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Hereby I assure that this thesis has been written autonomously and that no sources other than the ones indicated have been used. This work has not been part of another examination procedure; other attempts to obtain a PhD have not been undertaken.

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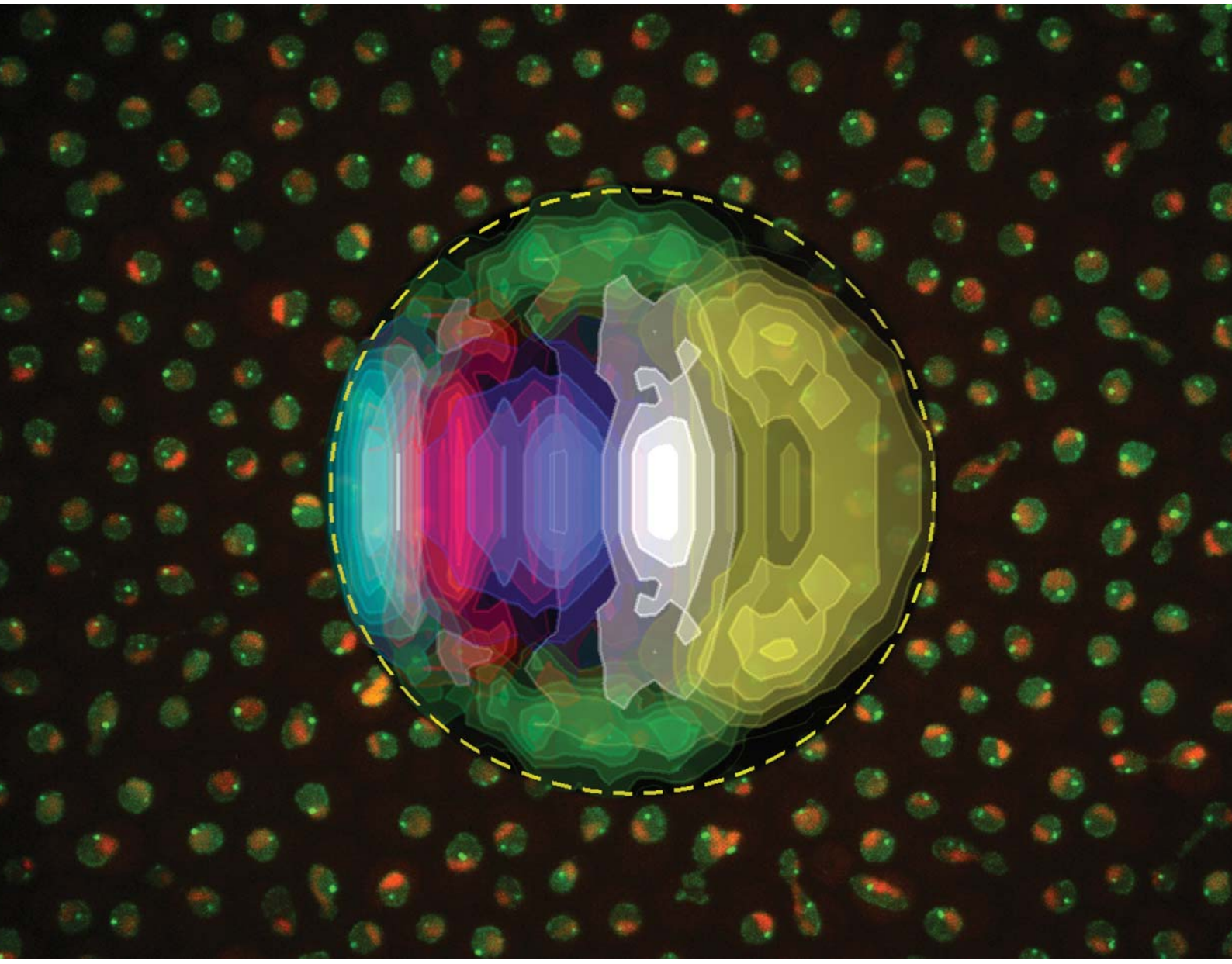

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SUMMARY

Chromatin is distributed non-randomly within the cell nucleus. Its spatial organization has been demonstrated to be important for nuclear metabolism such as, DNA replication, reparation or transcription. However, little is known about the functional implications resulting from its organization or the molecular driving forces responsible for chromatin organization.

