EFFECT OF ACTIVE TRANSCRIPTION ON PREREPLICATIVE COMPLEX FORMATION AND ORIGIN FUNCTION IN SACCHAROMYCES CEREVISIAE

Master’s Thesis

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**Abbreviations**

ACS - ARS consensus site

ARS - autonomously replicating sequence

BRE - B recognition element

CDK - cyclin-dependent kinase

CUT - cryptic unstable transcript

DPE - downstream core promoter element

matα - mating type α

matA - mating type A

MCM - mini chromosome maintenance

ncRNA - non-coding RNA

ORC - origin recognition complex

ORF - open reading frame

Pre-RC - pre-replicative complex; pre-replication complex

qPCR - quantitative PCR

RNAP - RNA polymerase

SUT - stable unannotated transcript

TSS – transcription start site

UTR - untranslated region

YPD - yeast peptone dextrose
Introduction

Transcription is a constant process in that doesn’t cease even during hectic replication in S phase. Recent studies have shown that *S. cerevisiae* genome is widely transcribed beyond annotated ORFs and that a vast majority of the genome is transcribed to some extent.

It has been known for over two decades that an active promoter upstream from an origin of replication can effectively hinder its work. Previously it was thought that origins, or ARS-elements, are situated in intergenic regions and are thus able to avoid transcription. However, recently it has been noted that as many as one third of *S. cerevisiae* origins are located in regions that are more or less actively transcribed by newly discovered ncRNAs. With this kind of new information on yeast transcriptome coming to light it is obvious that origins must have some sort of mechanism to counter transcriptional stress.

The objective of this study was to examine the effects of active transcription to origins of replication.

To study the influence of transcription on replication complex formation, I used different ARS-elements that were inserted into a galactose-inducible *GAL-VPS13* gene 3 kb downstream from the transcription start site and monitored the binding of essential pre-RC proteins to these loci by chromatin immunoprecipitation (ChIP).

Results showed that MCM is dislocated from the origin under active transcription but can be reloaded back onto the origin once transcription is turned off. This is a novel discovery and can explain how *S. cerevisiae* is able to maintain a sufficient number of active origins to ensure DNA replication in S phase, even under transcriptional stress.
1 Review of literature

1.1 Introduction to Saccharomyces cerevisiae genome

The genome of budding yeast (Saccharomyces cerevisiae) comprises of 16 chromosomes and is 12,052 kb in length with some 6000 known genes. The sizes of chromosomes in yeast range from 230kb (chromosome I) to 1522kb (chromosome IV) (Feldmann 2005, 1).

Centromeres in S. cerevisiae are fairly short in comparison with other eukaryotes. These are about 200 bp long sequences in the middle of each chromosome responsible for binding to the mitotic and meiotic spindle during the M phase of the cell cycle. In comparison, the evolutionally close relative Schizosaccharomyces pombe has centromeres between 35 and 110 kilobases in length.

The two ends of each chromosome, packed with repeated (TG1-3 or C1-3A) sequences, form the telomeres, each about 300bp in length. Their function is to prevent the ends of chromosomes from fusing and to protect the genetic material in the course of replication. (Feldmann 2005, 2).

1.2 Origins of replication in budding yeast

Origins are the locations in the yeast chromatin from where DNA replication initiates during the S phase of cell cycle. At origins, the multi-protein replication apparatus forms the replication bubble and begins two-directional DNA replication, in which replication forks are sent down both directions of the double stranded DNA. A haploid 12 Mb genome of S. cerevisiae contains approximately 400 unique origins of which 337 have been pinpointed as of April 2010 (yeastgenome.org). Historically these sites are called ARSs (Autonomously Replicating Sequences) because inserting this genomic sequence into a plasmid gave it the ability to autonomously replicate (i.e. if placed into a plasmid that plasmid would have a high chance to carry itself to next generations). Later research confirmed that these regions coincided with the now known sites for replication origins. Today origins are named according to this fashion and the numbers in the nomenclature often refer to the location of the origin within the genome i.e.
ARS609 and ARS1412 are located in chromosomes 6 and 14 respectively.

1.2.1 Structure of origins

*Saccharomyces cerevisiae* is unique because it is the only known eukaryote to have defined sequences that form the origins of replication (reviewed in Sclafani and Holzen 2007).

Until this day, only a handful of individual origins of the total 300-400 have been thoroughly studied and thus it’s not possible to point out a definite structure that makes for an origin. Still, some common characteristics do exist.

All ARS elements share an 11bp long AT-rich ARS consensus sequence (ACS), also called the A-element (Feldmann 2005, 4). The structure of the consensus sequence is as follows: WTTTAYRTTTW, where W is A or T, Y is T or C and R is A or G. Any mutation to the A-element cripples the origin. It is believed that the weaker bonds between A and T nucleotides facilitate chromatin unwinding and help to initiate replication. However, the A-element alone is not adequate for an origin to function. It is estimated that *S. cerevisiae* genome has about 12 000 ACS sequences but only about 300 of those are active (Nieduszynski et al 2006). Also, some origins may have multiple sequences highly similar to the A-element and disabling the origin requires mutating all of these elements (Theis and Newlon 2001). Higher eukaryotes, while lacking a defined origin sequence also seem to show preference to initiate replication at certain sites (reviewed in Masai et al. 2010).

In addition to ACS sequences, all *S. cerevisiae* origins have a number of B-elements that serve a series of functions, perhaps most important being the participation in binding of the origin recognition complex (ORC) to the origin (Rao and Stillman 1995; Lee and Bell 1997; Rowley et al., 1995). The B-elements are a heterogenic group “in terms of structure, number and distance from the A-element” (Weinreich et al. 2004). In addition to A- and B-element, many ARSs also contain a site for the replication enhancer protein Abf1p (ARS binding factor 1) to bind to (Lipford and Bell 2001).
1.3 Process of replication initiation in budding yeast: the assembly of prereplicative complex and the transition to replication

The process of replication in *S. cerevisiae* can be considered to begin long before the actual S-phase takes place. The first preparations for the eventual origin initiation ("firing") in S phase take place already in the change of M/G1 when the levels of cyclin dependent kinases (CDKs) decrease. This allows the formation of the prereplicative complex (Pre-RC), a multi-protein complex needed for the origin initiation, to commence at the sites of replication origins. At this point, the replication origins are already bound with ORC to mark the site for replication. Further down the G1 phase the origins are “licensed” by adding specific proteins to the pre-RC. Licensing is a term given to the process that gives an origin the ability to fire in the following S phase of the cell cycle. By the time cell enters the S phase, most part of the pre-RC is already in place. Entry to the S phase is regulated by CDK activity in the cell (Blow and Dutta 2005).

