seems to be flexible. However, the position of S220 in the structure is remarkably on the outermost part of the protruding A43/A14 heterodimer (*i.e.* of the stalk, see 1.2.3.4) (Figure 22).

The A34.5 triple-cluster is positioned at the N-terminal end of the protein. Not much is known about the domain organization or structure of this subunit, and due to the lack of a counterpart in the Pol II crystal structure, these sites could not be localized in the homology model. The position of A34.5, derived from the difference between cryo-EM structures of the complete Pol I and a variant lacking the A34.5/A49 heterodimer (Kuhn *et al*., 2007), is on the lower left in the front view of the polymerase (Figure 22).

The single phosphorylation site found on the common subunit ABC23 (S102) falls within the conserved C-terminal assembly domain, which is ordered in the Pol II crystal as opposed to the N-terminal tail-domain and which is involved in the interactions with Rpb1 and Rpb7 in the Pol II complex (Cramer *et al*., 2001; Armache *et al*., 2003). One published mutation of the close-by Q100 to arginine results in a cold sensitive (cs)- and ts-phentoype accompanied by reduced mRNA and tRNA levels along with a destabilization of the association of the Rpb4/7 heterodimer to the Pol II complex (Tan *et al*., 2003). A mutant with an exchange of S102 to phenylalanine (rpo26-17) was found in a screen for ABC23-mutations which result in a synthetic lethal phenotype with a ts-mutant of the largest Pol II subunit [rpo21-4 (W954(LELE)P)] (Nouraini *et al*., 1996). However, the yeast strain carrying the mutation ABC S102F without the Rpb1-mutation exhibited no growth defect. In the homology model the phosphorylation site on this common subunit (ABC23 S102) is located at the C-terminal end of α-helix 1 (Cramer *et al*., 2001), which is positioned between the largest polymerase subunit and A43, Rpb7 or C25, respectively. Detailed structural analyses of this region in the Pol II complex can be found in several publications (Cramer *et al*., 2001; Armache *et al*., 2003; Tan *et al*., 2003; Armache *et al*., 2005). Strikingly, S102 is not directly in one of the protein-protein interphases but rather in a narrow free space between the Rpb1-ABC23 and ABC23-Rpb7 contacts.

Finally, the phosphorylation sites of AC19 (T33 and T51/T54/S55) are located in the tail domain and at the transition to the dimerization domain, respectively.

2.3 Mutants of the Pol I phosphosites

2.3.1 Creating the mutants

In order to gain insight into the functions of the identified Pol I phosphorylation sites, mutations of the single sites to either alanine or aspartate were created to mimic constitutively dephosphorylated or phosphorylated states, respectively.

Site-directed mutagenesis was conducted on the cloned Pol I subunit genes (RPA-genes) using a variation of the recombination-based mutagenesis method described by Muhlrad and co-workers (Muhlrad *et al*., 1992; Cormack and Castaño, 2002). Briefly, the cloned RPA-gene was cut near the target site to create a gapped vector. Subsequently this 'DNA-damage' was repaired with a short PCR-product containing the desired mutation, upon co-transformation into a wild-type yeast strain (used as a cloning-tool in this case). Clones bearing a gap-repaired plasmid could be selected through the marker gene contained on the vector and screening for RPA-genes with the mutation was facilitated by additionally integrating a new restriction site into the mutagenesis-primers (which results in a silent mutation, *i.e.* no additional alteration of the protein sequence) (Figure 25). This strategy was chosen over more common oligonucleotide-directed mutagenesis methods like the QuikChange-technique (Stratagene) (Cormack and Castaño, 2002; Sambrook and Russell, 2001) due to the large size of the used vectors (*e.g.* pRS314-RPA190 is ~11.4 kb, which is difficult to amplify full length in PCR reactions without introducing random mutations).

The final mutant yeast strains were obtained by introducing the mutagenized vector into the corresponding plasmid shuffle-strain for the respective Pol I subunit gene using either canavanine- or 5-FOA-counterselection (depending on the genotype of the strain) (Sikorski and Boeke, 1991). As A34.5 is a non-essential protein, the vectors containing mutant alleles of this gene were simply transformed into a Δrpa34 deletion-strain.

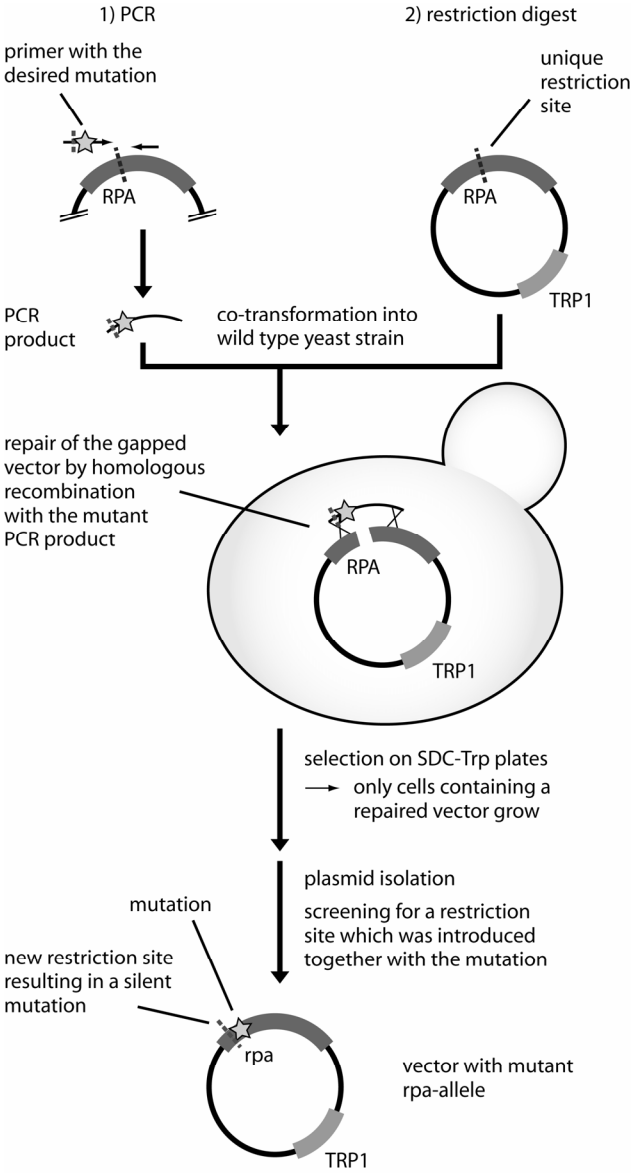

Figure 25. Strategy for site-directed mutagenesis.

The single phosphosites were mutated one at a time to create strains with the exchange of one specific phosphosite to alanine or aspartate. Only the two triple-clusters present in A190 (S1413/S1415/S1417) and A34.5 (S10/S12/S14) were each mutated in combination. In the cases with an ambiguous localization of the phosphosite on the corresponding peptide, all possible amino acids were exchanged in one mutant. Table 3 lists all phosphosite mutants created during this study. Note, that aspartate mutants were not generated for all the phosphosites and that the site AC19 T51/T54/S55 due to the ambiguous assignment was not included in the analyses yet.

Table 3. Pol I phosphorylation site mutations.

Pol I subunit	Mutation
A190	S354A
	S354D
	S685A
	S685D
	S936/941A
	S936D
	S941D
	S936E
	S941E
	S1413/1415/1417A

Pol I subunit	Mutation
A43	S208A
	S208D
	S220A
	S208/220A
	S262A
	S262/263/265A
	S285A
A34.5	S10/12/14A
ABC23	S102A
	S102D
AC19	T33A

2.3.2 In vivo analyses of the Pol I phosphorylation site mutants

The consequences of the phosphosite mutations on Pol I function were first studied by *in vivo* growth analyses. Cell growth depends on the efficient production of ribosomes and the rRNA production is tightly regulated in response to environmental conditions through signal transduction pathways (Warner *et al*., 1999; Rudra and Warner, 2004) (see 1.1). Thus, severe defects in Pol I transcription or the failure to respond to such regulatory signals should be detectable through changes in the growth behavior of the mutant strains compared to wild-type yeast.

The ability of the mutated Pol I subunits to complement the loss of the wild-type protein in the plasmid-shuffle strains was checked on full medium (*i.e.* on YPD agar-plates) at 16, 24, 30 and 37 °C. Isogenic wild-type strains carrying the same vector without mutations of the respective RPA-gene served as a control in each case. In parallel the plasmid-shuffling was controlled on different synthetic media lacking one defined amino acid, to check for the presence of the mutant copy vector, the loss of the wild-type plasmid and the maintenance of the original RPA-gene deletion. One example of these growth complementation experiments is shown in Figure 26. All mutated Pol I subunits fully complemented for the loss of their wild-type counterparts. The plasmid-shuffling was complete in all cases, while growth of the shuffle-strains still depended on the vector, *i.e.* the chromosomal copy was not restored by homologous recombination. These results clearly show, that all single Pol I phosphorylations investigated are non-essential post-translational modifications. Unexpectedly, there are even no detectable differences in growth for any of the phosphomutant strains compared to their corresponding wild-type.

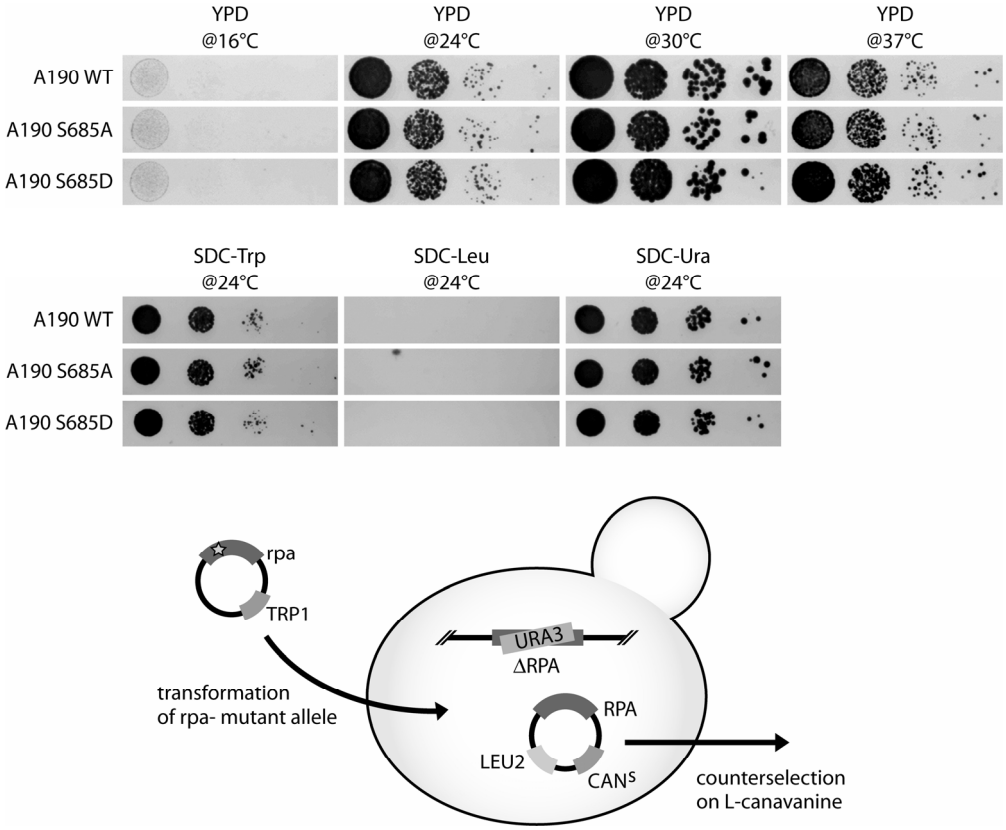

Figure 26. Mutation of the single phosphorylated amino acids has no effect on cell growth. Growth of yeast strains carrying the A190 S685A or S685D mutations on YPD plates at different temperatures are shown as an example. No growth phenotype compared to the corresponding isogenic wild-type strains was detectable for any of the tested mutants. Plasmid shuffling and maintenance of the chromosomal deletion was controlled via the respective auxotrophic markers. The A190 plasmid-shuffle strain shown in the example uses the canavanine-shuffle system. All other shuffle strains for the phosphorylated Pol I subunits use the 5-FOA-shuffle system.

In the stationary phase, cells down-regulate the production of new ribosomes to a minimal level. Upon re-entering the cell cycle from the G_0-phase some signal mediates the full activation of rRNA transcription by Pol I. A possible involvement of one of the identified phosphorylation in this switch was investigated by inoculating liquid cultures of phosphosite-mutants from stationary phase pre-cultures and observing the growth behavior. In the same experiment characteristics like the doubling time of the mutant strains relative to their isogenic wild-types could be analyzed by measuring the OD_{600} of the cultures.
Neither the lag phase from inoculation to detectable growth of the culture nor the growth rate in the logarithmic phase was altered for any of the strains investigated relative to their wild-type.

Furthermore the growth behavior of the mutant strains in the presence of rapamycin or 6-azauracil (6AU) was investigated by spotting serial dilutions of the cultures on agar plates containing these drugs.

Rapamycin inhibits the TOR-pathway (*T*arget *O*f *R*apamycin), which signals the availability of nutrients to the ribosome biogenesis apparatus (De Virgilio and Loewith, 2006) including Pol I (see 1.2.5.2). Changes in the rapamycin sensitivity for any of the mutant strains would point towards an involvement of the mutated site in these signaling events, but no such differences were detectable.

6AU is commonly used to test for defects in transcription elongation (Hampsey, 1997; Van Mullem *et al*., 2002; Schneider *et al*., 2007). It lowers the pool of UTP and GTP by interfering with their synthesis pathways and is best known from a screen for 6AU sensitive mutants which identified the Pol II elongation factor TFIIS (Exinger and Lacroute, 1992; Hampsey, 1997).

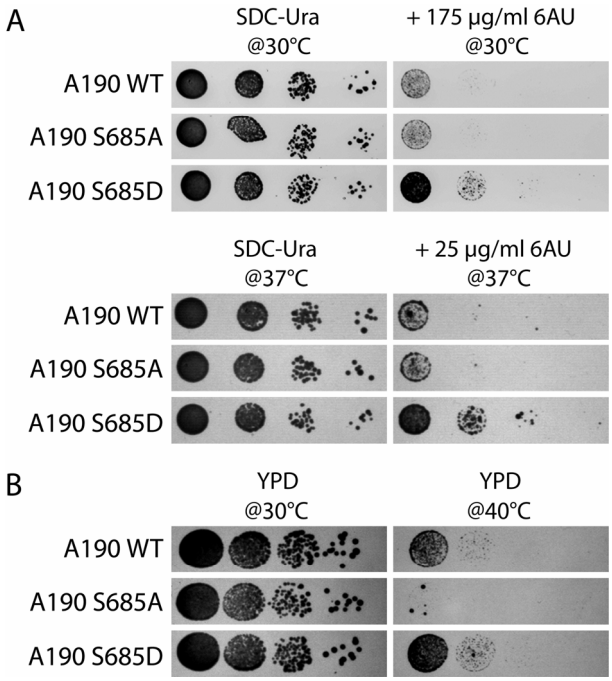

Figure 27. Sensitivity of A190 S685 mutants to 6AU and to extreme temperatures. (A) A yeast strain carrying the mutation A190 S685D is less sensitive to the NTP-pool depleting drug 6-azauracil (6AU) than the isogenic wild type or a strain with the analogous mutation to alanine. **(B)** The same mutant (A190 S685D) grows better on YPD at elevated temperatures (40° C) than the corresponding wild type strain, while the alanine variant is growth inhibited.

Remarkably, mutation of A190 S685 to aspartate resulted in a lowered 6AU sensitivity as compared to the strain expressing the wild-type subunit (Figure 27A). In contrast the analogous mutation to alanine did not alter the growth behavior on 6AU-plates. To rule out that some random mutations in the strain carrying the vector with the A190 S685D-allele caused this decreased sensitivity (*e.g.* mutations in the uracil transporter involved in the uptake of 6AU), the phenotype was confirmed by repeated plasmid-shuffling and assay on 6AU.