In the course of this thesis, I studied the budding yeast HMG-box protein Hmo1. An initial screen demonstrated that this chromatin-associated protein is genetically linked to the RNA polymerase (Pol) I, to genes coding for ribosomal proteins (RPGs) as well as to genes implicated in stress response. I could show that Hmo1 physically interacts with the rRNA coding gene transcribed by Pol I and with a subset of RPG promoters. Global expression analyses showed a clear dependence on Hmo1 for the expression of a sub-set of RPGs. An *hmo1* deletion strain is also largely alleviated in repressing RPG transcription after TOR complex 1 inhibition. These results suggested that Hmo1 is implicated in Pol I transcription as well as RPG regulation. Preliminary *in vitro* transcription assays suggest a role of Hmo1 in Pol I initiation and elongation events.

Since Hmo1 is a *bona fide* nucleolar factor, I wanted to test the hypothesis if Pol II transcribed RPGs associated with Hmo1 are localized in the proximity of the nucleolus, as previously reported for Pol III encoded tRNA genes. Due to the small size of the yeast nucleus and the stochastic, sub-diffusive movement of DNA, we first needed to develop a new method allowing determination of gene localization probabilities with very high accuracy and with respect to the nucleolus. As a result of this collaborative approach, we could demonstrate by analyzing thousands of cells, that genes are confined into sub-nuclear volumes. These 'gene territories' show a locus specific size and can be remodeled upon transcriptional activation. Applying this new method to Pol II transcribed genes required for ribosome biogenesis, such as the RPGs, indicates that the localization of the gene on the chromatin fiber has important implications for its three dimensional positioning. Genes in proximity to the centromere localize in front of the spindle pole body while RPGs further away from the extremities of the chromosome arm can also occupy a nucleolar-close territory. Furthermore, it also seems that neighboring genes are important positioning determinants.

All together, these results show that Hmo1 participates in mediating Pol I and RPG transcription regulation in response to growth conditions and that this important cross-talk between different RNA polymerases could be mediated by spatial co-positioning of the genes. Further analyses will be required to reveal the potential function of Hmo1 in this interplay.

RESUME

Au sein du noyau des cellules eucaryotes, la chromatine, support de l'information génétique, n'est pas distribuée de façon aléatoire. Son organisation spatiale est étroitement liée aux métabolismes nucléaires, tels que la réplication de l'ADN, la réparation ou la transcription. Cependant, les mécanismes gouvernant l'organisation tridimensionnelle de la chromatine restent mal connus. De même, les rôles fonctionnels de cette architecture restent à explorer.

Au cours de mon travail de thèse, j'ai étudié la protéine Hmo1, une protéine à boîte HMG de la levure *Saccharomyces cerevisiae*. Grâce à un crible génétique, nous avons pu mettre en évidence que cette protéine devenait essentielle à la croissance cellulaire dans trois contextes mutants: des mutants de l'ARN polymérase (Pol) I, des invalidations de gènes codant pour des protéines ribosomiques (RPG), ainsi que des invalidations affectant des gènes impliqués dans la réponse aux stress. J'ai pu montrer que Hmo1 interagit physiquement avec la région transcrite de l'ADN ribosomique et avec un sous-ensemble de promoteurs de RPG. De plus, un mutant présentant une invalidation de *HMO1* perd la capacité de répression de la transcription des RPG consécutive à une inhibition de la voie TORC1. Ces résultats indiquent que Hmo1 est impliquée dans la régulation de la transcription par la Pol I, ainsi que la régulation de l'expression des RPG par des voies de transduction du signal comme la voie TORC1. Des expériences d'analyse globale de la transcription (type transcriptome) confirment le rôle de Hmo1 dans la régulation de l'expression des RPG. De plus, mes données préliminaires sur l'effet de la protéine Hmo1 dans des systèmes d'étude de la transcription *in vitro* permettent de proposer un rôle de Hmo1 lors de l'initiation et de l'élongation de la transcription par la Pol I.