1.3.1 Replication factors and initiation of replication

Prereplication complex is the basis of replication initiation and most its proteins are highly conserved in all eukaryotes. The complex is assembled on site by a cascade of recruiting reactions started by ORC which is bound to the origin for the whole duration of the cell cycle (Bell and Dutta 2002). The assembly of Pre-RC continues through G1 phase and is finalized just before origin firing. By the time of origin initiation at least 20 different proteins have participated in the formation of pre-RC (Weinreich et al. 2004). The main components of the pre-RC will be discussed here in the order of introduction to the complex. In the introduction, more stress will be put on ORC and MCM as these complexes will be under scrutiny in my work.

1.3.1.1 The Originator, ORC – origin recognition complex

The origin recognition complex (ORC) is a six-subunit (Orc1-6p) complex that is highly conserved among all eukaryotes. Its function is to mark the site of replication origin and to further recruit other factors to the origin. Only origins that are marked with ORC are capable for replication. Unlike other replication factors ORC is bound to DNA through ARS-elements for
whole duration of the cell cycle (Figure 1; reviewed in Gilbert 2001). This is in contrast with most other eukaryotes where ORC is believed to be rotated according to the “ORC cycle” hypothesis (DePamphilis 2003).

![Figure 1. Illustration of an ARS-element with ORC bound to it (from Gilbert 2001).](image)

In addition to origin initiation, ORC is also linked to heterochromatin. Namely, ORC has a binding site for Sir1, a known heterochromatin-forming protein. It is not clear whether ORC recruits Sir1 to form heterochromatin or if heterochromatin recruits ORC to ease the replication of this hard-to-replicate region, different views on the matter exist (Leatherwood and Vas 2003).

ORC also has an important function in regulating the origin activity. While origin sequence is sufficient to keep the region free of nucleosomes, ORC participates in nucleosome positioning near the origins. Correct nucleosome positioning is required for replication factors to achieve their maximum efficiency (Eaton et al. 2010). A positive regulatory role of ORC was indicated in a study examining the nucleosomal arrangement near ARS1. ORC, along with Abf1p, positions the nucleosomes around ARS1 so that the origin remains active and that the initiation is not disturbed by nucleosomes bound near the origin (Lipford and Bell 2001).

1.3.1.1.1 Structure and interaction with DNA

The size of ORC subunits range from 120 kDa of Orc1p, to Orc6p’s 50 kDa. The molecular mass of ORC altogether equals approximately 412 kDa. The six-subunits of ORC form a tube-like structure around the origin DNA which envelop origin sequences (Chen et al. 2008).

ORC requires ATP hydrolyzation to bind origin. Two sites of ORC have the ability to bind ATP but only Orc1p also has the site for Mg$^{2+}$ and thus is able to hydrolyze ATP. Likewise, the ATP-binding activity of just Orc1p subunit is adequate for origin binding (reviewed in Bell and Dutta 2002). While ATP is needed for ORC to function properly, its exact mechanism of interaction with ORC is unknown (Klemm et al. 1997).

When analyzing the area of DNaseI protection, footprint analyses show that ORC
encompasses a 38-44 bp long stretch of DNA (Speck and Stillman 2007). If Cdc6p and WHD (winged helix domain) DNA-binding motifs are included however, the total length of ORC-DNA interaction is believed to stretch over 80 bp (Speck et al. 2005).

WHD is a protein domain that acts as a clamp to bind to DNA. Five of ORC’s six subunits are predicted to carry these domains.

Of the origin elements ORC binds to A1 and a part of B1 (Rao and Stillman 1995). On DNA ORC forms a close complex with Cdc6, which is also predicted to use its WHD to bind DNA (Figure 2; Chen et al. 2008).

![Figure 2](image.png)

**Figure 2.** The proposed structure of ORC and Cdc6p with origin DNA and protein domains for DNA binding. ORC engulfs most of the A-element and also parts of the B1-element of replication origin (Chen et al. 2008).

### 1.3.1.2 Cdc6p and Cdt1p

The first protein summoned by the ORC to the pre-RC complex is Cdc6p (Cell Division Cycle 6). Cdc6p forms a tight complex with ORC and is presumed to also participate in DNA binding (Chen et al. 2007). ORC-Cdc6p-complex then further recruits Cdt1p (Cell division cycle 1). Cdt1p is able to bind DNA nonspecifically but for further pre-RC formation it needs the cooperation of Cdc6p. These two proteins together with ORC are needed to recruit the MCM (mini chromosome maintenance) complex to the pre-RC but apparently not required to keep MCM in place once bound onto the origin (Gillespie et al. 2001; Sivaprasad et al. 2006).
1.3.1.3 MCM - *Mini chromosome maintenance*

While a few other proteins also share the initials Mcm, the most commonly referred are the six proteins Mcm2-7p, which together form the eukaryotic helicase, the MCM complex.

MCM proteins were first discovered when the deletion of some of the proteins led to inability to maintain small circular chromosomes (mini chromosome maintenance) with different ARS-elements in *Saccharomyces cerevisiae* (Maine et al. 1984).

MCM has an important role in initiation and elongation of DNA replication. The complex is loaded onto origin by other pre-RC elements in the G1 phase of the cell cycle. Together ORC, Cdc6p and Cdt1p act as a clamp loader that open the MCM complex and load it onto the DNA (Méndez and Stillman 2003). It functions as the eukaryotic helicase i.e. its purpose is to unwind DNA for the pursuing replicative complex that uses ssDNA as a template.

After its discovery, the MCM has been known to have an important role in eukaryotic replication and for a good while, it has been presumed that MCM has all the characteristics to be dubbed the eukaryotic helicase. However, few have yet dared to call it exactly that directly. In most cases MCM is referred to as the “putative” or “assumed” eukaryotic helicase. For most part this is because for a long time no one had been able to show DNA unwinding activity in vitro. At the time of writing it seems apparent that MCM is the bona fide helicase, while likely working together with different activators giving to its low processivity observed in studies (reviewed in Bochman and Schwacha 2008). The different activators that have been proposed for MCM to be fully active in vivo include Cdc45p and GINS-complex (Moyer et al. 2006).