Note that the same mutation A190 S685D, but not S685A, was found to be synthetic lethal with a deletion of the non-essential Pol I subunit A12.2 in a SL-screen by Alarich Reiter (Reiter, 2007). The RPA-gene phosphomutant vectors created in this study (see 2.3.1) were used for the SL-screen, *i.e.* the mutations were identical. In total 180 combinations of phosphorylation site mutants and deletions of non-essential components of the Pol I transcription machinery were tested. No other genetic interaction was evident (including all combinations of A190 S685D with deletions of the other non-essential Pol I subunits A49, A34.5 and A14), showing the specificity of the synthetic lethal effect between A190 S685D and Δrpa12 (Reiter, 2007). This indicates an involvement of the reversible phosphorylation at A190 S685 in modulating one of the functions of A12.2 which was described to participate in elongation, 3' RNA cleavage and termination (Van Mullem *et al*., 2002; Kuhn *et al*., 2007; Prescott *et al*., 2004).

Remarkably, mutants of this phosphorylation site (A190 S685) also showed different phenotypes compared to the wild-type at very high temperatures (Figure 27B). Growth on YPD plates at 40°C was tested due to reports of rpb1-mutants which grow almost like wild-type yeast at 36°C but exhibit drastic growth defects at temperatures higher than 38°C (Hampsey, 1997). All strains, including the wild-type, grew very slowly at this temperature, with the S685D-mutant showing a slightly higher growth rate. In contrast the A190 S685A mutant was severely inhibited under these extreme conditions.

2.4 The A12.2 paradox – A lethal mutation of a non-essential protein

In the course of the cooperation with the laboratory of Patrick Cramer a mutant of the Pol I subunit A12.2 was created to investigate the role of this subunit in the intrinsic Pol I RNA cleavage activity (Kuhn *et al*., 2007). A12.2 and its functions in Pol I transcription are of special interest due to its genetic interaction with the phosphosite mutant A190 S685D (Reiter, 2007) (see 2.3.2). The full knock-out of this non-essential subunit results in a ts-phenotype, while deletion mutants lacking only the C-terminal half exhibit no growth defects. However, this C-terminal part contains a highly conserved TFIIS-like motif (see 1.2.3.5).

Two acidic residues in the conserved motif which play a crucial role in the TFIIS-mediated Pol II RNA cleavage activity (Jeon *et al*., 1994; Kettenberger *et al*., 2003) were replaced by alanines in A12.2, resulting in the mutant A12.2 D105A E106A (A12.2 DE>AA). Mutagenesis was performed on the cloned RPA12 gene, but all attempts to transform the vector with the mutant allele into a Δrpa12 deletion strain failed, in contrast to the control vectors with the wild-type gene or the empty vector.

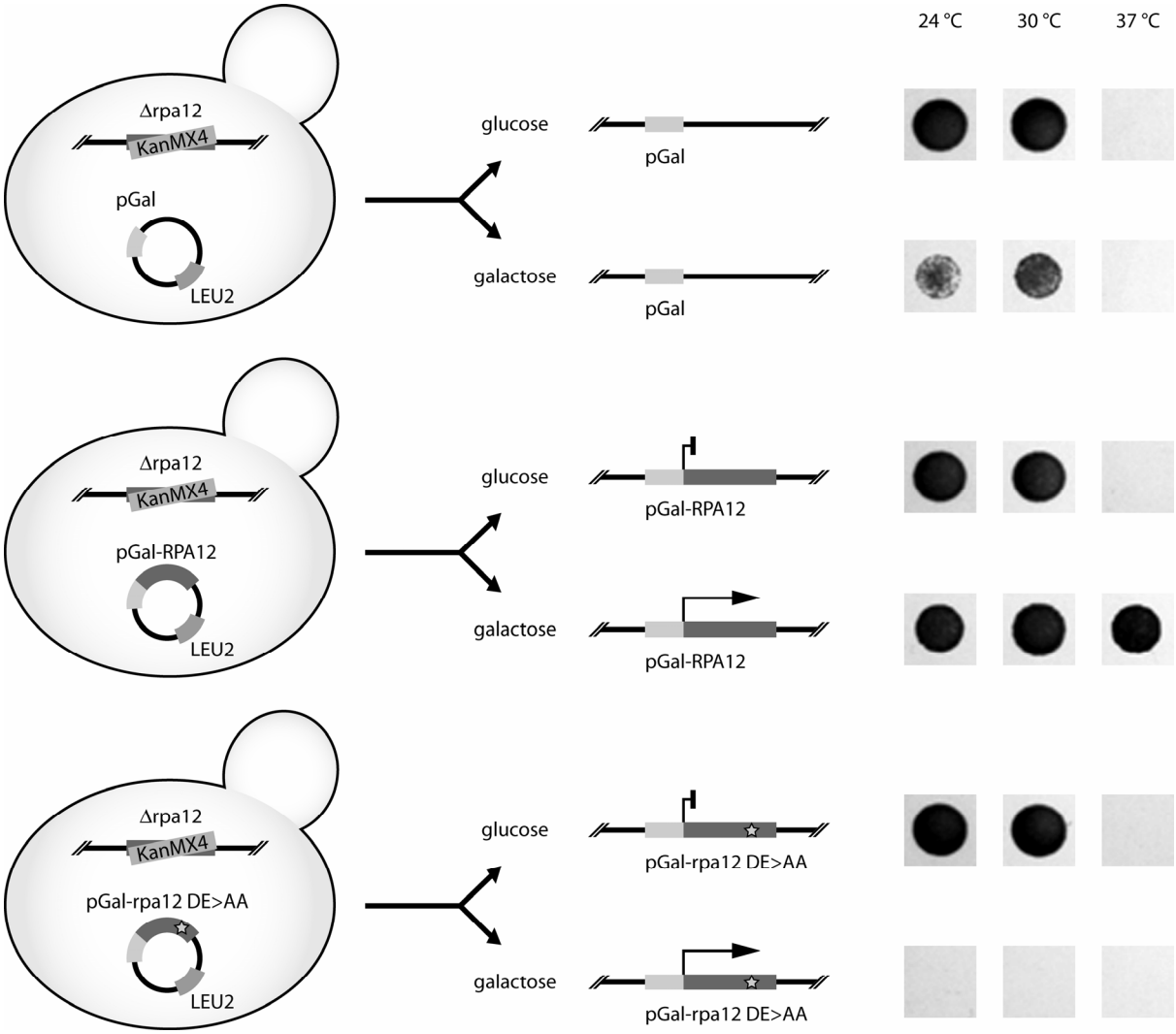

Figure 28. The mutation A12.2 D105A E106A is lethal. A strain expressing the mutant under the control of a galactose-inducible promoter and the corresponding wild-type and vector controls were spotted on plates containing either glucose (no expression) or galactose (expression) and grown at different temperatures. On glucose all strains grew like a Δrpa12 full deletion strain which is growth inhibited at 37 °C (Nogi *et al*., 1993). The A12.2 DE>AA mutant is unable to form colonies at any temperature on the galactose plates.

Thus an A12.2 plasmid-shuffle strain was constructed by transforming the wild-type allele encoded on a plasmid carrying the URA3 marker-gene into the Δrpa12 deletion strain. However, upon transformation of the mutant, wild-type or empty control vectors into this shuffle strain and counterselection on 5-FOA, no viable clones for the A12.2 DE>AA mutant could be obtained. These results suggested that paradoxically the mutation of two amino acids in a dispensable part of a non-essential protein leads to a lethal phenotype.

To further investigate this possibility, the genes for the wild-type A12.2 and the mutant A12.2 DE>AA were cloned under the control of an inducible galactose-dependent promoter,

i.e. their expression is repressed on glucose-containing media and induced upon switching to galactose as carbon-source. Single clones obtained by transforming these plasmids into the Δrpa12 strain and culturing on standard glucose containing YPD-plates, were diluted in sterile water and spotted on different glucose and galactose-plates. The results of the incubation of these plates at different temperatures are summarized in Figure 28. Under the repressing glucose conditions all strains behaved like the Δrpa12 deletion strain. On galactose plates, the knock-out was complemented by the wild-type A12.2 as judged by the ability to grow at 37 °C. In contrast, when inducing the expression of the A12.2 DE>AA mutant, no colonies were formed at any incubation temperature, confirming the observation that this mutation apparently results in a lethal phenotype.

3 Discussion

3.1 Pol I purification

3.1.1 The new yeast Pol I purification method

A new yeast RNA polymerase I purification procedure was developed which yields an active enzyme preparation suitable for the determination of Pol I phosphorylation sites and structure determination.

Six different Pol I purification methods have already been described in the literature (Ponta *et al*., 1972; Buhler *et al*., 1974; Van Keulen *et al*., 1975; Valenzuela *et al*., 1976a; Hager *et al*., 1977; Keener *et al*., 1998). Most of these were published in the years directly following the first descriptions of Pol I as one of three distinct nuclear RNA polymerases (Roeder and Rutter 1969). However, some of these apparently resulted in incomplete polymerase complexes as judged from today's knowledge about Pol I subunit composition (Ponta *et al*., 1972; Van Keulen *et al*., 1975; Hager *et al*., 1977). The protocols by Buhler *et al.* (1974) and Valenzuela *et al.* (1976a) contain basically the same steps (*i.e.* phosphocellulose batch > DEAE-cellulose batch > DEAE-cellulose or -sephadex chromatography > glycerol or sucrose gradient) and yielded pure complexes, which were used for most Pol I analyses including the identification of the *in vivo* phosphorylated subunits (Buhler *et al*., 1976b; Bell *et al*., 1976; Bell *et al*., 1977). The method by Buhler *et al*. (1974) was also used for most previous structural analyses (Schultz *et al*., 1990; Schultz *et al*., 1993; Klinger *et al*., 1996; De Carlo *et al*., 2003).

Only one method by Keener *et al*. (1998) used the efficiency of affinity chromatography (Bornhorst and Falke, 2000). In this publication the authors actually describe two procedures: the first one used four different chromatography columns with gradient elution (phosphocellulose > Q-sepharose > heparin-sepharose > MonoQ). In the second one, the initial phosphocellulose and Q-sepharose chromatography steps were replaced by one nickel-affinity chromatography using an N-terminal His_6 / HA_3-tagged A135.

The major advantage of the new Pol I purification procedure developed in this study is the low-salt precipitation as the first step. It is used to remove bulk proteins and to largely reduce the volume of the sample at the same time. This facilitated the upscaling of the procedure required to yield the quantities of highly purified protein needed for the crystallographic approach.

The subsequent Ni-NTA affinity and MonoQ anion exchange chromatographies are similar to two steps of the Keener protocol but other buffer conditions were employed. A Superose 6 gelfiltration was added as a final purification step.

3.1.2 Co-purifying proteins and Pol I interaction partners

In the final preparation three proteins of an eIF3-subcomplex (Rpg1p, Nip1p and Prt1p) (Phan *et al*., 2001) were present. Although co-purification is often a strong argument for protein-protein interactions, in this case a direct interaction with Pol I *in vivo* is contradicted by the cellular localizations. While Pol I is concentrated in the nucleolus, the translation initiation factor eIF3 is found in the cytoplasm (Kumar *et al*., 2002; Huh *et al*., 2003). Furthermore no indications for such an interaction was found in studies on the eIF3-components, including two hybrid screens (Placek *et al*., 2001; Asano *et al*., 2000; Uetz *et al*., 2000) and co-purification experiments (Asano *et al*., 2000; Phan *et al*., 1998; Phan *et al*., 2001).

The well-known interaction partner Rrn3p was not found in the Pol I preparation. However, the Pol I-Rrn3p-complex represents only about 2 % of the total Pol I population (Milkereit and Tschochner, 1998), thus no corresponding coomassie band was expected. With the only available Rrn3p antibody (Milkereit, 1999) no signals in western blot analyses were visible, but the Rrn3p associated with the purified Pol I might be present in amounts below the detection limit. On the other hand it might be possible, that the interaction of Rrn3p with the His_6-tagged A43 subunit prevents binding to the Ni-NTA-column, thus selectively depleting the initiation competent Pol I from the preparation. This possibility has not been tested.

3.2 Identification of Pol I phosphorylation sites

3.2.1 17 Pol I phosphorylation sites

A total of 13 Pol I phosphorylation sites could be successfully identified in this study. These complement four other Pol I phosphorylation sites found exclusively in proteome-wide phosphorylation analyses (Ficarro *et al*., 2002; Li *et al*., 2007). Phosphorylation at these 17 sites matches the amount of approximately 15 ± 3 phosphates per enzyme estimated by Bréant *et al.* (1983) for a Pol I preparation from logarithmically growing yeast (see 1.2.5.1). Also at the subunit level there is a good correlation between the numbers of calculated phosphate groups and of the sites determined so far (Table 4).

Table 4. The number of identified phosphosites approximately matches the calculated amount of phosphate groups per enzyme.

Subunit	Phosphosites (Ficarro *et al*., 2002; Gruhler *et al*., 2005; Li *et al*., 2007; this study)	Phosphates per Enzyme or Subunit (Bréant *et al*., 1983)
Total Pol I	17	15 ± 3
A190	7	6
A43	4	4
A34.5	3	2
ABC23	1	1 or 2
AC19	2	1 or 2

However, it should be noted that additional phosphorylation sites may exist which were not found due to limited amounts of the respective Pol I population (*e.g.* of the initiation-competent Pol I) or due to methodological constraints.

The phosphosubunits were digested with trypsin according to well established in-gel digestion protocols (Shevchenko *et al*., 2007; Cañas *et al*., 2007). While trypsin digestion is a standard procedure in mass spectrometric analyses and yields reliable and reproducible results, the sequence coverage is typically within the range of 40 – 60 %, mainly due to unfavorable peptide sizes and suppression effects related to differences in ionization efficiencies (Krause *et al*., 1999; Wa *et al*., 2006). The latter can be largely reduced by the reversed phase fractionation applied prior to the analysis, but in order to obtain almost full sequence coverage digestions with different specific proteases, producing different peptides, would be necessary (Wa *et al*., 2006).

The identified phosphosites apparently belong to the major phosphorylated residues in the total Pol I preparation. Their detection after chemical derivatization was facilitated by the high purity of the single gel-separated subunits and in some cases by the additional peptide fractionation (in the LC-MALDI approach), but no specific phosphopeptide-enrichment was applied. Thus, some low abundant phosphorylations might have escaped identification due to the detection limit.

The validity of the method employed in this study was verified by the five phosphorylation sites confirmed through two independent proteome-wide phosphorylation analyses which were reported during the course of this study, *i.e.* A43 S285, the triple-cluster A34.5 S10 / S12 / S14 and AC19 T33 (Gruhler *et al*., 2005; Li *et al*., 2007). As mentioned before, the methods used in these proteome-wide analyses were different from the chemical derivatization approach employed in this study. Rather, they relied on combinations of peptide fractionation and phosphopeptide enrichment via IMAC-columns.

The specificity of the chemical derivatization depends on the reaction conditions (especially the choice of the group II metal ions). O-linked carbohydrates can also be susceptible to β-elimination, but in the presence of Ba^{2+}-ions their reaction rate is two orders of magnitude slower than for the O-linked phosphates (Byford, 1991; Molloy and Andrews, 2001). Furthermore the apparent deamidation of asparagines or glutamines caused by the β-elimination reaction conditions need to be taken into account for the data evaluation (Karty and Reilly, 2005).
One drawback of the derivatization reactions used to identify the phosphorylation sites is that the phosphoryl group of phosphotyrosine is not β-eliminated in the presence of $Ba(OH)_2$ (Areces *et al*., 2004). Although tyrosine phosphorylation accounts for only about 0.05 % of the total phosphorylation in a mammalian cell, this modification is involved in many important cellular processes (Machida *et al*., 2003; Pawson and Scott, 2005). In yeast, however, no members of the protein tyrosine kinase-family were found so far (Zhu *et al*., 2000). Rather some tyrosine residues are phosphorylated by protein kinases with dual specificity, *i.e.* kinases which phosphorylate serine/threonine and tyrosine (Hunter and Plowman, 1997; Zhu *et al*., 2000).