La protéine Hmo1 étant une protéine nucléolaire, nous avons émis l'hypothèse d'une transcription péri-nucléolaire des RPG chez la levure, par analogie avec ce qui avait été montré pour les gènes codant les ARN de transfert, transcrits à proximité du nucléole. En raison de la petite taille du noyau de levure et de la nature stochastique des mouvements de la chromatine, nous avons choisi de mettre au point une nouvelle méthode permettant de déterminer de façon statistique et avec une très grande précision la position d'un gène dans le volume nucléaire par rapport au nucléole. Après analyse de plusieurs milliers de cellules, nous avons pu démontrer que, au sein d'une population cellulaire, les gènes sont confinés dans des sous-volumes nucléaires appelés 'territoires géniques'. Ces derniers ne sont pas distribués de façon aléatoire

par rapport à la périphérie du noyau et au nucléole et, pour certains gènes au moins, peuvent être remodelés en fonction de l'activité transcriptionnelle. La localisation de gènes nécessaires à la biogenèse du ribosome, tels que les RPG, semble influencée par leur distance génétique au centromère. En effet, chez la levure, les centromères sont physiquement attachés au centre organisateur des microtubules (le SPB) qui présente la particularité d'être localisé à l'opposé du nucléole. Les gènes proches des centromères occupent donc un territoire voisin du SPB et seuls les gènes plus éloignés des centromères peuvent occuper des régions nucléolaire / péri-nucléolaire. Nos résultats préliminaires suggèrent en outre que des gènes génétiquement liés occupent des territoires similaires.

L'ensemble de ces résultats indique que la protéine Hmo1 participe à la co-régulation de la production des ARN ribosomiques par Pol I et des ARN messagers des RPG en réponse à des altérations des conditions de croissance. Nous postulons que cette co-régulation pourrait être liée à la position intra-nucléaire des gènes codant les constituants du ribosome. D'autres études seront nécessaires pour mieux comprendre le rôle de Hmo1 dans ce mécanisme potentiel de régulation spatiale.

ZUSAMMENFASSUNG

Im Zellkern liegt die in Chromatin verpackte DNA nicht zufällig verteilt vor. Es wurde gezeigt, dass die räumliche Lokalisation von Chromatin einen wichtigen Einfluß auf nukleäre Prozesse wie DNA Replikation, Reparatur oder Transkription hat. Bisher gibt es jedoch nur wenige Hinweise auf die funktionellen Auswirkungen dieser Organisation sowie auf die molekularen Kräfte, die für die Chromatinorganisation verantwortlich sind.

In der vorliegenden Arbeit wurde das HMG-box Protein Hmo1 aus der Bäckerhefe untersucht. Ein anfänglicher 'Screen' zeigte, dass dieses Chromatin-assoziierte Protein mit Genen für RNA Polymerase (Pol) I, mit Genen, die für ribosomale Proteine (RPGs) kodieren, als auch mit Genen, die in der Stressantwort eine Rolle spielen genetisch in Verbindung steht. Des Weiteren konnte ich zeigen, dass Hmo1 physisch mit dem durch Pol I transkribierten rRNA-kodierenden Gen, sowie mit einer Untergruppe von RPG Promotoren interagiert. Mittels globaler Expressionsanalysen

konnte die Abhängigkeit der Expression von RPG-Untergruppen von Hmo1 bestätigt werden. Ein *hmo1* Deletionsstamm kann die RPG Transkription nur noch stark vermindert reprimieren wenn der TOR Komplex 1 inhibiert wird. Zusammengenommen deuten diese Ergebnisse darauf hin, dass Hmo1 sowohl in die Pol I Transkription, als auch in die RPG Regulation involviert ist. Erste *in vitro* Transkriptionassays deuten tatsächlich auf eine Funktion von Hmo1 bei der Pol I-Initiation und -Elongation.

Da Hmo1 primär im Nukleolus akkumuliert, wollte ich im Folgenden die Hypothese testen, ob Pol II transkribierte RPGs, die mit Hmo1 assoziieren, in der Umgebung des Nukleolus lokalisieren, so, wie es bereits für Pol III transkribierte tRNA Gene beschrieben wurde. Aufgrund der geringen Größe des Hefe-Zellkerns einerseits und der stochastisch, sub-diffusiven Bewegung der DNA andererseits, entwickelten wir zunächst eine Methode, die es ermöglicht höchst präzise die Aufenthalts-wahrscheinlichkeit eines Locus im Zellkern unter Berücksichtigung des Nukleolus zu bestimmen. Ein Ergebnis dieser kollaborativen Arbeit ist die Erkenntnis, dass Gene in definierten, sub-nukleären Volumina lokalisieren. Diese ‘Genterritorien’ weisen eine Locus-spezifische Größe auf und können durch transkriptionelle Aktivierung umorganisiert werden. Bei der Anwendung dieser Methode auf Pol II transkribierte Gene, die für die Ribosomenbiogenese benötigt werden, wie RPGs, wurden Hinweise dafür gefunden, dass die Lokalisation der Gene auf der Chromatinfaser, Auswirkungen auf deren dreidimensionale Positionierung hat. Gene in der Nähe des Centromers finden sich bevorzugt vor dem Spindelpolkörperchen, während RPGs, die weiter entfernt von den Enden des Chromosomenarmes liegen, ein ‘Territorium’ in der Nähe des Nukleolus einnehmen können. Zusätzlich scheinen benachbarte Gene eine wichtige Determinante dieser Positionierung zu sein.