For a two-way replication to work out, at least two MCM complexes would be needed to be loaded to an origin, one for each direction of the DNA strand. In reality however, it is believed that at least five head-to-head MCM complexes (a total of ten complexes) are loaded on each origin during licensing (Donovan et al. 1997). This is possibly because unlike prokaryotes (Heller and Marians 2006), eukaryotes lack the ability to restart replication fork in case of fork collapse. The extra MCMs would thus act as spares and could rescue the replication fork in case of stagnation. To support this, some preliminary studies have shown that a single MCM molecule has very limited processivity of around 200 bp in vitro (Bochman and Schwacha 2008).

As mentioned earlier, MCM complexes are loaded onto the origin in a head-to-head fashion with their C-terminal ends facing outside. The loading of two MCM complexes seems to
be highly synchronous as double-hexamers have not been observed prior to loading. MCM is loaded on double stranded DNA and origin melting doesn’t commence until MCM is activated in S phase (Remus et al. 2009). Although loaded as a double hexamer, the bond is broken at origin initiation and only single MCM complexes traverse with the replication fork (Reviewed in Forsburg 2004).

1.3.1.3.1 Licensing

The act of binding MCM onto the chromatin in G1 phase of the cell cycle denotes the act of licensing. (Blow and Dutta 2005).

For every proliferating cell to survive, it is crucial that DNA replication is carried out precisely only once per cell cycle. To follow this through, all eukaryotic cells have a mechanism dubbed licensing. Licensing in general means that the loading of origins is temporally separated from the firing of origins. Due to CDK activity, the loading of origins can only take place in G1 phase of the cell cycle. Once the cell has exited G1 phase all loading of origins is ceased. This is to make sure that the origin is not re-loaded after it has fired in the S phase as that would cause DNA to be re-replicated around that origin.

1.3.1.3.2 Structure of MCM and interaction with DNA

MCM consists of six subunits, Mcm2-7, which together form a circular (toroidal) six-subunit complex (Figure 3). MCM complex is highly conserved in all eukaryotes. MCM homologs have also been found in all sequenced archaea (Kelman and Kelman 2003; Kelman and White 2005), while curiously, completely lacking in all sequenced eubacteria.

Compared to ORC MCM seems to be in somewhat relaxed interaction with DNA. Whereas ORC binds to DNA through two of its subunits MCM seems to have no strong bonds with DNA. In vitro experiments show that MCM is able to “slide” freely along the DNA. This also happens even in NTP deprived conditions (Remus et al. 2009). This is notable because under normal circumstances when working as a helicase MCM requires ATP hydrolyzation to move along
the DNA (while at the same time unwinding DNA).

\[ \text{Figure 3. MCM double-helix structure side view (A) and top view (B) showing the head-to-head conformation of the complex as it appears on DNA (from Remus et al. 2009).} \]

1.3.1.4 The monitors of replication: the CDKs

It is clear that in order to ensure that the DNA is replicated only once per cell cycle the licensing reaction has to be strictly controlled. The cyclin dependent kinases (CDKs) have an important and diverse role in making this happen. The level of CDKs remains high from S phase all the way to the end of M phase. Outside the G1 phase CDKs have all of the following activities: inhibition of ORC, degradation of Cdc6 and exportation of Cdt1 and Mcm2-7 from the nucleus. The G1 phase constitutes the only window of opportunity for pre-RC formation and hence, re-replication of DNA is avoided (Blow and Dutta 2005). In the S phase in turn, CDKs have a facilitating role in replication as their activity is required for tight binding of Cdc45p onto the chromatin (Bell and Dutta 2002).

1.3.1.5 The transition to replication, Cdc45

Regarding to origin firing, Cdc45p (Cell Division Cycle 45) is the most decisive addition to the pre-RC complex (at this stage termed preinitiation complex, pre-IC). Soon after the inclusion Cdc45p to the pre-IC it recruits DNA polymerases onto the chromatin and the origin fires. The association of Cdc45p correlates with the initiation time of an origin and late origins attach Cdc45p to the pre-RC complex later than early firing origins (Aparicio et al. 1997). A scheme of the
process of pre-RC formation is pictured in Figure 4.

![Diagram of prereplication complex](image)

**Figure 4.** The assembly of prereplication complex. 

**a.** ORC is bound to the origin for the whole duration of the cell cycle with ATP (yellow star) bound to it. 

**b.** ORC recruits Cdc6 to the complex. 

**c.** and **d.** Origin is licensed when MCM complex, together with Cdt1 are bound onto DNA. 

**e.** Cdt1 is removed from the complex and degraded. 

**g.** Origin initiates after Cdc45 recruits DNA polymerase (both not shown) onto the origin. (modified from Masai et al. 2010).

### 1.4 Transcription in yeast

#### 1.4.1 Yeast RNA polymerases

Yeast utilizes three different RNA polymerases in transcription. RNA polymerases I and III mostly account for the transcription of tRNA and rRNA genes, while the 12-subunit RNAPII is responsible for the transcription of most part of *S. cerevisiae* DNA. In addition to transcribing ORFs and many annotated regulatory RNAs, recently RNAPII has been shown to be responsible for many short unannotated RNAs (Neil et al. 2009; Xu et al 2009). These ncRNAs are of a great interest in regards to yeast replication since they likely play a major role in transcriptional
interference experienced by replication origins.

1.4.2 Constant pervasive transcription in yeast

The classical view of transcription implies that only ORFs are transcribed in genomes while the rest of the genome is transcriptionally silent and has regulatory functions at best. In recent years it has been shown however, that transcriptional activity extends far beyond the traditional coding sites and many intergenic regions that were previously considered silent are actually transcribed (Wyers et al. 2005). Many of the non-ORF transcriptional sites can be attributed to known non-coding RNAs, but as one fairly recent article studying yeast transcriptome put it: “even this well studied genome has transcriptional complexity far beyond current annotation” (David et al. 2006). However, many articles have recently updated the conception of yeast transcriptome to the extent where ncRNAs are considered a fundamental group of RNAPII transcripts (Wyers et al. 2005).

1.4.3 Yeast promoters are inherently bidirectional

When studying yeast transcriptome many recent studies observed an increased and unexpected transcription away from annotated ORFs or ncRNAs. Later it was shown that these transcripts originate from particular regions of the genome rather than resulting from general transcriptional ‘noise’. These regions of active transcription coincided with the loci of known promoter regions, but surprisingly originated upstream from the promoters (Neil et al. 2009; Xu et al., 2009).