3.2.2 The total Pol I preparation used for phosphosite identification is a mixture of different Pol I populations

Previous experiments indicated that the phosphorylation pattern of Pol I changes through the transcription cycle (Fath *et al*., 2001). The enzyme preparation used in this study to identify the phosphosites was a total Pol I preparation from logarithmically growing yeast, *i.e.* a mixture of polymerases from different stages of the transcription cycle plus the pool of free Pol I complexes.
In each wild type yeast cell about 50 % of the approximately 150 rDNA copies are transcribed (Dammann *et al*., 1993) by an average number of 51 polymerases per gene (French *et al*., 2003). Accordingly about 3825 polymerases are engaged in elongation or termination which account for 25 % of the 15200 Pol I molecules of a yeast cell (Bier *et al*., 2004). Only about 2 % of Pol I is present in the initiation active complex with Rrn3p (Milkereit and Tschochner, 1998). The rest belongs to the large pool of Pol I molecules, which includes the complexes that are about to be assembled, imported or degraded.
Thus, the sites identified in this study are not necessarily phosphorylated at the same time; rather each one might be modified in any of these stages. Note that the same considerations need to be taken into account for the studies on the *in vivo* phosphorylated Pol I subunits by Bell *et al.* (1976; 1977) and Buhler *et al*. (1976b), as well as for the quantification of the phosphate groups per enzyme by Bréant and coworkers (1983).

The confirmed sites on subunits A43, A34.5 and AC19 were found in the proteome-wide analyses from α-factor arrested yeast cells (Gruhler *et al*., 2005; Li *et al*., 2007). However, in contrast to the effect of other inhibitors of the cell cycle 'start' checkpoint, the production of rRNA by Pol I is not downregulated upon α-factor treatment (Veinot-Drebot *et al*., 1989). These cells stop division but increase cell size, associated with continued RNA and protein synthesis (Throm and Duntze, 1970). Thus, the identified sites in these approaches also arose from a mixture of Pol I molecules throughout the transcription cycle and cannot for instance be assigned to an inactive state.

3.2.3 A similar motif in A190 and A34.5

Two triple clusters of phosphoserines were found among the Pol I phosphorylation sites: A190 S1413 / S1415 / S1417 (Ficarro *et al*., 2002) and A34.5 S10 / S12 / S14. The sequence comparison of the corresponding phosphopeptides revealed a striking similarity between the two clusters. In both cases the three phosphoserines are separated by aspartates and followed by four or three acidic amino acids, respectively (Figure 29). It is likely, that these sites are modified by the same kinase and phosphatase. The mutation of all three serines to alanine in either motif did not change the growth behavior of the yeast strains. Also a combination of alanine mutations in the A190 motif and a full deletion of the non-essential A34.5, which was created in the SL-screen by Alarich Reiter, showed no detectable growth phenotype (Reiter, 2007).

Phosphopeptide		Sequence
A190	1410-1430	**D**KE**S**D**S**D**S**E**DE**DVDMNEQ**I**NK
A34.5	7-31	**D**YV**S**D**S**D**S**D**DE**VISNEFS**I**PDGFKK
		* *****:** :. : .* .

Figure 29. Alignment of the A190 and A34.5 phosphopeptides containing the similar triple cluster motifs. Phosphorylated serines are highlighted.

3.3 Possible roles of phosphorylation at the identified sites based on the localization in the Pol I homology model and mutant phenotypes

3.3.1 All analyzed Pol I phosphorylations are non-essential post-translational modifications - general considerations and possible functions

The identified phosphosites apparently belong to the major phosphorylated sites in the Pol I complex. Surprisingly, *in vivo* analysis of mutants which mimic a constitutive unphosphorylated or phosphorylated state of the single sites showed that all Pol I phosphorylations analyzed are non-essential post-translational modifications. Furthermore the mutations have no apparent effect on cell growth. Thus, none of the single sites plays a crucial role in the regulation of Pol I transcription. In view of previous results indicating that Pol I activity is linked to its phosphorylation state (Fath *et al*., 2001; Fath *et al*., 2004), this was quite unexpected. As discussed above some additional sites might have escaped detection, *e.g.* sites which are required for complex formation with the transcription factor Rrn3 and are present in only about 2 % of the total Pol I population.
There are well known examples for non-essential components of the Pol I transcription machinery like the four non-essential Pol I subunits (Liljelund *et al*., 1992; Nogi *et al*., 1993; Smid *et al*., 1995; Gadal *et al*., 1997). Still their evolutionary conservation suggests important roles in Pol I transcription, which are about to be elucidated. There are also examples for non-essential phosphorylations like the modification at one of two sites in members of the AGC protein kinase family (Roelants *et al*., 2004). Detailed analyses of such phosphosites are rare in the literature but the existence of non-essential protein kinases (Ptacek *et al*., 2005) suggests that there are more.

Most of the Pol I phosphorylations involved in the regulation of the RNA polymerase activity apparently do not represent an ‘on-off switch’, but rather contribute to the fine-tuning of the enzyme. The kinetics of an assay measuring the *in vitro* transcription activity after different time points of alkaline phosphatase treatment showed that the removal of one or few phosphorylations has little effect or even leads to an increased activity (Fath *et al*., 2004). Only longer phosphatase incubation and the further dephosphorylation resulted in complete inactivation.
The consequences of the mutation of single sites involved in this fine-tuning would presumably not be detectable by a growth defect phenotype. A change in the Pol I transcription activity should result in a changed rate of rRNA production, which would influence the growth rate of the cell. However, apparently other regulatory mechanisms can compensate for lowered levels of rRNA production.

A well studied example for such mechanisms was found in a yeast strain with a reduced number of rDNA repeats. A wild-type yeast strain contains about 150 rDNA copies, approximately 50 % of which are actively transcribed in the logarithmic growth phase. Strikingly in a mutant strain the rDNA copy number was reduced to 42 repeats without affecting the cell growth rate or the rRNA synthesis rate (French *et al*., 2003). In this strain all 42 rDNA genes were found to be active and to possess a denser polymerase loading, resulting in a higher number of Pol I molecules per gene. As a consequence the number of polymerases engaged in transcription of the 35S rRNA precursor was almost identical to an isogenic wild-type strain, thus regulating the rate of rRNA production to wild-type levels.

Another possibility explaining the lack of growth defects in the analyzed mutant strains would be a role of the corresponding phosphorylation sites in redundant functions which are not necessarily related to the transcription activity. For instance, nuclear import or ubiquitination are known to be mediated by phosphorylation in many cases (Hood and Silver, 1999; Harreman *et al*., 2004; Hunter, 2007). The phosphorylation-dependent interaction of a polymerase subunit with an importin would be disturbed by the mutation, but it could still be co-imported in the assembled Pol I complex. Or there might be multiple ubiquitination domains on a subunit while the phosphosite mutation affects only one of them.

In all the cases discussed above the combination of several phosphorylation site mutations would be required to cause phenotypic consequences to elucidate the functions associated to the modification of these sites. With few exceptions only single phosphorylation site mutations have been analyzed in this study and further investigations are required. However, some of the phosphosite positions in the Pol I homology model are striking and implicate possible roles associated to the function of the corresponding parts of Pol I. These are discussed subsequently.

3.3.2 A190 S354 and A190 S1636 on the polymerase clamp

A190 S354 can be found on the top of the mobile clamp at the transition from the clamp head domain to the clamp core domain (Cramer *et al*., 2000; Cramer *et al*., 2001; Kuhn *et al*., 2007). The clamp forms one side of a positively charged cleft through which the DNA template enters the polymerase towards the active site (Cramer *et al*., 2000; Gnatt *et al*., 2001). It can adopt two different conformational states: the clamp swings between an open and closed form. In the open state the cleft is freely accessible and the RNA polymerase can be loaded on the DNA template during transcription initiation (Cramer *et al*., 2001). Upon promoter escape the clamp closes over the template and prevents dissociation of the

polymerase, thus facilitating a high transcription processivity (Gnatt *et al*., 2001; Cramer, 2002).
It was proposed, that the binding of nucleic acids (the downstream DNA and the DNA-RNA hybrid) causes the closure of the clamp by inducing the folding of five otherwise unordered 'switch' regions which connect the clamp to the polymerase body (Gnatt *et al*., 2001). During transcription termination the re-opening of the clamp needs to be triggered to release the RNA polymerase from the DNA template. It is tempting to speculate, that the phosphorylation at this particular position on top of the clamp could assist in these conformational changes or in the stabilization of one state. However, mutations of A190 S354 resulted in no detectable alterations of the yeast cell growth. Thus, this single phosphorylation site cannot be exclusively required for the regulation of the clamp closure / opening. The possibility of synergetic effects with the phosphorylation of A190 S1636 (Li *et al*., 2007) still needs to be investigated. This site is located more at the bottom of the clamp, in a small C-terminal part of A190 which contributes to the clamp core (Kuhn *et al*., 2007). This site has not been included in the mutational analyses yet.

3.3.3 A190 S936/941 in the funnel

A190 S936/941 is localized inside the funnel / pore beneath the active site. This cavity allows the entry of the nucleotides and apparently forms the exit path for the 3' end of the nascent transcript upon backtracking of the polymerase (Cramer *et al*., 2000; Gnatt *et al*., 2001). The phosphorylation site is directly adjacent to two overlapping NTP binding sites termed A- and E-site (Westover *et al*., 2004). Binding to the A-site (*A*ddition site) brings matching nucleotides into the correct position for phosphodiester bond formation in the active site of the enzyme (Gnatt *et al*., 2001; Westover *et al*., 2004). The function of the E-site (*E*ntry site) seems to be primarily required to overcome the restriction of NTP diffusion towards the active site by the narrow funnel / pore opening (Batada *et al*., 2004). Nucleotides bind to the E-site in a base unspecific manner with low affinity, thus largely expanding their lifetime in the active center region. Furthermore binding of a NTP to any of the two overlapping sites apparently restricts unfavorable backtracking of the polymerase under normal transcription conditions (Batada *et al*., 2004). Negatively charged amino acid side chains in the vicinity of the E-site may additionally raise the barrier height for backtracking (Batada *et al*., 2004).
The close proximity of A190 S936/941 to a lysine (A190 K934) which is involved in NTP binding to both sites and the strikingly high degree of conservation suggests a role of this phosphoserine related to the functions described above. The mutant phenotypes however contradict this assumption. A190 S936 or S941 were mutated to alanine, aspartate or the larger glutamate with no effects on cell growth on either full media or on minimal media complemented with the nucleotide pool depleting drug 6-azauracil. Thus, phosphorylation at

A190 S936/941 neither interferes with the passage of nucleotides through the funnel nor with their binding to the E-site, because this was calculated to be especially important under conditions of low NTP concentration (Batada *et al*., 2004). Furthermore the movement of the trigger loop is apparently not restricted by the phosphosite mutations. This highly mobile loop closes off the active site beneath a correctly positioned matching NTP in the A-site and forms interactions which finally 'trigger' the phosphodiester bond formation (Wang *et al*., 2006).

Interestingly, a number of mutations in the homologous region of the largest Pol II subunit result in a *sit* phenotype (*S*uppressor of *I*nitiation of *T*ranscription defect), including a mutation of the amino acid corresponding to A190 S941 (rpb1-A759P) (Archambault *et al*., 1998; Trinh *et al*., 2006). There are two reasonable explanations for the *sit* phenotype: either the mutant polymerases could be recruited to the promoter more efficiently or the mutations could contribute to more efficient transcription elongation (Archambault *et al*., 1998). Consistent with the latter, a mutation in the homologous region of Pol III (rpc160-L750S) was found to cause a higher polymerization rate (Rozenfeld and Thuriaux, 2001; Trinh *et al*., 2006). A similar effect of the mutations of A190 S936/941 could presumably be easily compensated by the cell as discussed above (3.3.1). The rate of rRNA synthesis could be adjusted to wild-type level by reducing the number of active rDNA repeats or by lowering the polymerase loading per gene, which would result in the observed wild-type growth. This possibility could be investigated by *in vitro* transcription assays.

3.3.4 A190 S685 at the backside of the Pore1-domain

The phosphorylation site A190 S685 is localized on the part of the Pore 1-domain which contributes to the backside of the polymerase complex, in the vicinity of ABC14.5, the AC40/AC19 heterodimer and a conserved loop of the hybrid binding-domain of A135. The mutation of S685 to either alanine or aspartate (mimicking constitutively unphosphorylated or phosphorylated states, respectively) resulted in no detectable growth defect on full media. In contrast the growth behavior changed depending on the mutation on media containing the NTP pool-depleting drug 6-azauracil (6AU): while the growth of the alanine mutant (A190 S685A) was similar to the wild-type yeast strain, the analogous mutation to aspartate (A190 S685D) resulted in a lowered sensitivity. 6AU-sensitivity is most often associated with defects in transcription elongation (Hampsey, 1997; Van Mullem *et al*., 2002; Schneider *et al*., 2007) like the inability to cleave the 3' end of the nascent RNA chain from a backtracked Pol II upon a lack of the elongation factor TFIIS (Wind and Reines, 2000; Kettenberger *et al*., 2003). Strikingly most of the mutations of Rpb1 conferring 6AU sensitivity were found in the TFIIS interaction interface, but others were found *e.g.* on the clamp (Trinh et al. 2006). Additional mutations resulting in a 6AU sensitive phenotype were found in Rpb2 (the second

largest Pol II subunit), including two mutations in the hybrid binding-domain (Powell and Reines, 1996; Trinh *et al*., 2006). Interestingly the corresponding amino acids in the Pol I homology model are near the region of A135 which is in the vicinity of the phosphosite A190 S685. On the other hand mutations leading to lowered 6AU sensitivity are rare. One example is the deletion of the gene coding for the 3'-5' exoribonuclease Not3 (Riles *et al*., 2004), which was described to be involved in Pol II elongation as part of the CCR4-NOT complex (Denis *et al*., 2001).

Another striking difference between the alanine and aspartate mutations of A190 S685 is the genetic interaction with the non-essential Pol I subunit A12.2: A190 S685D was found to be synthetic lethal with the deletion of RPA12 (Reiter, 2007). In contrast the growth behavior of the analogous A190 S685A – Δrpa12 strain was similar to a Δrpa12 control strain. Furthermore no other synthetic growth defects between an A190 S685 mutation and a gene-deletion of any other non-essential Pol I subunit was apparent. Synthetic lethality indicates a functional linkage between the two genetic interaction partners. Accordingly reversible phosphorylation at A190 S685 seems to be linked to one of the functions of the TFIIS-like subunit A12.2 which was described to be involved in transcription elongation (Van Mullem *et al*., 2002), termination (Prescott *et al*., 2004) and 3' RNA cleavage (Kuhn *et al*., 2007). Moreover interaction of A12.2 with A190 seems to be important for the correct conformation of a part of the largest subunit (Nogi *et al*., 1993; Van Mullem *et al*., 2002). Interestingly the A12.2 binding site is localized in the jaw region of A190, which is on the opposite side of the polymerase with respect to the phosphorylation site A190 S685 (Figure 30). Thus a direct interaction seems to be rather unlikely.

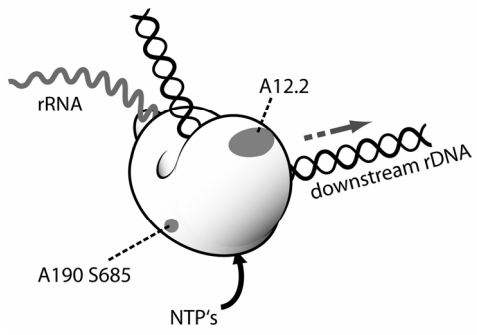

Figure 30. Scheme of Pol I showing the localization of A190 S685 and A12.2.

The cleavage of the 3' end of the nascent rRNA from a backtracked Pol I during the elongation phase apparently occurs in the active center (Kuhn *et al*., 2007). It is unknown how the C-terminal part of A12.2 participates in this process (Kuhn *et al*., 2007). The stimulation of the Pol II RNA cleavage activity by TFIIS is facilitated by an acidic hairpin which contains the conserved motif present in all TFIIS-like subunits. This hairpin enters the polymerase

through the funnel / pore and positions a metal ion required for cleavage in the active site, while conformational changes realign the RNA (Kettenberger *et al*., 2003). The location of the TFIIS-like conserved motif of A12.2 is too far from the funnel / pore to stimulate the intrinsic Pol I cleavage activity in a similar way. On the other hand, A190 S685 is located on the backside of the Pore 1-domain. Phosphorylation at this site might allosterically induce a conformational change of the pore and the adjacent active center (Johnson and O'Reilly, 1996; Johnson and Lewis, 2001), thus possibly stimulating the intrinsic Pol I RNA cleavage activity. The same conformational rearrangement by the phosphomimetic mutation to aspartate might facilitate more efficient elongation in the presence of 6AU, possibly through efficient binding of NTPs to the E-site, easier passage of NTPs through the pore or better control of unfavorable backtracking of the polymerase. However, in this conformational state the loss of A12.2 might lead Pol I into a dead end situation resulting in the synthetic lethal phenotype.

Another possible explanation for the synthetic lethality is the occurrence of two different effects of A190 S685D and Δrpa12 on transcription elongation which finally corrupt the transcription system. The 6AU sensitivity of a Δrpa12 strain can be rescued by the N-terminal domain of A12.2 (Van Mullem *et al*., 2002), indicating that the full deletion has an impact on transcription elongation independent from the RNA cleavage activity. Synthetic lethality of A190 S685D with a deletion of the C-terminal domain of A12.2 instead of the full knock-out could be tested to address this question.

Finally an involvement of A190 S685 phosphorylation in transcription termination is possible. For example an allosteric change could assist in transcript release.
Further experiments are required to investigate to which of the functions of A12.2 the reversible phosphorylation of A190 S685 is linked. Because the alanine and aspartate mutations exhibit no growth defect in the presence of A12.2, a conditional mutant could be constructed by cloning the gene RPA12 under the control of an inducible galactose-dependent promoter. On galactose containing medium these cells would grow like a wild-type strain, but upon shifting to glucose the expression of A12.2 would be stopped and thus the synthetic effect induced.

3.3.5 A43 S220 on the outermost part of the stalk and other sites in the A43 OB-domain

The four phosphorylation sites found in subunit A43 are localized in the C-terminal OB-domain. It contains an OB-protein fold (*O*ligonucleotide/oligosaccharide *B*inding protein fold) (Murzin, 1993) which enables the A43/A14 heterodimer to bind RNA *in vitro* (Meka *et al*., 2003). The *in vivo* relevance of this feature which can also be found in the homologous

subunits of Pol II, Pol III and the archaeal RNA polymerase (Meka *et al*., 2003; Orlicky *et al*., 2001; Jasiak *et al*., 2006) is unclear. Apparently, phosphorylation of the four sites is not required for the oligonucleotide interactions of the OB-domain since the *in vitro* RNA binding studies were performed with recombinant expressed subunits (Meka *et al*., 2003).

The crucial interactions of A43 include the complex formation with Rrn3p, which renders Pol I competent for transcription initiation (Peyroche *et al*., 2000). This complex formation is accompanied by a change in the Pol I phosphorylation pattern *in vivo* (Fath *et al*., 2001). Furthermore the *in vitro* interaction seems to depend on the phosphorylation status of Pol I but not of Rrn3p (Fath *et al*., 2001). The interaction interface has not been mapped yet; most information on the binding site of Rrn3p came from the ts-mutant rpa43-6 (Peyroche *et al*., 2000) which contains three different mutations, resulting in an unstable Pol I-Rrn3p complex. The three amino acid exchanges were mapped to a more conserved region of A43 spanning from positions 42 to 172 in the primary structure (Peyroche *et al*., 2000). In the recently solved crystal structure of the A43/A14 heterodimer two of the three mutations are located on the upstream surface of A43 (Figure 31) (Kuhn *et al*., 2007).

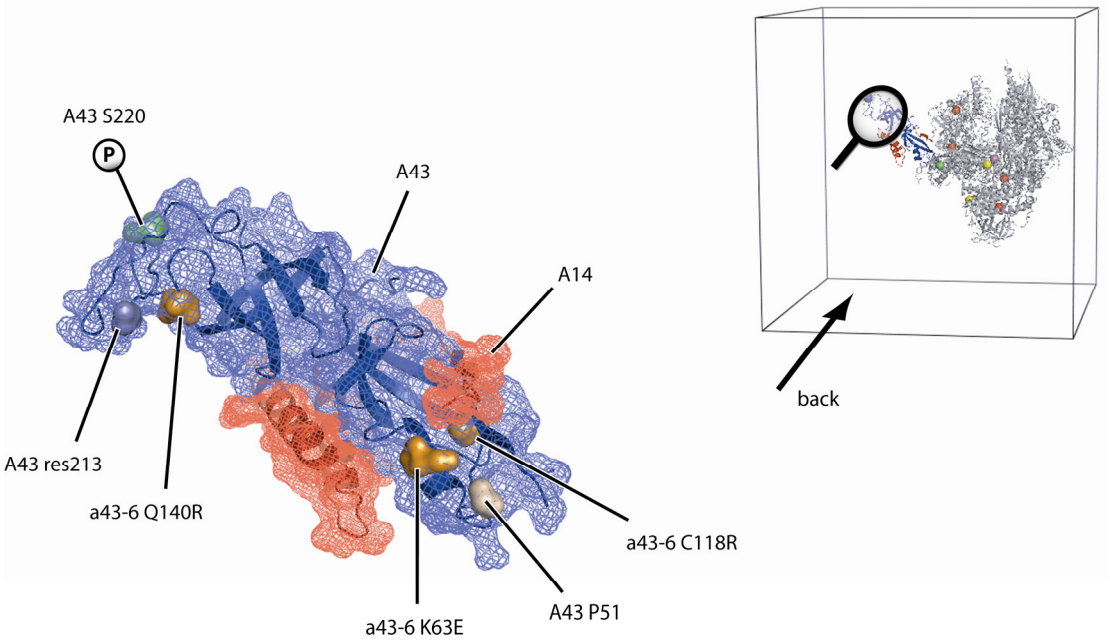

Figure 31. Positions of the phosphosite A43 S220 and of the residues mutated in rpa43-6 in the A43/A14 crystal structure. A43 S208 is not included in the structure; the position of the next ordered residue (213) is shown. Furthermore the conserved P51 is depicted, which was used to dock the crystal structure into the Pol I homology model (Kuhn *et al*., 2007).

Only one (S220) of the four A43 phosphosites is included in the structure. The others lie in regions which were deleted from the recombinant proteins in order to facilitate crystallization.

A43 S220 is located on the outermost part of the protruding A43/A14-stalk and in close proximity to one of the mutations of the rpa43-6 mutant. Thus, it is nearby but not within the proposed Rrn3p binding site and would be freely accessible to modifying enzymes in the initiation-competent complex.

The mutants of the identified phosphosites showed no detectable growth defect, arguing that the complex formation between A43 and Rrn3p as a key regulatory step of rDNA transcription is not negatively affected. However, an involvement of phosphorylation at these sites in the mechanism of Rrn3p-binding / -release can not be excluded. As discussed, only about 2 % of the total cellular Pol I is present in the initiation-competent form, the rest belongs to the pools of transcribing or free polymerases, and the identified phosphosites most probably originated from these latter two (see 3.2.2). Dephosphorylation might render Pol I competent for complex formation, while phosphorylation might be involved in the release of Rrn3p upon promoter escape. Accordingly the alanine mutations of the phosphorylation sites could stabilize the Rrn3-association beyond the transcription initiation step. As shown by the CARA-mutant strain which expresses a fusion protein of A43 and Rrn3, even the constitutive association of this transcription factor to the polymerase does not alter the growth behavior on full media (Laferté *et al*., 2006; Chédin *et al*., 2007). In this study only the alanine mutations of the single phosphosites have been investigated so far, except for one aspartate mutant (A43 S208D) and two combined alanine mutations of the neighboring phosphosites S208 and S220 (A43 S208A S2220A). Rrn3-association has not been tested for any of the mutants. Co-immunoprecipitation (Co-IP) experiments could be employed to compare the total amounts of Pol I-Rrn3p-complexes in wild-type and mutant strains. Furthermore the association of Rrn3 with the elongating Pol I could be tested by chromatin immunoprecipitation (ChIP) assays.

3.3.6 ABC23 S102 in the A190-ABC23-A43 interface

The common ABC23 subunit (= Rpb6) is involved in enzyme assembly (Nouraini *et al*., 1996; Minakhin *et al*., 2001) and forms the main interaction interface of the core polymerases to their respective ‘stalk’ heterodimers (Peyroche *et al*., 2002). ABC23 is phosphorylated in the three nuclear RNA polymerase complexes (Bell *et al*., 1977; Kolodziej *et al*., 1990) but it is unclear whether the same site is modified in all cases. The identified phosphorylation site in Pol I (ABC23 S102) can be found in the C-terminal assembly domain. In the Pol I homology model it is located in a narrow space between the interaction interfaces A190-ABC23 and ABC23-A43.
Neither the mutation of ABC23 S102 to alanine nor to aspartate resulted in any detectable growth defect phenotype in the assays employed in this study. The chromosomal wild-type

allele was deleted from the mutant yeast strains and accordingly Pol II and Pol III are not impaired by these mutations as well.

A number of experiments indicate that phosphorylation of S102 is not involved in the assembly function of ABC23 and not required for basal transcription activity. First, the yeast ABC23 can be fully functionally substituted by its mammalian counterpart (McKune and Woychik, 1994). Interestingly the C-terminal domains of these two homologous proteins are highly conserved but the mammalian subunit contains an alanine in place of the yeast S102 (McKune and Woychik, 1994). Second, the basal transcription activity of Pol I purified from a Δrpa14-strain which additionally lacks the subunits A43 and ABC23, can be restored by the recombinantly expressed ABC23 (Lanzendörfer *et al*., 1997). Third, Pol I immunoprecipitated from yeast strains carrying the single phosphosite mutations showed no significant difference in subunit composition to the corresponding wild-type, as analyzed by western blots (Reiter, 2007).

A mutation of the phosphosite S102 to phenylalanine in the ABC23-mutant rpo26-17 is synthetic lethal with the insertion mutation W954(LELE)P in the largest Pol II subunit Rpb1 (Rpb1 ts-mutant rpo21-4) (Nouraini *et al*., 1996). Strikingly this insertion mutation is positioned in a part of the foot domain of Rpb1 which is significantly different between Rpb1 and A190. The ‘truncated foot’ of A190 is 62 amino acids smaller than the Rpb1 counterpart (Kuhn *et al*., 2007). In contrast there are only minor differences between the foot domains of Rpb1 and C160 (the largest Pol III subunit) (Jasiak *et al*., 2006).
While all investigated mutations of ABC23 S102 exhibited no detectable growth defect phenotype, a mutation of the neighboring Q100 to arginine in the ABC23-mutant rpb6-151 resulted in a cs- and ts-phenotype, accompanied by reduced mRNA and tRNA levels (Tan *et al*., 2003). Apparently the effects on Pol II transcription are caused by the destabilization of the association of the Rpb4/7 heterodimer to the core complex (Tan *et al*., 2003). ABC23 Q100 was found be directly involved in this interaction through a hydrogen bond with glycine 66 of Rpb7 (Armache *et al*., 2005). The stability of the A43/A14 binding to ABC23 in the Pol I complex has not been investigated, but apparently the production of rRNA is not directly affected by the ABC23 Q100R mutation (Tan *et al*., 2003). In a similar manner phosphorylation of ABC23 S102 may have different effects on Pol I, Pol II and Pol III, thus possibly contributing to the specific binding of the stalk heterodimers A43/A14, Rpb4/Rpb7 or C25/C17 to their respective core polymerase. The ABC23 phosphosite would be freely accessible prior to the binding of A43/A14. However, the phosphomutant phenotypes show that it cannot be exclusively the trigger for such a mechanism.

3.4 A mutation in the conserved TFIIS-like motif of the non-essential A12.2 is lethal

The C-terminal domain of the TFIIS-like subunit A12.2 is involved in the intrinsic Pol I cleavage activity (Kuhn *et al*., 2007). It contains the highly conserved motif Q.RSADE..T.F shared with the other members of the TFIIS-like protein-family (Chédin *et al*., 1998; Hausner *et al*., 2000). Full deletion of this non-essential Pol I subunit results in a ts-phenotype and 6AU sensitivity, while truncation of the C-terminal half apparently has no consequences on cell growth (Nogi *et al*., 1993; Van Mullem *et al*., 2002). Thus it was surprising that mutation of the aspartate and glutamate of the conserved C-terminal motif to alanines is lethal (A12.2 D105A E106A). Apparently the full or partial deletions of A12.2 can be compensated by the cell while the DE>AA mutation leads into a dead end situation. Possibly some protein binding or conformational change can still occur but the loss of function prevents the release from this state.
In a similar manner the analogous mutation in the homologous, but essential Pol III subunit C11 was found to be lethal (Chédin *et al*., 1998). The DE>AA mutation in the non-essential Pol II cleavage factor TFIIS has only been tested *in vitro* were it resulted in a loss of function (Jeon *et al*., 1994). Structural analysis of TFIIS bound to Pol II revealed that the two acidic amino acids of the conserved motif coordinate a metal ion in the active site required for RNA cleavage (Kettenberger *et al*., 2003). However, as discussed above the location of A12.2 in the Pol I complex contradicts an RNA cleavage mechanism analogous to the one described for TFIIS (see 3.3.4).

But the involvement of an additional free A12.2 in the cleavage mechanism might be possible. While one A12.2 is stably associated to the Pol I complex as a polymerase subunit, another one could bind through the funnel / pore as a cleavage factor and stimulate RNA 3' trimming in a TFIIS-like manner. This would require an additional pool of free A12.2, which should be apparent in quantifications of the cellular amount of A12.2, compared to other Pol I subunits. This subunit was not included in the quantification of Pol I complexes yet (Bier *et al*., 2004). However, the *in vitro* assays to test the intrinsic Pol I RNA cleavage activity were performed with a highly purified enzyme preparation (Kuhn *et al*., 2007). As described above, this Pol I purification procedure involves an affinity chromatography step via a His_6-tagged A43 subunit and a size exclusion chromatography step (see 2.1). Thus, a potential additional A12.2 would need to be stably associated to the polymerase, but no such extra mass was apparent in the cryo-EM analyses. Furthermore the stoichiometric ratio of A12.2 to other Pol I subunits in the polymerase complex is 1 (Paule, 1998). The disintegration of a part of the polymerase complexes in the *in vitro* assay reaction batch would be necessary to provide the single subunit in a free form.
Another possible mechanism enabling A12.2 to enter the active center through the funnel / pore and stimulate RNA cleavage similar to TFIIS includes its release from the jaw-position

when the polymerase becomes stalled. However, Pol I is a very stable complex (Schneider and Nomura, 2004) and partial disassembly was not observed so far.
This leads to a third possibility: a complete free Pol I complex might function as a cleavage factor for another DNA bound, backtracked Pol I, *i.e.* the C-terminal domain of A12.2 bound to the jaw-position of one polymerase could access the active center of another polymerase and facilitate RNA cleavage. Apparently only the N-terminal part of A12.2 is involved in the interaction of this subunit with the polymerase jaws, the C-terminal part seems to be more loosely associated (Van Mullem *et al*., 2002). Such a mechanism could at least partially provide a reason for the large pool of free Pol I (about 70 %; see 3.2.2) which is highly concentrated in the nucleolus, while the transcription machinery apparently uses an efficient re-initiation system.