These newly discovered non-coding RNAs are divided into two classes: stable unannotated transcripts (SUTs) and cryptic unstable transcripts (CUTs). Both mainly originate from promoter regions, normally about 200nt upstream from the TSS (Neil et al. 2009). As their names imply, CUTs are quickly degraded while SUTs have a longer life span. Also their average size differs CUTs being somewhat shorter that SUTs median lengths being 440 and 761 bases respectively (Xu et al. 2009).
The function of these newly discovered transcripts is still unknown although it has been shown that CUTs can potentially interfere with the 5’ untranslated regions (UTR) at the start of genes, and thus inhibit transcription. Thus, it is possible that CUTs hold a self-regulatory role in cells but to some extent they might just be transcriptional noise originating from nucleosomes free regions (Neil et al. 2009; Xu et al. 2009).

While it is still no completely clear of how big a part ncRNAs form of the total transcriptome, in a compact genome as that of *S. cerevisiae*, SUTs and CUTs among others might form a substantial part of the total transcriptional stress experienced by origins. The compact nature of the yeast genome and the widespread transcription of non-coding RNA might indicate that a large proportion of origins might be actively transcribed. In fact, the data analyzed from CUT data from Neil et al. revealed that at least one third of all origins are under transcriptional stress (Lõoke et al. unpublished data). Taking into account the difficulties of identifying ncRNAs (degradation, low levels of transcription) it is likely that this number is still an underestimate.

1.4.4 Effect of transcription on replication

Active transcription affects replication initiation in two distinct ways. Firstly, both transcription and replication require an area that is cleared of nucleosomes for corresponding factors to access DNA. This way active transcription may facilitate origin firing by clearing the DNA strand of nucleosomes. On the other hand, transcription and replication complexes both need to be in close contact with DNA to fulfill their functions. Thus, the replication and transcription machineries are in constant competition over free strands of DNA.

It has been observed in mammals that regions, which are actively transcribed are also replicated early in the S phase (Hatton et al. 1988). This is likely because active transcription keeps the strand clear of nucleosomes which might deter any replication factors. In yeast however, no such correlation has been found. On the other hand, ARS-elements are typically situated in intergenic or subtelomeric (Y’ and X elements) regions of the chromosome, which would suggest that origins prefer loci that see less active transcription. (Wyrick et al. 2001).

To comply with the fact that origins tend to locate in transcriptionally quiet loci, a recent study showed that active transcription on the site of origin can completely cripple its activity in *S. cerevisiae*. When studying ARS605, an active origin under normal conditions, it was noticed
that it loses all of it origin activity in meiosis. This is because ARS605 lies within the ORF of MSH4, a gene that is only active during meiosis and the activated transcription completely abolished ARS605’s origin activity (Mori and Shirahige 2006).

While most origins tend to locate away from the sites that see active transcription, studies have shown that even intergenic regions are not safe from transcription. Because a major part of origins experience transcriptional stress, from time to time it is essential for origins to have mechanism to counter transcriptional stress.

With the knowledge that the yeast transcriptome contains a substantial share of the genome including a large number of origins, the objective of my study was to find out how cells cope with transcriptional stress. Further, I also wanted to confirm the underlying causes of replication inhibition that had previously been presented in literature.
2 Materials and methods

Growing media and solutions.

5-FOA (5-Fluoroorotic Acid) plates. The existence of URA3 selection marker was controlled on FOA-5 plates that contained (final concentrations), ammonium sulfate 5.0 g yeast nitrogen base without amino acid 1.7 g, Dextrose 20 g and 5-FOA 1.0 g.

FA500. Hepes-KOH pH 7.5 50 mM, NaCl 500 mM, EDTA 1 mM, Triton X-100 1%, Na deoxycholate 0.1%.

FA-lysis buffer. 50 mM HEPES [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and a mix of protease inhibitors [1 mM PMSF (phenylmethanesulphonyl fluoride), 1 μg/ml leupeptine, 1 μg/ml pepstatine]).

His plates. Plates for HIS gene selection contained [YNB (Yeast nitrogen base, Applichem) 6.7 g/l, glucose 2% (Medipharmia), agar 2% (LAB MTM), amino acids and nucleotides – Adenine 40 μl/ml, Uracil 40 μl/ml, Leucin 80 μl/ml, Tryptophane 40 μl/ml, Lysine 80 μl/ml (Serva)].

LiCl. Tris-HCl, pH 8 10 mM, LiCl 250 mM, NP-40 0.5%, Na deoxycholate 0.5%, EDTA 1 mM.

PBS (phosphate buffer solution). pH 7.4; NaCl 8 g/l, KCl 2.7 g/l, Na₂HPO₄ 1.44 g/l, KH₂PO₄ 1.76 g/l,

Sporulation plate (SPA). Contents (final concentrations), Difco yeast extract, potassium acetate 10 g/l, glucose 0.5 g/l, agar 20 g/l + nucleotides and amino acids (adenine 10 mg/l, uracil 10 mg/l, L-histidine 10 mg/l, L-tryptophan 10 mg/l, L-leucine 20 mg/l)

TES. Tris-HCl, pH 7.5 10 mM, EDTA 1 mM, NaCl 100 mM.

YPD (Yeast extract, Peptone, Dextrose). YPD medium contained (final concentrations), Difco yeast extract 10 g/l, Difco peptone 20 g/l, glucose/galactose/raffinose 20 g/l, (for plates agar 20 g/l).

Yeast strains. All S. cerevisiae strains were congenic with strain W303 (Thomas and Rothstein 1989). To create GAL-VPS13-ARS loci, the ARS sequences were inserted into the coding region of the GAL-VPS13 (Kristjuhan and Svejstrup 2004) 3-kb downstream from the beginning of VPS13 coding sequence. The inserted chromosomal positions of amplified ARS sequences were as follows:
ARS605 (Chr6:135943-136180); ARS607 (199329-199515); ARS609 (Chr6:256257-256446); ARS409 (Chr4:212368-212673) (Saccharomyces Genome Database). A C-terminal triple E4-tag was fused to ORC2 or MCM4 loci in W303 and the resulting strains were crossbred with the set of GAL-VPS13-ARS strains to obtain a panel GAL-VPS13-ARS strains with E4-tag added to ORC2 or MCM4 loci. For efficient α-factor arrest, the BAR1 gene was also deleted in some of those strains. For reference, E4-tagged ORC2 strain with no ARS insertion in GAL-VPS13 gene was also made.