Further experiments are required to elucidate the exact function of A12.2 in the intrinsic RNA cleavage activity of Pol I and whether the C-terminal domain with the conserved motif enters the active center similar to TFIIS. The A12.2 DE>AA mutant expressed under the control of a galactose-inducible promoter could provide a useful tool for these investigations. Upon induction the polymerase molecules apparently become trapped in the 'dead end situation' which presumably resembles an intermediate state of the A12.2-dependent RNA cleavage process. This would enable purification and structural analysis. Furthermore transcription assays could be used to investigate whether these 'dead end situation' polymerase complexes are still active but stalled or completely inactive, thus resulting in the observed lethal phenotype.

3.5 Outlook

The activity of Pol I was previously found to be linked to its phosphorylation state (Fath *et al*., 2001; Fath *et al*., 2004). The phosphorylation sites identified in this study and in three proteome-wide phosphorylation studies (Ficarro *et al*., 2002; Gruhler *et al*., 2005; Li *et al*., 2007) form the basis for a detailed analysis of this correlation.

Surprisingly, the mutations of single phosphorylation sites had no apparent impact on the growth behavior of the resulting yeast strains *in vivo*. However, it is possible that other regulatory mechanisms of the ribosome biogenesis machinery compensate for slight changes in the activity of Pol I. The kinetics of *in vitro* transcription assays with partially dephosphorylated Pol I indicated a more complex regulatory mechanism than an 'on-off switch' caused by a single phosphorylation (Fath *et al*., 2004). Combinations of different phosphosite mutations will need to be tested to obtain more information about their contribution in the regulation of Pol I. Furthermore *in vitro* assays could be employed to test

the effects of the phosphomimetic mutations of single phosphorylation sites on the enzymatic activities of Pol I.
Furthermore additional Pol I phosphorylation sites might have escaped detection. The mass spectrometric analyses should be extended with the use of other specific proteases to obtain more complete sequence coverage (Wa *et al*., 2006). Low abundant phosphorylations could be analyzed after phosphopeptide enrichment, *e.g.* via TiO_2 chromatography (Pinkse *et al*., 2004).

The phosphorylation sites identified in this study were obtained from total Pol I preparations similar to the preparations originally used to identify the *in vivo* phosphorylated subunits by ^{32}P-labeling (Bell *et al*., 1976; Buhler *et al*., 1976b; Bell *et al*., 1977). A comparison with the phosphorylation sites from Pol I preparations enriched in single steps of the transcription cycle would be very interesting and could reveal important regulatory sites. Comparison of the autoradiographies from ^{32}P-labeled initiation-competent Pol I and total Pol I showed that there are apparent differences in the phosphorylation patterns (Fath *et al*., 2001). Initiation-competent Pol I could be enriched via two different affinity tags on Rrn3 and on a Pol I subunit. Furthermore it might be possible to enrich elongating polymerase via its association to the DNA in a chromatin fraction after cross-linking.

The apparent connection of one of the identified phosphoserines (A190 S685) to transcription elongation should be further investigated. A mutation of this phosphosite to aspartate resulted in a lowered sensitivity to 6AU. The same mutation was found to be synthetic lethal with a deletion of the non-essential Pol I subunit A12.2 (Reiter, 2007), which was described to function in transcription elongation (Van Mullem *et al*., 2002), RNA cleavage (Kuhn *et al*., 2007) and termination (Prescott *et al*., 2004). This synthetic lethality with Δrpa12 could be used to create a conditional mutant by expressing A12.2 under the control of an inducible promoter. Such a conditional mutant would enable to investigate which of the functions of A12.2 is apparently connected to the reversible phosphorylation at A190 S685, by e.g. analyzing the effects on transcription elongation or on termination upon switching from permissive to restrictive conditions, using suitable assays (Schneider *et al*., 2006; Schneider *et al*., 2007; Kuhn *et al*., 2007; Prescott *et al*., 2004; Gadal *et al*., 2002).

Furthermore a lethal mutation of the non-essential A12.2 (A12.2 D105A E106A) was found in this study. Possibly these mutants are trapped in an intermediate state of 3' RNA cleavage from a backtracked polymerase. It would be very interesting to investigate the details of this lethal effect. The mutant under the control of the galactose-dependent promoter could also be a very useful tool for the studies on the mechanism of the intrinsic Pol I RNA cleavage activity.

4 Materials & Methods

4.1 Materials

4.1.1 Saccharomyces cerevisiae strains

Name	Genotype	Origin
GPY2	*leu2-Δ1 ade2-101 trp1-Δ63 ura3-52 his3-Δ200 lys2-801 RPA43Δ::LEU2 pAS22 (TRP1)*	Fath *et al*., 2000
NOY222	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pNOY20 (LEU2 CANs)*	Wittekind *et al*., 1988
ToY605	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-RPA190 (TRP1)*	this study
ToY778	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S354A (TRP1)*	this study
ToY781	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S354D (TRP1)*	this study
ToY779	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S685A (TRP1)*	this study
ToY780	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S685D (TRP1)*	this study
ToY677	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S936/941A (TRP1)*	this study
ToY1030	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S936D (TRP1)*	this study
ToY1031	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S941D (TRP1)*	this study
ToY1032	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S936E (TRP1)*	this study
ToY1033	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S941E (TRP1)*	this study
ToY245	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S1413/1415/1417A (TRP1)*	this study
D101-I2	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 yCPA43 (URA3)*	Thuriaux *et al*., 1995
GPY9	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pGP5 (TRP1)*	Peyroche *et al*., 2000
ToY523	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S208A (TRP1)*	this study
ToY612	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S208D (TRP1)*	this study
ToY678	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S220A (TRP1)*	this study

ToY679	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S208/220A (TRP1)*	this study
ToY561	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S262A (TRP1)*	this study
ToY611	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S262/263/265A (TRP1)*	this study
ToY562	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S285A (TRP1)*	this study
T4-1C	*ura3-52 ade2-1 lys2-801 his3-Δ200 trp1-Δ1 rpa34-Δ::HIS3*	Gadal *et al*., 1997
ToY1034	*ura3-52 ade2-1 lys2-801 his3-Δ200 trp1-Δ1 rpa34-Δ::HIS3 pRS314-RPA34*	this study
ToY1035	*ura3-52 ade2-1 lys2-801 his3-Δ200 trp1-Δ1 rpa34-Δ::HIS3 pRS314-rpa34-S10/12/14A (TRP1)*	this study
JAY444	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRPO26 (URA3)*	Archambault *et al*., 1990
ToY607	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRS314-RPO26 (TRP1)*	this study
ToY568	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRS314-rpo26-S102A (TRP1)*	this study
ToY1036	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRS314-rpo26-S102D (TRP1)*	this study
DLY200	*ura3-52 his3-Δ200 trp1Δ lys2-801 ade2-101 rpc19::HIS3 pLS135 (URA3)*	Lalo *et al*., 1993
ToY608	*ura3-52 his3-Δ200 trp1Δ lys2-801 ade2-101 rpc19::HIS3 pRS314-RPC19 (TRP1)*	this study
ToY609	*ura3-52 his3-Δ200 trp1Δ lys2-801 ade2-101 rpc19::HIS3 pRS314-rpc19-T33A (TRP1)*	this study
BSY420 (1n) [1]	*ade2-1 can1-100 his3Δ200 leu2-3,112 trp1-1 ura3-1*	Milkereit *et al*., 2001
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann *et al*., 1998
Y06861	*BY4741 Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR063w::kanMX4*	Winzeler *et al*., 1999
ToY1264	*BY4741 Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR063w::kanMX4 YCplac33-RPA12 (URA3)*	this study
ToY1265	*BY4741 Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR063w::kanMX4 YCplac111Gal (LEU2)*	this study
ToY1266	*BY4741 Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR063w::kanMX4 YCplac111Gal-RPA12 (LEU2)*	this study
ToY1267	*BY4741 Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR063w::kanMX4 YCplac111Gal-rpa12 DE>AA (LEU2)*	this study

[1] Wildtype yeast strain BSY420 was used as a cloning tool in site-directed mutagenesis

4.1.2 Escherichia coli strains

Name	Genotype	Origin
XL1-Blue	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)*	Stratagene
DH5α	*F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ–*	Grant et al., 1990

4.1.3 Plasmids

Plasmid	Gene	Description	Derived from	Origin
pRS314	–	TRP1, Amp^r, CEN6, ARSH4	pBluescript	Sikorski and Hieter, 1989
pRS413	–	HIS3, Amp^r, CEN6, ARSH4	pBluescript II	Sikorski and Hieter, 1989
YCplac33	–	URA3, Amp^r, CEN4, ARS1	pUC19	Gietz and Sugino, 1988
YCplac111Gal	–	LEU2 , Amp^r, CEN4, ARS1	YCplac111	Ferreira-Cerca *et al*., 2005
pNOY16	RPA190	TRP1, CEN1, ARS3	pTC3	Wittekind *et al*., 1988
pRS314-RPA190	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S354A	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S354D	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S685A	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S685D	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S936/941A	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S936D	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S941D	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S936E	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S941E	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa190-S1413/1415/1417A	RPA190	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pGP5	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	Peyroche *et al*., 2000
pRS314-rpa43-S208A	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa43-S208D	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa43-S220A	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa43-S208/220A	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa43-S262A	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study

pRS314-rpa43-S262/263/265A	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa43-S285A	RPA43	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-RPA34	RPA34	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpa34-S10/12/14A	RPA34	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-RPO26	RPO26	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpo26-S102A	RPO26	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpo26-S102D	RPO26	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-RPC19	RPC19	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS314-rpc19-T33A	RPC19	TRP1, Amp^r, CEN6, ARSH4	pRS314	this study
pRS413-RPA12	RPA12	HIS3, Amp^r, CEN6, ARSH4	pRS413	this study
pRS413-rpa12 DE>AA	RPA12	HIS3, Amp^r, CEN6, ARSH4	YCplac33	this study
YCplac33-RPA12	RPA12	URA3, Amp^r, CEN4, ARS1	YCplac33	this study
YCplac111Gal-RPA12	RPA12	LEU2, , Amp^r, CEN4, ARS1	YCplac111Gal	this study
YCplac111Gal-rpa12 DE>AA	RPA12	LEU2, , Amp^r, CEN4, ARS1	YCplac111Gal	this study

4.1.4 Oligonucleotides

Oligonucleotide	**Sequence**	**Length**	**Gene**	**Position (rel. to ATG)**
A190 -801for +XhoI	5′- GCG CAC TCG AGC CAC GTT TGT CTC TTA TC –3′	29 nt	RPA190	-801– -784
A190 +69rev	5′- CTC TTT AGC TGT TAG GAT C –3′	19 nt	RPA190	69 – 88
RPA34 -424for +XhoI	5′- GCG CTC TCG AGT GAA TGT ATC AAA TGG GAA GGA G -3′	34 nt	RPA34	-424 – -402
RPA34 1067rev +BamHI	5′- GCG CTG GAT CCA CAG GTC AAC CTT GGA AGT G -3′	31 nt	RPA34	1067 – 1048
RPO26 -234for +XhoI	5′- GCG CTC TCG AGT TGA CAG CGA TTT CAA CAG TTA C -3′	34 nt	RPO26	-234 – -211
RPO26 806rev +BamHI	5′- GCG CTG GAT CCT TTA CTA CAA ATC AGC AAG CGA C -3′	34 nt	RPO26	806 – 784
RPC19 -717for +XhoI	5′- GCG CTC TCG AGG ATC TGT TCG CTC TGA TGC -3′	30 nt	RPC19	-717 – -698
RPC19 844rev +BamHI	5′- GCG CTG GAT CCG TGC TTA ATT CTG TTA CAG GTT G -3′	34 nt	RPC19	844 – 822
A190 S354A MluI for	5′- GGT CAA GGC TGA CGC GTT TTT TAT GGA TGT TCT TG -3′	35 nt	RPA190	1046 – 1080
A190 S354D XhoI for	5′- GAG ACC CAA TCT CTC GAG AAA ATT GGT CAA GGC TGA CGA TTT TTT TAT GGA TG – 3′	53 nt	RPA190	1022 – 1074
A190 S354A EcoRI rev	5′- GGA TGT AAC AGC GTC AAG AAT TCC TGA C -3′	28 nt	RPA190	2690 – 2663

A190 S685A MluI for	5′- GGC TAA CAA GTA AGG ACG CTT TTT TCA CGC GTG AAC AAT ACC AG- 3′	44 nt	RPA190	2035 – 2078
A190 S685D MluI for	5′- GGC TAA CAA GTA AGG ACG ATT TTT TCA CGC GTG AAC AAT ACC AG- 3′	44 nt	RPA190	2035 – 2078
A190 S936/941A KasI for	5′- CTG GCG CCA AAG GTG CTA ACG TTA ATG TTG CTC AAA TTA TG -3′	41 nt	RPA190	2791 – 2831
A190 S936/941A BglII rev	5′- GCA AAT AAC CAG ATC TAG ATG TTT TAA CGG -3′	30 nt	RPA190	3057 – 3028
A190 S936D KasI for	5′- CTG GCG CCA AAG GTG ATA ACG TTA ATG TTT CTC AAA TTA TGT G -3′	43 nt	RPA190	2791 – 2833
A190 S941D KasI for	5′- CTG GCG CCA AAG GTT CTA ACG TTA ATG TTG ATC AAA TTA TGT G -3′	43 nt	RPA190	2791 – 2833
A190 S936E KasI for	5′- CTT TCT GGC GCC AAA GGT GAG AAC GTT AAT GTT TCT CAA ATT ATG TG -3′	47 nt	RPA190	2787 – 2833
A190 S941E KasI for	5′- CTT TCT GGC GCC AAA GGT TCT AAC GTT AAT GTT GAG CAA ATT ATG TG -3′	47 nt	RPA190	2787 – 2833
A190 mut4237-49 SalI rev	5′- CTC TTA TTA ATT TGT TCA TTC ATG TCG ACG TCT TCG TCC TCA GCG TCA GCA TCA GCT TCT TTA TCG -3′	66 nt	RPA190	4292 – 4226
A190 3038 BglII for	5′- CAT CTA GAT CTG GTT ATT TGC AAC GTT GTC TAA C -3′	34 nt	RPA190	3038 – 3071
A43 S208A NsiI for	5′- CAT TGG TCT TTT GAT TCA TGA TGC ATT TAA TGC TAG -3′	36 nt	RPA43	432 – 467
A43 S208A AvrII rev	5′- CCC AGT GGC CTA GGG ATC TGT TTC CAA AGG CAA ATT TGC C -3′	40 nt	RPA43	652 – 613
A43 S208D AvrII rev	5′- CCC AGT GGC CTA GGG ATC TGT TTC CAA AGT CAA ATT TGC C -3′	40 nt	RPA43	652 – 613
A43 S220A BsiWI rev	5′- GAA CAT TTC GTA CGG TGA ACC TCA ATT TAC CGT CAA TGG GTT CAC CAT TAG CAT CTA CCC AGT -3′	63 nt	RPA43	709 – 647
A43 S262A AatII for	5′- GAA GAT GCT GAC GTC ATA AAC ACA GAT G -3′	28 nt	RPA43	523 – 550
A43 S262A MluI rev	5′- CTT TCA GCT TGG GAA CGA GAC GCG TTA TAG CC -3′	32 nt	RPA43	806 – 775
A43 S262/263/265A XmaI rev	5′- CTT TCA GCT TGG GCC CGG GCG GCG TTA TAG CCA TTG C -3′	37 nt	RPA43	806 – 769
A43 S285A MscI for	5′- GTA TTT GAT GAC GAA GTG GCC ATC GAA AAC AAA GAG AGC C -3′	40 nt	RPA43	835 – 874
A43 S285A NcoI rev	5′- GTT GAT ATA TTG TCG AAT TTG CCA TGG TTT AGC TAC G -	37 nt	RPA43	1128 – 1092