**Electroporation.** Strains were grown in 25ml YPD medium overnight and grown to a (late-log) density of 1×10^8 per ml and cells were collected with centrifugation (1500 rpm, 5 min, Sigma 4K 15C, rotor 272/F) and suspended in 10ml TE buffer with an added 1ml 1M LiAc and incubated for 45 min at 30°C. 250 µl of 1M DTT was added and the cells were incubated for another 15 min. 40 ml of H2O was added and cells were collected with centrifugation (1500 rpm, 5 min). The cells were then washed with cold H2O and centrifuged (1500 rpm, 5 min) twice. Cells were suspended with 1 ml cold 1M sorbitol and centrifuged (6000 rpm, 1 min) twice. Electroporation was carried out in 2 mm cuvettes with 40 ml of cell suspension and 5 µl of DNA. *Bio Rad GenePulser Xcell* was used for electroporation with following parameters, C = 25 µF; PC = 200Ω; V = 1500 V. After electroporation 1 ml of 1M sorbitol was added and cells were plated on YPD plates for 24 hours and then stamped to either 5-FOA or -His plates to control fragment insertion.

**Tetrad dissection.** Haploid cells were plated on -His-Trp plates crosswise to select for diploid cells with both HIS and TRP inserts. Diploid cells were grown on sporulation plates to induce the formation of haploid spores. The tetrads from sporulation plates were dissected using Singer Instruments MSM Manual dissection microscope.

**ChIP assay.** Cells were grown in yeast extract-peptone (YP) medium containing 2% glucose, galactose or rafinose as a carbon source to the density of about 7×10^6 per ml. For cell cycle arrest experiments, α-factor mating pheromone (Zymo Research) was used with the final concentration of 5 µM and cell cycle status during the experiment was confirmed by fluorescence-activated cell sorting (FACS) analysis and visually by light microscopy. Cells were then fixed in 1% formaldehyde for 10 min at room temperature before adding glycine at 200mM. Cells were washed with PBS and centrifuged twice (5 min, 1500 rpm, Sigma 4K 15C, rotor 272/F) and lysed in FA-
lysis buffer (with protease inhibitors) with glass beads (0.5 mm Glass Beads (Soda Lime) BioSpec Products Inc.) at 4°C for 15 minutes using Disruptor Genie\textsuperscript{TM} (Scientific Industries). Lysate was then collected by breaching 1.5 ml Eppendorf tubes and placing them inside 2 ml Eppendorf tubes and centrifuging them for 1 min 5000 rpm (Eppendorf Centrifuge 5415R, rotor F45-24-11). 1 ml of FA-lysis was then added to the lysate and transferred to 15 ml tubes for electroporation. Chromatin was then fragmented by extensive sonication (Diagenode – Bioruptor\textsuperscript{TM}) 3 min (6 x 30 sec sonication, 1 min pause, 200 W, at 0˚ C). The lysate was then transferred to Eppendorf 1.5 tubes and centrifuged for 5 minutes at 13200 rpm at 4°C (Eppendorf Centrifuge 5415R, rotor F45-24-11). Supernatant was then transferred to a new similar tube and the centrifuging was repeated for 15 minutes at 4°C. This supernatant from now on will be called WCE (whole cell extract). 500 µl of WCE was then moved to 1.5 ml tubes and incubated with 1 µl (1 mg/ml, Quattromed) antibodies directed against Mcm4p or Orc2p anti-E4 tag (1E2; Icosagen) overnight at 4°C. Samples were then centrifuged for 5 and 15 min (Eppendorf Centrifuge 5415R, rotor F45-24-11) at 4°C transferring the supernatant to a new tube after each time. Protein A beads [ProteinA Sepharose\textsuperscript{TM} Fast Flow (GE Healthcare Bio – Sciences AB)] were incubated for 15 minutes with BSA [BSA (Bovine serum albumine, BioTop, final concentration 1 mg/ml) + FA-lysis] at 4° C. 15 µl of the incubated beadsand 30 µl of buffer (BSA+FA-lysis) was added to samples and incubated 1.5 hours at 4° C. Samples were washed consecutively with 1 ml of FA-lysis, FA500, LiCl and TES each for 5 min at 4° C and centrifuged after each wash for 1 min at 6000 rpm at 4˚ C (Eppendorf Centrifuge 5415R, rotor F45-24-11) after what the supernatant was extracted. 200 µl of H\textsubscript{2}O was then added with 1 µl ProteinaseK (18.7 mg/ml, Fermentas) and was incubated at 65° C for 8 hours. DNA was purified with phenol-chloroform treatment. 400 µl of phenol-chloroform (1:1) was added and the sample was mixed with vortex and centrifuged for 5 min at RT at 13200 rpm (Eppendorf Centrifuge 5415R, rotor F45-24-11). Water phase was transferred to a new tube and the procedure was repeated, this time with 500 µl of chloroform. Water phase was then transferred to a 2 ml Eppendorf tube and the DNA was precipitated using 50 µl NaAc (3 M), 1 µl glycogen (20 mg/ml) and 1.5 ml of 96% EtOH. Samples were placed at -20˚C overnight. Samples were then centrifuged at 4° C for 15 min (13200 rpm; Eppendorf Centrifuge 5415R, rotor F45-24-11). DNA was washed with 500 µl of 70% EtOH and centrifuged again at 4° C for 15 min (13200 rpm; Eppendorf Centrifuge 5415R, rotor F45-24-11). DNA was dissolved in 40 µl of H\textsubscript{2}O. 1 µl of DNA was used with qPCR. Coprecipitated DNA was analyzed by quantitative real-time PCR using an ABI Prism 7900HT real-time PCR system under standard conditions (40 cycles; 95° C for 15 s and 60° C for 1 min). 5x Hot FIREPol EvaGreen qPCR mix (Solis BioDyne) was used. PCRs were done with primer pairs covering coding region of VPS13 gene (3-kb), ARS106, ARS609, ARS409 and the FBA1 gene for
normalization. Results were analyzed with programs SDS (Sequence Detection Systems) version 2.2.2 and Microsoft Excel.