	3′			
A34 S10-14A for	5′- ATG TCG AGA TTA TTA TAA CCT TAC AG -3′	26 nt	RPA34	-305 – -280
A34 S10-14A PflFI rev	5′- GTT TGA TAT GAC TTC GTC ATC AGC GTC TGC ATC TGC TAC GTA ATC -3′	45 nt	RPA34	63 – 19
ABC23 S102A BsiWI for	5′- CTG ATG AGG AGA CGT ACG AGG AAA AAC C -3′	28 nt	RPO26	147 – 174
ABC23 S102A AgeI rev	5′- CTA CAA AAA CCG GTG CAT TCA TGG CAA TTT GTA GG -3′	35 nt	RPO26	404 – 370
ABC23 S102D AgeI rev	5′- CTA AAT CTA CAA AAA CCG GTG CAT TCA TGT CAA TTT GTA GG- 3′	41 nt	RPO26	410 – 370
AC19 T33A AatII for	5′- AGA AGA ACA GGA CGT CGA TAT GGC TGG CGA TG -3′	32 nt	RPC19	75 – 106
AC19 T33A MluI rev	5′- GCC CTT TTT GTA ACG CGT CCA CCG CGG TCG -3′	30 nt	RPC19	361 – 332
RPA12 -336for +EcoRI	5′- GCG CTG AAT TCG TCA CGA TAG AGT TAT CGC TG – 3′	32 nt	RPA12	-336 – -316
RPA12 697rev +BamHI	5′- GCG CTG GAT CCG GAT GAT AGC TGT TAT TAC TTT GAG – 3′	36 nt	RPA12	697 – 673
RPA12 +1for +BamHI	5′- GCG CTG GAT CCA TGT CTG TTG TAG GAT CGT T – 3′	31 nt	RPA12	1 – 20
RPA12 697rev +SphI	5′- GCG CTG CAT GCG GAT GAT AGC TGT TAT TAC TTT G – 3′	34 nt	RPA12	697 – 675
A12 D105A-E106A NotI for	5′- CAG TTA AGA TCT GCG GCC GCA GGT GCT ACT GTC TTC – 3′	36 nt	RPA12	298 – 333

All oligonucleotides were purchased from MWG Biotech AG, Ebersberg, Germany.

4.1.5 Peptides

Peptide	**MH^+**	**Comment**	**Origin**
EAIpSAAPFAK -NH_2	1083.524 Da	Synthetic Phosphopeptide (see Klemm *et al*., 2004)	Bachem
TVMENFVAFVDK	1399.6931 Da	BSA-sequence 569-580	Bachem

4.1.6 Model proteins

Protein	**Molecular Weight**	**Origin**
BSA (Fraction V)	66 kDa	Sigma
α-Casein	24,5 kDa	Sigma

4.1.7 Enzymes

Enzyme	Origin
Antarctic Phosphatase	New England Biolabs
Calf Intestinal Phosphatase	New England Biolabs
iProof high-fidelity DNA polymerase	Bio-Rad
Restriction Endonucleases	New England Biolabs
T4 DNA ligase	New England Biolabs
Taq DNA polymerase	
Trypsin, modified, sequencing grade, from bovine pancreas	Roche

4.1.8 Antibodies

Antibody	Species	Dilution	Origin
α-A135	rabbit	1:50000	A. Sentenac, Paris (Buhler *et al*., 1980)
α-A49	rabbit	1:50000	A. Sentenac, Paris (Buhler *et al*., 1980)
α-A43	rabbit	1:50000	A. Sentenac, Paris (Buhler *et al*., 1980)
Pol I antiserum	rabbit	1:10000	A. Sentenac, Paris (Buhler *et al*., 1980)
α-Rpb1 (8WG16)	mouse	1:1000	BAbCO
α-Rpc53	rabbit	1:100000	
α-Rrn3-NT	rabbit	1:400	Milkereit, 1999
goat-α-rabbit IgG (H+L)-POD	goat	1:3000	Dianova
goat-α-mouse IgG (H+L)-POD	goat	1:10000	Dianova

4.1.9 Chemicals

Chemicals were purchased at the highest available purity from Sigma-Aldrich, Merck, Fluka, Roth or J.T.Baker, except 5-FOA (Toronto Research Chemicals), agarose, electrophoresis grade (Invitrogen), bromine phenol blue (Serva), G418/Geneticin (Gibco), milk powder (Sukofin), Nonidet P-40 substitute (NP40) (USB Corporation), Tris ultrapure (USB Corporation) and Tween 20 (Serva).

.Ingredients for growth media were purchased from BD Becton, Dickinson and Co. (Bacto Agar, Bacto Peptone, Bacto Tryptone and Bacto Yeast Extract), Q-Biogene, Bio101,Inc. or Sunrise Science Products (Complete supplement mixtures (CSM), Yeast nitrogen base (YNB), amino acids and adenine) and Sigma-Aldrich (D(+)-glucose, D(+)-galactose, amino acids and uracil).

The matrix for MALDI-MS was α-cyano-4-hydroxycinnamic acid (>98%, TLC) (Sigma-Aldrich).

4.1.10 Other Materials

Material	**Origin**
Broad Range Protein Markers	New England Biolabs
DNA ladders	New England Biolabs
Yeast genomic DNA (strain S288C)	Invitrogen
Salmon sperm DNA (10 mg/ml)	Invitrogen
PVDF membrane Immobilion P 0.45µm	Millipore
3MM filter-papers	Millipore
BM Chemiluminescence Blotting Substrate (POD)	Roche
SimplyBlue SafeStain	Invitrogen
Pro-Q Diamond Phosphoprotein stain	Invitrogen
Spectra/Por 2 dialysis tubing (MWCO 12-14 kDa)	Spectrum Laboratories
Ni-NTA Agarose	Qiagen

4.1.11 Media

Medium	Composition
YPD (*Y*east extract, *P*eptone, *D*extrose)	1 % (w/v) Bacto Yeast Extract 2 % (w/v) Bacto Peptone 2 % (w/v) Glucose
YPD + Geneticin	YPD +200 µg/ml geneticin (G418)
YPAD	YPD +100 mg/l adenine
SDC-His, -Leu or -Trp (*S*ynthetic *D*extrose *C*omplete minus His, Leu or Trp)	0,67 % (w/v) YNB + Nitrogen 0,063 % (w/v) CSM -His -Leu -Trp w/ 20 mg/ml Ade 2 % (w/v) Glucose supplemented with 20 mg/l L-histidine, 100 mg/l L-leucine and/or 50 mg/l L-tryptophane
SDC-Ura (*S*ynthetic *D*extrose *C*omplete minus Ura)	0,67 % (w/v) YNB + Nitrogen 0,065 % (w/v) CSM -His -Leu -Ura 2 % (w/v) Glucose supplemented with 20 mg/l L-histidine, 100 mg/l L-leucine

SDC -Trp +5FOA	SDC -Trp +0,1 % (w/v) 5-FOA
SDC -Arg -Trp	0,67 % (w/v) YNB + Nitrogen 0,06 % (w/v) CSM -Arg -His -Lys -Trp -Ura 2 % (w/v) Glucose supplemented with 20 mg/l L-histidine, 50 mg/l L-lysine, 20 mg/l L-uracil
SDC -Arg -Trp +Can	SDC -Arg -Trp +6 mg/l L-canavanine
LB (*L*uria *B*roth)	1 % (w/v) Bacto Tryptone 0,5 % (w/v) Bacto Yeast Extract 0,5 % (w/v) NaCl
LB_{Amp}	LB + 100 µg/ml ampicilin

Media for agar plates were supplemented with 2 % (w/v) Bacto Agar. All growth media were autoclaved for 20 min at 120 °C. If required antibiotics, fungicides, counterselection drugs or other chemicals (*e.g.* 6AU) were added after cooling to about 60 °C.

4.1.12 Equipment

Device	**Manufacturer**
4700 Proteomics Analyzer MALDI-TOF/TOF	Applied Biosystems
Alpha 2-4 lyophilizer	Christ
Biofuge Fresco refrigerated tabletop centrifuge	Hereaus
Biofuge Pico tabletop centrifuge	Hereaus
C412 centrifuge	Jouan
Centrikon T-1170 ultracentrifuge	Kontron Instruments
Centrikon T-324 centrifuge	Kontron Instruments
CT422 refrigerated centrifuge	Jouan
Electrophoresis system model 45-2010-i	Peqlab Biotechnologie GmBH
FPLC-System (Pumps P-500; Controller LCC-501+; Fraction collector FRAC-100)	Pharmacia Biotech
Gel Max UV transilluminator	Intas
IKA-Vibrax VXR	IKA
Incubators	Memmert
LAS-3000 chemiluminescence imager	Fujifilm
MicroPulser electroporation apparatus	Bio-Rad
Mini-PROTEAN 3 electrophoresis system	Bio-Rad
NanoDrop ND-1000 spectrophotometer	Peqlab Biotechnologie GmBH

Optima L-80 X ultracentrifuge	Beckman Coulter
PCR Sprint thermocycler	Hybaid
Power Pac 3000 power supplies	Bio-Rad
Pulverisette 6 planetary mono mill	Fritsch
Roto-Shake Genie	Scientific Industries
Shake incubators Multitron / Minitron	Infors
Speed Vac Concentrator	Savant
Thermomixer compact	Eppendorf
Trans-Blot SD Semi-dry transfer cell	Bio-Rad
UltiMate 3000 NanoHPLC	Dionex
Ultrospec 3100pro spectrophotometer	Amersham
XCell SureLock Mini-Cell electrophoresis system	Invitrogen

4.1.13 Software

Software	**Producer**
4000 Series Explorer v.3.6	Applied Biosystems
Acrobat 7.0 Professional v.7.0.9	Adobe
Chromeleon v.6.70	Dionex
Data Explorer v.4.5 C	Applied Biosystems
GPS Explorer v.3.5	Applied Biosystems
Illustrator CS v.11.0.0	Adobe
Image Reader LAS-3000 v.1.12	Fujifilm
Mascot	Matrix Science
Microsoft Office 2003	Microsoft
ND-1000 v.3.5.2	Peqlab Biotechnologie GmBH
Photoshop CS v.8.0.1	Adobe
PyMOL v.0.99	DeLano Scientific

4.2 Methods

4.2.1 Cultivation of S. cerevisiae strains

Strains of the budding yeast *Saccharomyces cerevisiae* (4.1.1) were cultured using standard microbiological methods (Sherman, 2002).
Liquid cultures were grown in YPD- or SDC-media in shake flasks at 30 °C, except for Δrpa12 deletion strains which were grown at 24 °C. Culture volumes were chosen to be 40 % of the flask volumes and cell growth was monitored by measuring the optical density at 600 nm (= OD_{600}).
For cultivation on solid agar plates containing either YPD- or SDC-media, single colonies or small aliquots of glycerol stocks were spread out with sterile disposable inoculation loops (Sarstedt) in order to obtain colonies derived from single yeast cells. Plates were incubated upside down at the respective temperatures.
Yeast strains were stored for short periods on agar-plates at 4 °C. Long-term preservation was accomplished by storing small aliquots of liquid cultures mixed with glycerol to a final concentration of 15 % (v/v) at -80 °C (= glycerol stock).

4.2.2 Preparation of yeast whole cell extracts (WCE)

4.2.2.1 Protein extraction on a small scale

For protein extraction on a small scale, cells from 20-50 ml liquid cultures were harvested by centrifugation in 50 ml tubes (Sarstedt) for 3 min at 2000 g (Jouan). The cells were washed with 1 ml cold H_2O transferred into 1.5 ml tubes and resuspended in an equal volume of high-salt extraction buffer [400 mM $(NH_4)_2SO_4$, 150 mM HEPES pH 7.6, 10 mM $MgCl_2$, 20 % (w/v) glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, 2 mM benzamidine]. Cold glass beads (Ø 0.75–1 mm, Roth) were added to a level with the liquid and the tubes vigorously shaken on an IKA Vibrax at 14000 rpm and 4 °C. Afterwards the protein extracts were mixed with additional 150 µl ice-cold high-salt extraction buffer and the glass beads and cell debris pelleted in a tabletop centrifuge (15 min, 13000 rpm, 4 °C). The cleared yeast WCEs were transferred to new tubes and if required frozen in liquid nitrogen before storage at -20 °C.

4.2.2.2 Preparation of WCE for Pol I purification

Strain GPY2, carrying the gene for an N-terminal His6/HA-tagged A43 on a plasmid (Fath et al., 2000), was cultivated to OD_{600} = 1-2 at 30 °C in 12 x 800 ml YPD media (= ~10 l) in 2 l shake flasks. The cells were harvested by centrifugation (4000rpm, 5 min, 4 °C; Herolab A6.9) and combined during a final wash step with cold H_2O. The cell pellet was resuspended in 0.5 ml ice-cold lysis buffer (150 mM HEPES pH 7.8, 60 mM $MgCl_2$, 60 % glycerol, 3 mM DTT, 1 mM PMSF, 2 mM benzamidine) per gram yeast cells and frozen in liquid nitrogen for storage at -80 °C if required.
Frozen cells were thawed on ice and $(NH_4)_2SO_4$ added to a final concentration of 400 mM (from a 2.5 M stock solution) and mixed with an equal volume of ice-cold dilution buffer (400 mM $(NH_4)_2SO_4$, 100 mM HEPES pH 7.8, 20 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2 mM benzamidine). 120 ml of this cell suspension were added to 120 ml pre-cooled glass beads (Ø 0.75–1 mm, Roth) and the cells were lysed using a Pulverisette 6 planetary mono mill (Fritsch) (3 cycles of 4 min 500 rpm and 1 min cooling). Glass beads were separated by filtration prior to centrifugation (20 min, 8000 g, 4°C; Herolab A6.9) to remove the cell debris and residual glass beads. The final whole cell extract (WCE) was obtained after centrifugation at 100,000 g for 90 min (Kontron TFT55.38 or Beckman Ti45) by separating the clear middle phase from the turbid insoluble matter in the lower phase and the top layer of lipids. Protein concentrations were determined using the method by Bradford (1976) (see 4.2.4.1). If required, the WCE was frozen in liquid nitrogen and stored at -80 °C.

4.2.3 Pol I purification

A scheme of the purification strategy is given in Figure 15. All steps were performed at 4 °C. 50 ml of the GPY2-WCE (see 4.2.2.2) were dialyzed against buffer B (50 mM KAc, 20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 10 % glycerol, 1 mM PMSF, 2 mM benzamidine, 5 mM β-mercaptoethanol) over night, using Spectra/Por 2 dialysis tubing (MWCO 12-14 kDa) (Spectrum Laboratories). After centrifugation at 30,000 g for 1 h (Beckman Ti45), the pellet was resuspended in buffer C (1.5 M KAc, 20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 10 % glycerol, 0.1 % NP40, 5 mM β-mercaptoethanol, 1 mM PMSF, 2 mM benzamidine) and applied to 2 ml equilibrated Ni-NTA Agarose (Qiagen). Binding was performed in batch for 4 hours on a turning wheel, before pouring the suspension into disposable 20 ml Econo-Pac columns (Bio-Rad) for subsequent wash-steps with buffer C and buffer D (300 mM KAc, 20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 10 % glycerol, 0.1 % NP40, 5 mM β-mercaptoethanol, 1 mM PMSF, 2 mM benzamidine) and elution with buffer E (buffer D + 50 mM imidazole). The eluting proteins were loaded onto a Mono Q anion exchange column (Mono Q HR5/5,

Pharmacia) and fractionated by applying a salt gradient from 300 mM to 2 M KAc with buffers F (20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 10 % glycerol) and G (buffer F + 2 M KAc). 0.5 ml aliquots of the pooled Pol I containing fractions (eluting at 1.1 M KAc) were applied to gelfiltration chromatography on a Superose 6 column (Superose 6 HR10/30, Pharmacia) with buffer H (1.5 M KAc, 20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 10 % glycerol).