**Primers.** Following primer sets were used in the study.

*FBA1*: F 5’-TTGCACCCCAATCTCTCCTCAAAACTT R 5’-ATTTCTGGTCTCAAAGCGATGTCA

*VPS13 3k*: F 5’-TGATTCTATAAGCTGGCAAC R 5’-CTAAATACCGAATCCCTGGAA

*ARS609*: F 5’-CCGATCTTTGTCTGGAGGATCA R 3’-TGCCGAGAGCTGACAATTTAGT

*ARS409*: F 5’-CCCATTCTTGACTGAGGTACA R 5’-GACTTACTTTGTTGTAAGGGCTTCA

*ARS106*: F 5’-GGCCGACTTGCCATAATATCA R 5’-TCAAAAGCCGAAAAAGGAGTT

**Flow cytometry.** For flow cytometry analysis 0.5 ml of yeast culture was fixed in 10 ml of 70% ethanol overnight at 4°C and stained with with 20 mg/ml propidium iodide in the presence of 50 mg/ml of RNaseH for 30 min at room temperature in the dark and washed afterwards in PBS. Cells were subsequently analyzed using a FACS Calibur flow cytometer.
3 Results

3.1 Construction of the yeast strains used

To study the effect of transcription on replication I needed to build a gene construct in which transcription could be induced through an ARS-element. To be able to control transcription a \textit{GAL10} promoter was also included upstream from the construct (Figure 5). This promoter in its natural context participates in galactose metabolism and can be activated by the introduction of galactose to medium. This way it was possible to turn transcription on and off and to study its effect on protein binding and pre-RC formation.

![Figure 5](image.png)

\textbf{Figure 5.} The construct of the \textit{GAL-VPS13-ARS} gene. An ARS element of interest (the black rectangle) was inserted inside the \textit{VPS13} gene. Upstream of the \textit{VPS13-ARS} gene a \textit{GAL10} promoter is placed to control transcription. A primer situated 3 kb from transcription start site was used to evaluate protein interactions with qPCR.

3.1.1 Insertion of ARS605 into \textit{GAL-VPS13}

To study ORC binding to the gene construct I used a strain which had all the above-mentioned properties. To engineer the planned gene construct an ARS605 origin was first amplified from genomic DNA using PCR and these fragments were then introduced to the designated strain using electroporation. Primers used to amplify the ARS-element also contained 50nt long sequences which were homologous to fractions of the native \textit{VPS13} gene so that ARS605 would assemble to the construct as shown in Figure 5. Thus, when electroporated with the cells, some of the ARS fragments were expected to enter the native \textit{VPS13} through homologous recombination. For selection purposes, the strains used for inserting the fragments also contained a \textit{URA3} inside the
VPS13 gene. The 50nt long sequences were designed so that after homologous recombination URA3 was to be replaced by ARS605.

The electroporated cells were then plated on YPD plates for 24 hours and after that stamped onto 5-FOA plates. URA3 gene product converts 5-FOA to 5-fluorouracil, a compound lethal to the cell. Thus, only cells where homologous recombination has taken place are able to grow on 5-FOA plates. However, a spontaneous mutation of the URA3 gene can produce false positive colonies where 5-FOA is not degraded although recombination has not occurred. For this reason the colonies were also controlled using PCR to verify the existence of the insertion. Out of six colonies that were able to grow on 5-FOA plates, PCR confirmed the insertion of ARS605 in five colonies (Figure 6.)

Of the five controlled positive colonies the cells represented on lane 1 were chosen to continue with the gene construction.

![Figure 6](image)

**Figure 6.** A picture of a gel electrophoresis of PCR-amplified fragments from colonies grown on FOA-5 plates. Colonies from the plates were examined for ARS insertion inside the VPS13 gene. Lanes 1-5 show bands indicating successful insertions of the ARS fragments inside the VPS13 gene. The lane 6 showing a longer fragment size is negative indicating an apparently spontaneously mutated URA3 gene.

Previous ChIP experiments in our group with the GAL-VPS13-ARS gene construct observing RNAPII location on the gene had previously confirmed that transcription runs through the gene and is not interrupted by the ARS-element insertion (Lõoke et al. unpublished data).
3.1.2 Tagging of Orc2p

To be able to analyze the binding of ORC to the origin an epitope tag first needed to be added to Orc2p. An E4 tag was inserted on the C-terminal end of the protein for antibody targeting.

This time a mat α strain (for later crossing with mat A) was used for adding an epitope tag to Orc2p. To insert the tag the natural stop codon of ORC2 was replaced with an E4 tag together with a new stop codon. The inserted fragment also contained a HIS3 marker gene for selection. After cells had been electroporated with the fragment, the colonies from -His plate were controlled for tag insertion with PCR. Primers were designed so that the reverse primer was homologous with a strand downstream from ORC2 and the forward primer with the electroporated fragment. Together these primers would produce a 500bp fragment that could be detected with PCR. Of the six colonies tested, three were confirmed to have the tag insertion in PCR (Figure 7). Colony represented on lane 1 was chosen for further strain construction.

![PCR gel electrophoresis](image)

**Figure 7.** A picture of gel electrophoresis of PCR-amplified fragments showing the 500bp long fragment signifying the insertion of the E4 tag to ORC2 gene (lanes 1-3) or lack of it (lanes 4-6).

At this point, two different strains had been constructed: one with the origin inserted to the VPS13 gene and one with a tagged Orc2p. To get a strain which would have both of these properties, the GAL-VPS13-ARS605 gene and a tagged Orc2p, these two strains had to be combined. For this purpose, the two strains were of a different mating type, one being A and the other being α. These two strains were then mated to achieve a diploid cell with both characteristics. Diploid cells were sporulated and the haploid spores were selected for TRP1 (added for GAL-VPS13 selection earlier) and HIS3 markers so that they would carry a copy of each of the inserts.
3.2 ORC binds to replication origins at the GAL-VPS13-ARS constructs

ORC binding is the initial step of pre-RC assembly and therefore of utmost importance for origin function. For this reason, it was important to find out whether ORC is able to mark the origins that were inserted into the VPS13 gene. To do this, I used the GAL-VPS13-ARS605 strain together with three other similarly constructed strains. These four strains were grown in YPD medium with glucose as carbon source. Glucose was used to repress the GAL promoter and thus inhibit transcription. All strains carried a different ARS element inside the GAL-VPS13-ARS construct. After being grown to desired density, ChIP (chromatin immunoprecipitation) assay was used to evaluate ORC binding. All the strains analyzed showed a 3 to 9 fold increase in ORC binding at the GAL-VPS13-ARS construct in comparison with the reference region. The same cell extracts used for measuring ORC binding at inserted ARS sequences were also used for reference to measure the ORC binding on natural ARS106 origin within different strains. These natural origins showed patterns of ORC binding similar to origins in the GAL-VPS13-ARS construct (Figure 8). The FBA1 gene was used to set the background level since no replication factors were expected to be found there.

![ChIP Orc2p binding](image-url)

**Figure 8.** ChIP determined Orc2p binding levels on the GAL-VPS13-ARS construct (blue bars) and within a natural origin ARS106 (green bars) in different strains used. Samples from same lysates are placed next to each other. The Y-axis shows the fold increase in Orc2p on GAL-VPS13-ARS compared to the level of Orc2p found on FBA1 gene. The X-axis denotes the inserted ARS-element (blue bars) or the natural unmodified ARS106 within the same strains.
This test proved that the different ARSs placed inside the GAL-VPS13-ARS construct are able to bind ORC and thus are viable for origin marking. No major difference in ORC binding was observed between natural origins and ARSs placed inside the gene construct (Figure 8).

3.3 Replication origins are licensed in GAL-VPS13-ARS context

The addition of MCM to origins is of critical importance as it enables origins to fire in the ensuing S phase. Licensing takes place strictly in G1 phase only and unlicensed origins are nonfunctional. After confirming that origins were marked by ORC it was equally important to study whether the GAL-VPS13-ARS construct could also recruit MCM complex to the origin.

Two strains, with ARS609 and ARS409 inserts, were chosen to examine if the inserted origins could bind MCM. These two strains were similar to the strains from previous experiment, only difference being that Mcm4p was tagged instead of Orc2p in order to study the binding of MCM complex to the origin.

For this purpose, the strains were grown with glucose as carbon source to inhibit transcription. The ChIP assay revealed slightly lower levels of MCM binding compared to that of ORC. However, the levels were still over two fold higher than those of the reference region FBA1, which confirms that licensing at the GAL-VPS13-ARS construct has taken place. Again, MCM binding at natural origins ARS409 and ARS609 was also studied for reference. The natural origins showed somewhat higher levels of MCM binding compared to GAL-VPS13-ARS (Figure 9). Although the levels of MCM were a little higher at natural origins compared to the constructed GAL-VPS13-ARS gene, the MCM levels on the gene construct compellingly showed that MCM is recruited to these origins as well.

The next important question was if MCM would stay in place after transcription is turned on within the GAL-VPS13-ARS constructs. To examine this, cells were grown in a medium containing galactose, which activates the GAL promoter and induces transcription. Again, the level of Mcm4p was observed within the GAL-VPS13-ARS gene and a natural ARS409 origin and compared to the background level of FBA1.

After inducing transcription through the gene construct, the levels of Mcm4p dropped significantly and fell to the level of the reference region FBA1 on both strains (Figure 9). This
clearly indicates that MCM complexes which had prior been bound to the origins were removed by the activated transcription.

As expected, no decline in Mcm4p binding levels was observed with the natural ARSs where the conditions remained unchanged after the introduction of galactose. The rise in Mcm4p binding levels in galactose compared to those in glucose, may partly be due to the fact that cells grow more slowly in galactose and thus a larger proportion of them reside in G1 phase where licensing takes place.

![Mcm4p ChIP](Figure 9) ChIP determined Mcm4p binding levels to GAL-VPS3k-ARS609, GAL-VPS3k-ARS409 and to genuine ARS609 and ARS409 loci. Cells were grown overnight in glucose or galactose containing media.

3.4 Relicensing of origins after transcription inhibition

Last experiment confirmed that MCM is removed from origins in the GAL-VPS13-ARS gene construct under galactose-induced transcription. As it has been shown that a major part of origins are under transcriptional stress, I went on to test if MCM could be brought back to the origin once it has been dislocated. To do this, a strain with a GAL-VPS13-ARS609 insert and a tagged
Mcm4p was grown in three different media each containing a different sugar. All media except for the initial raffinose also contained α-factor, a yeast pheromone that prevents mat A cells from entering S phase. Alpha-factor had to be included because MCM binding (i.e. licensing) only occurs in G1 phase and also to make sure that origins are given the chance to be relicensed instead of going through another round of cell cycle to be licensed ‘normally’ in the next G1 phase. Cells were first grown in a medium containing raffinose overnight to achieve desired density. After that, the cells were incubated in raffinose for another 2 hours and α-factor was added for G1 arrest. Secondly, the cells were transferred to galactose-containing medium for 2 hours to activate the GAL-VPS13 promoter and induce transcription. Lastly, the cells were put to grow in a glucose-containing medium for 1 hour to repress transcription and to see whether MCM would be re-bound to the origin after transcription is turned off. For the whole duration of the experiment cells were arrested with α-factor which prevented them from entering S phase. An illustrative picture of the growing procedure can be found in Figure 10A. The G1 arrest induced by α-factor was also verified with flow cytometry (Figure 11A) and visually with light microscopy.

After each of the three stages (raffinose, galactose and glucose) a sample was taken from the culture to undergo ChIP assay. Similarly to previous experiment, the levels of MCM binding dropped considerably inside the GAL-VPS13-ARS609 after the induction of transcription with galactose. The levels of binding declined more than 60% after activation of transcription compared to initial levels of incidence. However, after transcription was turned back off with the introduction of glucose the levels quickly rose back to their original states. As expected, the natural origin ARS609 used as reference showed no discrepancy between different sugars used (Figure 10B). The reappearance of Mcm4p confirmed that MCM complexes can be rebound to GAL-VPS13-ARS complexes once transcription is turned off.
Figure 10. (A) The procedure of growing cells in media with different carbon sources. (B) ChIP assay used to measure Mcm4p binding in GAL-VPS13-ARS609 construct after being grown consecutively in three different media for different periods of time: raffinose (grown for 2 hours), galactose (2 hours) and glucose (1 hour), in that order. The Y-axis shows the percentage of the level of Mcm4p binding in the initial medium (raffinose).
Figure 11. (A) Cells arrested in G1 phase with α-factor. Cells had been grown consecutively in media containing (from left to right) raffinose, galactose and glucose. The cells form only one peak populating the G1 phase and thus form a synchronous population. (B) For reference, S. cerevisiae cells under normal asynchronous conditions with uninhibited cell cycles form two peaks indicating the G1 and G2 phases with a higher peak at the G2 phase.
Discussion

The licensing of replication origins takes place in G1 phase of the cell cycle. After licensing has been carried out the origins stand by for replication initiation in the ensuing S phase. During this period from licensing to origin initiation they are especially vulnerable to traversing transcription bubbles. This study gives the first overview of the dynamics of origin licensing under transcriptional stress.

Most of the origins in budding yeast tend to locate in more transcriptionally quiet neighborhoods on intergenic regions of the chromosomes. However, in spite of this, most of them likely have to endure a degree of transcriptional stress as recent studies have shown that the transcription of non-coding regions is widespread in yeast (David et al. 2006; Neil et al. 2009; Xu et al. 2009). In fact, the CUT data analyzed from Neil et al. revealed that at least one third of all yeast origins are regularly transcribed (Lõoke et al. submitted for publication). Considering that these newly found ncRNAs are extremely hard to track down due to their short lifespan and low levels of expression, it is likely that the number of origins that are transcribed is considerably higher in reality. For this reason it is important to understand the origins’ mechanisms of countering transcriptional stress.