4.2.4 Protein analysis

4.2.4.1 Determination of protein concentrations

Protein concentrations were determined using the Bio-Rad Protein Assay which is based on the method by Bradford (1976). Briefly, 1-10 µl of each protein solution to be tested were mixed with 1 ml of the protein assay dye (Bio-Rad) after diluting the reagent to the working concentration according to the manufacturer's instructions. The approximate protein concentrations in µg/µl were calculated by dividing the absorbance at 595 nm (= OD_{595}, corrected for the absorbance of the dye) by the sample volume and multiplying with the factor 23 which was determined using a BSA standard curve.

4.2.4.2 Protein precipitation for analysis

4.2.4.2.1 TCA precipitation

The volume of the protein sample to be analyzed was adjusted to 100µl with cold H_2O prior to mixing with 10 µl ice-cold 100 % (w/v) TCA (final concentration approximately 10 % TCA) and 2 µl 2 % (w/v) DOC (Desoxycholate) (Bensadoun and Weinstein, 1976). Precipitation was conducted for about 30 min on ice. The proteins were pelleted by centrifugation in a refrigerated table top centrifuge (13000 rpm, 20 min, 4 °C) and the supernatant carefully removed with a suction pump. The pH of the pellet was neutralized under a stream of NH_3, taken from the gaseous phase of a NH_4OH-bottle with a Pasteur-pipette. Finally the proteins were resolubilized in an adequate volume of SDS-sample buffer for separation by SDS-PAGE.

4.2.4.2.2 Chloroform/methanol precipitation

Protein precipitation for subsequent mass spectrometric analyses was performed using the chloroform/methanol precipitation method by Wessel and Flügge (1984). The volume of the sample was adjusted to 150 µl with H_2O, followed by the addition of four volumes (450 µl) methanol, one volume (150 µl) chloroform and 3 volumes (450 µl) H_2O. After each of these addition steps the sample was mixed well by vortexing. The resulting phases were separated by centrifugation in a table top centrifuge for 5 min, 13000 rpm. The upper phase was discarded while carefully avoiding loss of the interphase which contains the precipitated proteins. Upon addition of another three volumes of methanol (450 µl) and vortexing, the proteins were pelleted by centrifugation (5 min, 13000 rpm). The supernatant was completely removed and the protein pellet dried for 10 min in a vacuum centrifuge (Speed-Vac Concentrator). After resolubilization with NuPAGE sample buffer (Invitrogen) the proteins were either directly loaded on a polyacrylamide gel or stored at -20°C.

4.2.4.3 Separation of proteins by SDS-PAGE

The proteins contained in the samples were separated according their molecular weight using the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method by Laemmli (1970). Vertical electrophoresis was performed on 10-12.5 % polyacrylamide gels using either the Mini-PROTEAN 3 system (Bio-Rad) or a Peqlab electrophoresis system (Peqlab Biotechnologie GmBH, model 45-2010-i). Broad range protein marker (NEB) or pre-stained broad range protein marker (NEB) was used for size-determination.

Samples for mass spectrometric analyses were separated on 4-12 % gradient NuPAGE Bis-Tris gels (Invitrogen) using the NuPAGE MOPS SDS running buffer (Invitrogen) complemented with NuPAGE antioxidant (Invitrogen). Gel electrophoresis was performed according to the manufacturer's instructions except for the voltage applied. Instead of running the gels at constant 200 V, a constant current of 50 mA was applied while limiting the voltage to maximum 180 V.

4.2.4.4 Western Blot

In order to detect specific proteins via immunodetection in the samples separated by SDS-PAGE, the proteins were transferred to PVDF-membrane (Polyvinyldifluoride) (Immobilion-P 0.45µm, Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad).

The gel and the PVDF membrane (pretreated with methanol) were placed into the semi-dry blot apparatus between two piles of three 3MM filter-papers (Millipore) soaked with transfer-buffer (20 % (v/v) methanol, 47.8 mM Tris, 38.6 mM glycine, 0.037 % (w/v) SDS) and a constant voltage of 24 V applied for 60-75 min. All filter-papers and the membrane had the same size as the SDS-gel and air bubbles between the layers were rolled out, because these would interfere with the transfer.
To control the blotting of the proteins before immunodetection, the total protein content was reversibly stained with Ponceau S. The membrane was incubated for 5 min at RT in the Ponceau S-staining solution (0.5 % (w/v) Ponceau S, 1 % (v/v) acetic acid) and slightly destained with H_2O until the protein bands were visible.

Prior to specific immunodetection of defined proteins, the free space on the membrane was blocked with non-related proteins from bovine milk to avoid unspecific binding of the antibodies by incubating the blot-membrane with 5 % (w/v) milk powder in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4) for 1 h at RT or over night at 4 °C on a shaker. The antibodies were diluted to an adequate working dilution (see 4.1.8) in PBST (PBS plus 0.05 % (v/v) Tween 20) supplemented with 5 % (w/v) milk powder. The incubations were performed in small bags made of sealed plastic foils on a turning wheel at RT for 1 h (primary antibodies) or 40 min (secondary antibodies). Following each antibody incubation step, the membrane was washed three times for 5 min in about 50 ml PBST on a shaker.
To detect the specifically bound antibodies on the blot, the membrane was incubated for 1 min at RT with 2-4 ml BM Chemiluminescence Blotting Substrate (POD) (Roche) which was prepared according to the manufacturer's instructions. This reagent contains hydrogen peroxide (H_2O_2) and luminol which is a substrate for the horseradish peroxidase conjugated to the secondary antibodies used in this study. The light which is emitted during this reaction at the corresponding specific positions of the blot-membrane was detected with a LAS-3000 chemiluminescence imager (Fujifilm) using the software Image Reader (Raytest).

4.2.4.5 Polyacrylamid gel staining

4.2.4.5.1 Coomassie staining

To visualize the total protein content of a polyacrylamide gel, it was stained with SimplyBlue SafeStain (Invitrogen) according to the manufacturer's instructions. This stain is a commercially available pure (*i.e.* keratin-free) Coomassie G-250 stain. Briefly, the gel was washed 3 times 5 min with H_2O to remove SDS and buffer salts which interfere with the

binding of the stain to the proteins. Staining was performed for 1 h at RT on a shaker, before destaining with H_2O.

4.2.4.5.2 Silver staining

To stain polyacrylamide gels with low protein content, the more sensitive silver staining was preferred over the coomassie staining. The proteins were fixated in the gel by incubation in fixation-solution (50 % (v/v) methanol, 12 % (v/v) acetic acid, 0.02 % (v/v) formaldehyde) for 1 h or over night (RT). Afterwards the gel was washed in 50 % (v/v) ethanol for 20 min and incubated in 0.8 mM $Na_2S_2O_3$ for 1 min, directly followed by three 20 seconds wash steps with H_2O. Next, the gel was incubated in staining-solution (12 mM $AgNO_3$, 0.03 % (v/v) formaldehyde) for 20 min and washed two times for 20 seconds with H_2O. The stained protein bands became visible upon incubation with developing solution (566 mM Na_2CO_3, 0.02 % (v/v) formaldehyde, 0.016 mM $Na_2S_2O_3$). The development was stopped with 1 % acetic acid.

4.2.4.5.3 Phosphoprotein staining

SDS-polyacrylamide gels were stained with Pro Q Diamond Phosphoprotein stain (Invitrogen) according to the manufacturer's instructions and visualized on a 315 nm UV transilluminator. The same gel was then stained with SimplyBlue SafeStain (Invitrogen) for total protein staining (see 4.2.4.5.1).

4.2.5 Unspecific transcription assay

12.5 µl of the RNA polymerase preparation to be tested were incubated with 12.5 µl ^{3}H-transcription mix for 30 min at RT in 15 ml tubes. The ^{3}H-transcription mix contains nicked calf thymus DNA as template as well as all four NTPs including ^{3}H-UTP. RNA polymerases are able to initiate transcription in a promoter-independent way at the DNA nicks and thus produce ^{3}H-labeled RNA according to their enzymatic activity in this assay. The reaction was stopped by mixing with 200 µl of a saturated pyrophosphate solution containing 0.1 mg/ml salmon sperm DNA (Invitrogen), followed by precipitation of the proteins with 10 ml 5 % TCA for 30 min on ice. To remove the surplus of radiolabeled nucleotides, the solution was filtered through GFC-filters (Millipore) which were pretreated with a saturated

pyrophosphate solution. The filters were washed three times with 5 % TCA and once with ethanol before drying at RT. The amount of ^{3}H-labeled RNA bound to the filters was measured after adding 1 ml Ultima Gold scintillation mix (Packard) by measuring the counts per minute for 1 min in a liquid scintillation analyzer 1600 TR (Packard).

^{3}H-transcription mix

1.5 ml nicked calf thymus DNA	1 mg/ml in TE-buffer, including 5 mM $MgCl_2$ and 20µg/ml BSA; nicked by limited digested with DNase I
1 ml ^{3}H-NTP-mix	3.3 mM ATP, CTP and GTP, each; 0.01 mM UTP; 100 µCi 5-^{3}H-UTP (Amersham); 125 mM KCl; 25 mM $MgCl_2$

4.2.6 Protein identification using MALDI-TOF/TOF mass spectrometry

Samples of the purified Pol I preparations were precipitated by methanol/chloroform and loaded on a 4-12 % NuPAGE Bis-Tris gel (Invitrogen). After electrophoresis using NuPAGE MOPS SDS running buffer (Invitrogen), the gel was stained with SimplyBlue SafeStain (Invitrogen) and the bands of interest were excised. The proteins were digested in gel with modified sequencing grade trypsin (Roche) (Shevchenko *et al*., 1996; Shevchenko *et al*., 2007). Briefly, the excised gel pieces were cut into small cubes (edge length about 1 mm) and subsequently washed with 50 mM NH_4CO_3, 50 mM NH_4CO_3 / 25 % (v/v) acetonitrile, 25 % (v/v) acetonitrile and 50 % (v/v) acetonitrile, followed by lyophilization. The dried gel pieces were rehydrated with an equal volume of trypsin in 50 mM NH_4CO_3 (endconcentration 4 µg trypsin per 100 µl gel) for 30 min at RT. After addition of another volume 50 mM NH_4CO_3 and incubation at 37 °C for 16 h, the resulting tryptic peptides were eluted by diffusion upon shaking the gel cubes in two volumes of 100 mM NH_4CO_3 (two times), 100 mM NH_4CO_3 / 25 % (v/v) acetonitrile and 50 % (v/v) acetonitrile / 0,1 % (v/v) TFA at RT. The supernatants of these elution steps were pooled in a fresh Eppendorf 1.5 ml Tube and the solvents removed by lyophilization.

Prior to analysis, the peptides were desalted using ZipTip C_{18} pipette tips (Millipore) according to the manufacturer's instructions. The peptides were eluted with 2 µl 50-80 % (v/v) acetonitrile / 0,1 % (v/v) TFA, mixed with 1 µl α-cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich) (6 mg/ml in 50 % (v/v) acetonitrile / 0,1 % (v/v) TFA; final concentration: 2 mg/ml CHCA) and manually spotted on the MALDI target plate using the dried-droplet method (Cañas *et al*., 2007).

Peptide mass fingerprints and MS/MS analyses were performed on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer operated in positive ion reflector mode and evaluated by searching the NCBInr protein sequence database with the

Mascot search engine (Matrix Science) implemented in the GPS Explorer software (Applied Biosystems).

4.2.7 Identification of phosphorylation sites

Following in-gel digestion (see 4.2.6), approximately 37.5 pmol of the tryptic peptides of any phosphorylated Pol I subunit were used for the chemical derivatization at a time. The sulfhydryl groups of the cysteine residues were protected by reduction with 10 mM DTT at 37 °C and carboxymethylation with 35 mM Iodoacetic acid at 24 °C for 1 h each. The β-elimination and Michael-addition reactions were performed in a single step by incubation of the alkylated peptides in the presence of 64.5 mM $Ba(OH)_2$ (taken from a freshly prepared saturated $Ba(OH)_2$ solution) with either 450 mM ethanethiol or 400 mM pentanethiol in 30 % acetonitrile at 50 °C for 90 minutes. The reactions were stopped by precipitation of $BaCO_3$ after the addition of NH_4HCO_3 to a final concentration of 100 mM. Residual alkanethiols, NH_4HCO_3 and acetonitrile were removed by lyophilization. The modified peptides were either analyzed after desalting with ZipTip C_{18} pipette tips (Millipore) as described for protein identification (4.2.6) or fractionated via C_{18} reverse phase liquid chromatography. The latter was performed using a Dionex UltiMate NanoHPLC applying a gradient from 15 to 60 % acetonitrile / 0,05 % TFA at a flow rate of 300 nl/min within 90 min. Fractions were mixed with CHCA matrix and collected directly on the MALDI-target using a Dionex Probot system. All samples were analyzed on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer. Identification of the originally phosphorylated residues is based on the unique mass shift of + 44.0085 Da (for ethanethiol) or + 86.0554 Da (for pentanethiol) compared to the expected mass of an unmodified serine or threonine. A customized Pol I sequence database (including trypsin and all co-purifying proteins) was used to allow more variables in the search parameters and the results were confirmed by searching against the NCBInr protein sequence database.

4.2.8 Creation of mutant yeast strains

4.2.8.1 Cloning of genes coding for Pol I subunits (RPA-genes)

For the mutational analyses, the genes coding for the *in vivo* phosphorylated Pol I subunits were cloned into vector pRS314 (TRP1) (Sikorski and Hieter, 1989) (see 4.1.3) including their endogenous promoter and terminator regions.

Molecular biological techniques (including restriction digestion, ligation, PCR and DNA analyses by agarose gel electrophoresis) were performed according to standard protocols (Sambrook and Russell, 2001). *E. coli* strains XL1-Blue or DH5α (see 4.1.2) were used for plasmid amplification. Transformation of competent *E. coli* cells was conducted by electroporation using a MicroPulser Electroporation apparatus (Bio-Rad) with 2 mm cuvettes. Plasmid mini-preps, PCR product purifications and agarose gel-extractions were performed using commercially available kits by Invitrogen or Qiagen according to the manufacturers' instructions.

RPA190 was subcloned from plasmid pNOY16 (Wittekind *et al*., 1988) which contains an 11 kb fragment of chromosome XV including RPA190 and RPA43. First, a short 870 bp fragment spanning the region from position -801 to +69 of RPA190 was amplified by PCR and cloned into pRS314 via a new Xho I site from the forward primer and a Bam HI site at position +49 of RPA190. This construct was cut with Bam HI and Spe I and ligated with a 5.8 kb Bam HI - Avr II fragment of pNOY16 which contains the remaining part of RPA190 including the 3'UTR. Spe I and Avr II can be efficiently ligated due to the compatible overhangs, but the resulting sequence can not be recognized by either one of the two restriction enzymes.
RPA34, RPO26 and RPC19 were amplified from yeast genomic DNA (strain S288C, Invitrogen) via PCR. The primers contained additional Xho I sites (forward primers) or Bam HI sites (reverse primers) which were used for cloning into vector pRS314.
Plasmid pGP5 (RPA43 cloned into pRS314) (Peyroche *et al*., 2000) was used for the analyses of the A43 subunit.

For the cloning of RPA12 into vectors pRS314 (TRP1) and YCplac33 (URA3), the ORF plus promoter and terminator regions was amplified from yeast genomic DNA (strain S288C, Invitrogen) using PCR primers with additional Eco RI (forward primer) or Bam HI (reverse primer) restriction sites. Cloning into vector YCplac111Gal (LEU2) was performed using primers Bam HI (forward primer) or Sph I (reverse primer) sites. In this case the gene was cloned starting from +1 (ATG); the RPA12 promoter was replaced by the Gal1-10 promoter included in the vector (Ferreira-Cerca *et al.*, 2005).

4.2.8.2 Site-directed mutagenesis

A scheme of the mutagenesis strategy is given Figure 25. PCR reactions were performed with primers carrying the desired mutations (see 4.1.4) on plasmids containing the gene for the respective Pol I subunit. The length of the PCR product was chosen to span a unique restriction site, which in turn was used to cut another aliquot of the same plasmid. 45-75 ng of

the PCR product were transformed together with 15 ng of the linearized plasmid into a BSY420 (1n) wildtype yeast strain (see 4.2.8.3). Transformants were selected for the TRP1 marker contained on the vector. As a consequence only cells carrying a plasmid repaired by homologous recombination could form colonies. After plasmid isolation, vectors containing the mutation were selected by restriction analysis screening for a new restriction site, which was also introduced in the PCR primer, but results in a silent mutation. All constructs were verified by sequencing.

4.2.8.3 Yeast transformation

Yeast cells were transformed with plasmids (carrying mutant or WT alleles of RPA-genes) or linear DNA fragments (for homologous recombination in gap repair or chromosomal integration) using the DMSO-enhanced lithium acetate (LiAc) method by Hill *et al*. (1991) with some slight modifications.

50 ml liquid YPD cultures were grown to OD_{600} = 0.5-1.5 in shake flasks. The cells were gently pelleted by centrifugation (about 2000 g, 3 min, RT) and resuspended in 15.3 µl LiT-buffer (100 mM LiAc, 10 mM Tris/Cl pH 7.4) per OD_{600} and ml. 100 µl of this cell suspension were used per transformation batch.

Each 100 µl cell suspension-batch was supplemented with 40-200 ng plasmid DNA or up to 1 µg linear DNA to be transformed, 10 µl salmon sperm DNA (10 mg/ml, Invitrogen) and 500 µl LiT-PEG-buffer (50 % (w/v) PEG 4000 in LiT-buffer), followed by incubation for 45 min at RT on a turning wheel. Afterwards the transformation-batches were gently mixed with 50 µl DMSO, heat-shocked at 42 °C for 15 min and pelleted by centrifugation in a table top centrifuge (3000 rpm, 30 sec).

For selection of auxotrophic markers (*e.g.* the TRP1 marker contained on the mutant vectors) the cells were directly resuspended in 100 µl H_2O and plated on SDC-plates lacking the corresponding amino acids. If selection of the dominant marker $G418^R$ was required, the cells were first resuspended in 1 ml YPD medium and incubated for about 2 h at 30 °C while shaking to allow the expression of the marker before plating on the selection media as described for auxotrophic markers (Mount *et al*., 1996). Because the selection on G418-containing YPD-plates often results in a high number of transient transformants, these plates were replica-plated on fresh G418 plates before cultivating single clones.

4.2.8.4 Yeast plasmid shuffle

Plasmid shuffle yeast-strains were used to replace the essential RPA-genes for the mutant alleles. In these strains the chromosomal locus of the gene of interest is knocked-out with a marker gene and the deletion complemented by a wild-type copy of the gene of interest on a plasmid containing a counterselectable marker. The mutant copy is introduced on another plasmid and the strain grown on the corresponding selection-medium. If the mutant allele is able complement the chromosomal deletion, the plasmid containing the wild-type copy can be lost during cultivation. Finally growth on the respective counterselection medium is lethal for all cells still containing the wild-type plasmid.

Single clones derived from LiAc-transformations of the mutant vectors (see 4.1.3) into the corresponding shuffle-yeast strains (see 4.2.8.3) were streaked on the counterselection-plates with sterile disposable inoculation loops (Sarstedt). A portion of the same clones was streaked on control-plates containing the same medium except for the counterselection drug. Transformants of the RPA190-shuffle strain NOY222 (Wittekind *et al*., 1988) (see 4.1.1) were cultivated on plates containing L-canavanine to select against the Can^S-allele of the CAN1-gene which codes for a functional arginine permease and thus allows for the uptake and incorporation of this lethal arginine-derivate (Grenson *et al*., 1966; Whelan *et al*., 1979; Broach *et al*., 1979). The transformants of all other RPA-shuffle strains (see 4.1.1) were cultivated on plates with 5-FOA (5-Fluoro-orotic acid) which facilitates counterselection against strains carrying a functional URA3-gene. URA3 codes for the enzyme orotidin-5'-phosphate decarboxylase of the uracil-biosynthesis pathway which also converts 5-FOA into the toxic 5-fluorouracil (Boeke *et al*., 1984; Boeke *et al*., 1987).
Single clones were controlled for 1) the presence of the mutant vector, 2) the loss of the wild-type plasmid and 3) the maintenance of the chromosomal deletion via the respective auxotrophic markers. Single clones were further cultivated on YPD plates to obtain the mutant strains listed in 4.1.1.

4.2.9 In vivo phenotyping of mutant yeast strains

4.2.9.1 Spot tests on agar-plates

Equally sized colonies of yeast strains carrying the phosphorylation site mutations or the corresponding wild type genes were resuspended in 1 ml sterile H_2O and diluted to $OD_{600} = 0.1$. 7-10 µl of this cell suspension and of serial 1:10, 1:100 and 1:1000 dilutions were spotted on the test plates and on the corresponding control plates in parallel.

6-Azauracil (6AU)-phenotyping was performed with SDC-Ura plates supplemented with 25-200 µg 6AU (up to 300 µg 6AU in single experiments). SDC-Ura and YPD plates were used as controls. Phenotypes were monitored after incubation at either 30 °C or 37 °C for 2 days (plates without 6AU) or 4-6 days (plates with 6AU).
Rapamycin was used at concentrations of 0.1-0.2 mM in YPD plates.

4.2.9.2 Growth in liquid cultures, inoculated from stationary phase cells

Mutant yeast strains and the corresponding wild-type strains were grown in 20 ml YPAD (YPD + 100 µg/ml adenine) to stationary phase. From these cultures fresh 50 ml YPAD liquid cultures in shake flasks were inoculated to OD_{600} = 0.1. Growth at 30 °C was monitored by measuring the OD_{600} in 1 h intervals.

4.2.10 Figure preparation

Figures were prepared with Illustrator CS (Adobe) and Photoshop CS (Adobe). Mass spectra were reproduced using Data Explorer (Applied Biosystems) and figures of Pol I structures were prepared with PyMOL (DeLano Scientific).

5 References

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6 Abbreviations

6AU	6-azauracil
Å	Ångstrøm
ACN	acetonitrile
ARS	autonomous replication sequence
bRNAP	bacterial RNA polymerase
CF	core factor
CHCA	α-cyano-4-hydroxycinnamic acid
ChIP	chromatin immunoprecipitation
CID	collision induced dissociation
cs	cold sensitive
CSM	complete supplement mixture
CTD	carboxy-terminal domain
Da	Dalton
DFC	dense fibrillar component
EM	electron microscopy
ESI	electrospray ionization
ETS	external transcribed spacer
EtSH	ethanethiol
FC	fibrillar center
GC	granular component
IMAC	immobilized metal affinity chromatography
IP	immunoprecipitation
ITS	internal transcribed spacer
KAc	potassium acetate
kDa	kilo Dalton
LB	Luria Broth
LC	liquid chromatography
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption/ionization
MH^+	molecular mass in Da (= M) plus one dalton from the proton added during the ionization process (= MH^+)
mRNA	messenger RNA
MS	mass spectrometry
MS/MS	tandem mass spectrometry
nanoHPLC	nano high pressure liquid chromatography
NTS	non-transcribed spacer
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PeSH	pentanethiol

PKA	protein kinase A
PMF	peptide mass fingerprint
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
Pol IV	RNA polymerase IV
rDNA	ribosomal DNA
RP	ribosomal protein
rRNA	ribosomal RNA
SCX	strong cation-exchanger
SDC	synthetic dextrose complete
snoRNP	small nucleolar ribonucleoprotein
TBP	TATA-binding protein
TOF	time of flight
TOR	target of rapamycin
TORC	target of rapamycin complex
tRNA	transfer RNA
ts	temperature sensitive
UAF	upstream activation factor
WCE	whole cell extract
YNB	yeast nitrogen base
YPD	yeast extract, peptone, dextrose

7 Summary

A tight control of ribosome biogenesis in dependence of the growth conditions is crucial for the economics of a cell. Signal transduction cascades forward the status of environmental factors to several key regulatory steps via reversible phosphorylation. As one of the first steps of ribosome biogenesis, the transcription of the 35S rRNA precursor by RNA polymerase I (Pol I) is also one of the main targets for regulation. In logarithmically growing *Saccharomyces cerevisiae* cells, five of the fourteen Pol I subunits were found to be phosphorylated *in vivo* (A190, A43, A34.5, ABC23 and AC19) to a total content of about 15 ± 3 phosphate groups per enzyme. Further *in vivo* and *in vitro* studies showed that the activity of Pol I is linked to its phosphorylation status. However, little is known about the positions and functions of the single Pol I phosphorylation sites.

In this study the site-specific phosphorylation of yeast Pol I was analyzed using a combination of chemical derivatization and LC-MALDI-TOF/TOF mass spectrometry. Prerequisite was the development of a rapid method to purify Pol I while maintaining its phosphorylation status. The resulting enzyme preparations were active in promoter-independent transcription assays and the purity was suitable for structural analyses by the cooperating research group of Patrick Cramer. A total of 13 phosphoserines and –threonines were identified on the *in vivo* modified subunits. Five of these were confirmed by independent proteome-wide phosphorylation analyses which employed alternative methods and additionally provided four other sites. A Pol I homology model facilitated the three-dimensional localization of seven phosphorylation sites in the new cryo-EM structure.

The single phosphoresidues were systematically mutated to alanine or aspartate to mimic constitutively dephosphorylated or phosphorylated states, respectively, and the resulting yeast strains were analyzed using *in vivo* assays. Surprisingly all Pol I phosphorylations analyzed were found to be non-essential posttranslational modifications. None of the mutants showed a detectable growth phenotype with one exception: mutation of A190 S685 to aspartate resulted in a lowered sensitivity to the NTP-depleting drug 6-azauracil (6AU) compared to the isogenic wild-type or the analogous mutation to alanine. This drug is commonly used to investigate defects associated to the elongation phase of RNA transcription. Strikingly, in a related study by Alarich Reiter the same mutation of phosphorylation site A190 S685 to aspartate but not to alanine was found to be synthetic lethal with the non-essential TFIIS-like Pol I subunit A12.2 which was described to function in transcription elongation, 3’ RNA cleavage and transcription termination. The data suggest a role of the reversible phosphorylation at A190 S685 in modulating one these functions.

Furthermore a mutation of the TFIIS-like domain of A12.2 was created to analyze its role in the intrinsic Pol I RNA cleavage activity. Unexpectedly this mutation of the non-essential A12.2 was found to result in a lethal phenotype, possibly due to a dead end situation. Under the control of an inducible promoter, this mutant should provide a useful tool for the elucidation of the RNA cleavage process.

8 Zusammenfassung

Eine strikte Kontrolle der Ribosomen Biogenese in Abhängigkeit der Wachstumsbedingungen ist entscheidend für den Energiehaushalt einer Zelle. Signal-Transduktions-Kaskaden übermitteln die Umweltbedingungen an mehrere Schlüsselstellen mittels reversibler Phosphorylierung. Die Transkription der 35S Vorläufer rRNA durch die RNA Polymerase I (Pol I) ist, als einer der ersten Schritte der Ribosomen Biogenese, auch einer der Hauptangriffspunkte zur Regulation. In logarithmisch wachsenden Zellen der Hefe *Saccharomyces cerevisiae* werden fünf der vierzehn Pol I-Untereinheiten *in vivo* phosphoryliert (A190, A43, A34.5, ACB23 und A34.5). Insgesamt gibt es etwa 15 ± 3 Phosphatgruppen pro Enzym. *In vivo* und *in vitro* Studien zeigten, dass die Aktivität der Pol I mit ihrem Phosphorylierungsstatus verknüpft ist, jedoch gibt es nur wenige Informationen über die Positionen und die Funktionen der einzelnen Phosphorylierungsstellen.

In der vorliegenden Arbeit wurde die orts-spezifische Phosphorylierung der Pol I durch eine Kombination von chemischer Derivatisierung und LC-MALDI-TOF/TOF Massen-spektrometrie untersucht. Vorraussetzung hierfür war die Entwicklung einer Methode zur schnellen Reinigung der Pol I, welche den Phosphorylierungsstatus erhält. Die Enzympräparationen waren aktiv in Promoter-unabhängigen Transkriptions-Tests und der Reinheitsgrad war geeignet für Strukturanalysen, die in Zusammenarbeit mit der Forschungsgruppe von Patrick Cramer durchgeführt wurden. Insgesamt wurden 13 Phosphoserine und –threonine in den fünf *in vivo* modifizierten Untereinheiten identifiziert. Fünf davon wurden durch unabhängige proteomweite Phosphorylierungsanalysen bestätigt, welche alternative Methoden verwendeten und zusätzlich vier weitere Stellen identifizierten. Ein Pol I Homologie-Modell ermöglichte die dreidimensionale Lokalisierung von sieben Phosphorylierungsstellen innerhalb der neuen Cryo-EM Struktur.
Die einzelnen Phosphoaminosäuren wurden systematisch zu Alanin oder Aspartat mutiert um konstitutiv dephosphorylierte bzw. phosphorylierte Stadien zu imitieren, und die daraus hervorgehenden Hefestämme wurden mittels *in vivo* Analysen untersucht. Überraschenderweise sind alle untersuchten Pol I Phosphorylierungen nicht-essentielle post-translationale Modifikationen. Keine der Mutanten zeigte einen erkennbaren Wachstums-Phänotyp, mit einer Ausnahme: Verglichen mit einem isogenen Wildtyp Stamm oder einer analogen Mutation zu Alanin, resultierte die Mutation von A190 S685 zu Aspartat in einer geringeren Sensitivität gegenüber 6-Azaurazil (6AU), welches den zellulären NTP-Vorrat abreichert. Diese Chemikalie wird häufig verwendet um Defekte in der Elongationsphase der Transkription zu untersuchen. Auffallenderweise wurde in einer begleitenden Studie von Alarich Reiter die synthetische Letalität zwischen der gleiche Mutation der

Phosphorylierungsstelle A190 S685 zu Aspartat und der nicht-essentiellen Pol I Untereinheit A12.2 festgestellt, welche Funktionen in Transkriptions Elongation, 3' RNA Spaltung und Transkriptions Termination hat. Die Daten lassen eine Beteiligung der reversiblen Phosphorylierung von A190 S685 an einer dieser Funktionen vermuten.

Des Weiteren wurde eine Mutation in der TFIIS-ähnlichen Domäne von A12.2 generiert um deren Rolle in der intrinsischen 3' RNA-Spaltaktivität der Pol I zu untersuchen. Unerwarteterweise resultiert diese Mutation der nicht-essentiellen Untereinheit A12.2 in einem letalen Phänotyp. Unter der Kontrolle eines induzierbaren Promoters könnte diese Mutante ein wertvolles Werkzeug für weitere Untersuchungen des Mechanismus der Pol I RNA-Spaltaktivität darstellen.

9 Publications

Gerber J, Reiter A, Steinbauer R, Jakob S, Kuhn CD, Cramer P, Griesenbeck J, Milkereit P, Tschochner H. (2007) Site specific phosphorylation of yeast RNA polymerase I. *Nucleic Acids Res.,* in press. Published online December 15, 2007. 10.1093/nar/gkm1093.

Kuhn CD, Geiger SR, Baumli S, Gartmann M, Gerber J, Jennebach S, Mielke T, Tschochner H, Beckmann R, Cramer P. (2007) Functional architecture of RNA polymerase I. *Cell*, **131**(7):1260-72.

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