The discovery that transcription disrupts pre-RC formation at origin is well in line with earlier studies on the subject (Donato et al. 2006; Mori and Shirahige 2007, Snyder et al. 1988). Although this study concentrated on MCM it has been found that transcriptional interference also removes ORC from the origin, thus preventing the addition of MCM to pre-RC (Mori and Shirahige, 2007). Since both ORC and MCM are both components of pre-RC this study effectively complements my findings on MCM removal. This work is also a part of a study from our group that has been submitted for publishing.

Distinctive from previous studies, this is the first time to prove that S.cerevisiae cells have the ability to relicense origins after transcriptional stress. Further experiments conducted in our group have also confirmed that the process of relicensing is relatively swift the initial level of MCM being restored within ca. 30 minutes. The origins that have been relicensed accordingly are also able to initiate replication after being released to S phase (Lõoke et al. submitted for publication).

The interrelationship between transcription and replication is a complex one. It has been compellingly shown that direct transcription through origins greatly impairs their function. For this reason all eukaryotes have excess potential in terms of origin numbers to be able to carry out
replication despite a degree of transcriptional inactivation of origins. However, the chromatin remodeling associated with facilitating transcription has also been shown to assist replication in a similar fashion. It is possible that replication factors make use of the open euchromatin associated with transcription. The drawback would in this case be the dislocation of pre-RC components from the origins but as this study has shown this can be readily overcome with the relicensing of origins.

The results from our group together with recently published articles suggest that genome-wide transcription is prevalent in yeast and that a major part of replication origins have to endure significant transcriptional stress. My studies however, show that origins have the measures to overcome this stress by relicensing of origins once transcription has paused. This is likely the key mechanism that cells utilize to ensure that sufficient number of origins remain active for a successful completion of S phase.
Effect of active transcription on prereplicative complex formation and origin function in *Saccharomyces cerevisiae*

Lari Järvinen

Summary

Recent studies have shown that non-specific genome-wide replication is widespread in yeast. As replication origins are known to be inactivated by transcription, this suggests that origins need to have a method to counter the transcriptional stress posed by traversing transcription bubbles. The purpose of the present study was to examine the effects of transcription on pre-RC formation and origins’ ability to withstand transcriptional stress.

In this study I examined the responses of yeast origins to transcriptional stress by creating a gene construct in which different ARS-elements could be inserted. To be able to control transcription on the origin an inducible promoter was also placed in front of the gene. I also used two essential pre-RC proteins, Orc2p and Mcm4p, that were modified to include an epitope tag. This allowed me to study the binding of ORC and MCM complexes to replication origins under active or repressed transcription.

My results confirm earlier findings that RNAPII-dependent transcription inhibits the assembly of prereplicative complex at *S. cerevisiae* origins. My studies also show that active transcription at replication origins removes the MCM complex fundamental to origin function. This is well in line with earlier studies on transcriptional origin inhibition. The unique observation in this work however, is that this transcriptional inhibition is reversible. As soon as transcription is turned off origins can quickly be relicensed by the reintroduction of MCM. Relicensing takes place in the same G1 phase where MCM was removed and no additional round of cell cycle is needed. This process of relicensing is of significant importance because it is likely one of the vital mechanisms that allow cells to pass through S phase with a sufficient number of functional origins.
References


Internet resources used in the paper:

Saccharomyces Genome Database, http://www.yeastgenome.org/
Aktiivse transkriptsiooni mõju prereplikatiivse kompleksi moodustumisele ja origini funktsioonile pärmis *Saccharomyces cerevisiae*

Lari Järvinen

Kokkuvõte


Hiljutised uuringud on näidanud, et varem tuvastamata jäänud ncRNA (mittekodeeriv RNA) vormid, CUT ja SUT, on laidadelt levinud pärmi transkriptoomi osad. Meie töögrupis läbiviidud uuringutes leiti, et rohkem kui üks kolmandik pärmi originidest peab taluma CUT-ide poolt põhjustatud transkriptsioonilist stressi (Lõoke et al. saadetud avaldamisele). Kuna CUT-id on raskesti tuvastatavad oma lühikese eluea ning väikse transkriptsioonitaseme tõttu, on üsna tõenäoline, et põhiosa pärmi originidest peab taluma transkriptsioonilist stressi.

Käesoleva töö eesmärk oli selgitada mehhanisme, mis põhjustavad originide inaktivatsiooni transkriptsiooni poolt, ning uurida originide vastust transkriptsioonilisele stressile. Selleks sisestasin GAL10 promootori kontrolli all oleva mudeleeni *VPS13* kodeerivasse alasse homoloogilist rekombinatsiooni kasutades erinevaid pärmi originide järjestusi. Et uurida pre-RC
komponentide seondumist originile, olid rakus epitoobiga märgistatud kaks valku - Orc2p ning Mcm4p. Analüüsides nende valkude lokalisatsiooni oli mul võimalik kontrollida oluliste pre-RC komponentide, ORC- ning MCM-komplekside, originile seondumise dünamiikat sõltuvalt transkriptsioonist.

Minu tulemused kinnitasid eelnevaid kirjanduses esitatud andmeid, et aktiivne transkriptsioon eemaldab pre-RC komponendid originidelt. Esimest korda kui näidati minu katsetes seda, et pärast MCM kompleksi eemaldamist originilt, on see võimalik originile tagasi laadida ilma rakutsüklist uuesti läbimata. Seda protsessi nimetatakse origini uuesti litsentseeerimiseks (relicensing). Lisaks on teised meie töögrupis tehtud katsed näidanud, et uuesti laadimise protsess on üsna kiire (ca 30 minutit) ning nõnda uuesti litsentseeeritud originid on võimalised replikatsiooniks rakutsüklist S-faasis (Lõoke et al. saadetud avaldamisele).

Meie töögrupi katsed koos hiljuti kirjanduses avaldatud katsete tulemustega viitavad sellele, et transkriptsioon on pärimis laialdane nähtus kogu genoomi ulatuses. See aga seab nõudeid originide valmisolekule transkriptsioonilisest stressist ülesaamisel. Käesolev töö näitab, et pärmi originid suudavad transkriptsioonist tuleneva stressiga hakkama saada efektiivse originide relitsentseeerimise kaudu.