Zusammenfassend zeigen diese Resultate, dass Hmo1 bei der Regulation der Pol I- und der RPG Transkription als Reaktion auf wechselnden Wachstumsbedingungen mitwirkt. Weiterhin weisen die Daten darauf hin, dass der essentielle ‘crosstalk’ zwischen unterschiedlichen RNA Polymerasen durch die hier beschriebene räumlichen Co-Lokalisation von Genen vermittelt werden könnte. Weitere Analysen sind nötig, um die potentielle Aufgabe von Hmo1 in diesem Zusammenspiel aufzudecken.

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ABBREVIATIONS

3C	chromosome conformation capture
3D	three dimensional
Å	Ångström
AMP	adenosine monophosphate
bp	base pair
BrUTP	5-bromo-UTP
cAMP	cyclic AMP
CB	Cajal body
CE	core element
CF	core factor
ChIP	chromatin immunoprecipitation
cpm	counts per minute
CT	chromosome territory
DAM	DNA adenine methyltransferase
DAPI	4',6-diamidino-2-phenylindole
DFC	dense fibrillary component
DNA	deoxyribonucleic acid
EC	euchromatin
EM	electron microscopy
FISH	fluorescent in situ hybridization
FC	fibrillary center
FCS	fluorescence correlation spectroscopy
FLIP	fluorescent loss in photobleaching
FRAP	fluorescence recovery after photobleaching
GC	granular component
GEF	guanosine exchange factor guanosine triphosphate
GTP	guanosine 5'-triphosphate
HC	heterochromatin
hnRNP	heterogeneous RNP
HMG	high mobility group
IC	interchromatin compartment

ICD	interchromosome domain
ICN	interchromosomal network
IF	immunofluorescence
INM	inner nuclear membrane
m	meter
Mb	mega base pair
µm	micrometer
MAR	matrix attachment region
MNase	micrococcal nuclease
mRNA	messenger RNA
MTOC	microtubule organizing center
NE	nuclear envelope
nm	nanometer
NOR	nucleolar organizing region
NPC	nuclear pore complex
NTPs	nucleotide triphosphates
ONM	outer nuclear membrane
PKA	protein kinase A
Pol	DNA dependent polymerase
kb	kilo base pair
RNA	ribonucleic acid
RPG	ribosomal protein gene
rDNA	ribosomal DNA (DNA coding for rRNAs)
RNP	ribonucleoprotein
rRNA	ribosomal RNA
S	Svedberg
SILAC	stable isotope labeling of amino acids in cell culture
SIR	silent information regulator
SL	synthetic lethal
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SPB	spindle pole body
SPT	single particle tracking
TBP	TATA-binding protein

TF	transcription factor
tgm	tRNA gene-mediated
TOR	target of rapamycin
tDNA	transfer DNA (DNA coding for tRNAs)
tRNA	transfer RNA
ts	temperature sensitive
TSS	transcription start site
UAF	upstream activating factor
UBF	upstream binding factor
hUBF	human UBF
UE	upstream element
UTP	uridine 5'-triphosphate
WT	wild type

INTRODUCTION

1. TRANSCRIPTION OF RIBOSMOMAL COMPONENTS

1.1 Eukaryotic RNA polymerases

1.1.1 Enzymes decoding genetic information

DNA-dependent RNA polymerases are responsible for the first step in translating the genomic information encrypted in ~ 12 million letters in yeast or ~ 3 billion letters in mammals. In conjunction with basal and gene specific transcription factors (TFs), they are responsible to find the right chapter in front of a huge shelf full of books. Once found the beginning of a chapter i.e. the promoter, the RNA polymerase reads the genetic information and copies / transcribes it into an RNA molecule. The RNA is then translated from the nucleic acid code into the amino acid code forming poly-peptides with the help of an enzymatic complex, the ribosome. These polypeptides then form proteins, mostly enzymes, that are the basis for life.

However, not all RNAs are translated into proteins. Non-coding RNAs are for example used as integral, structural and functional components in RNA protein complexes, such as the ribosome, or they function in regulating transcription and translations events. Another level of complexity is added by the fact that eukaryotic cells harbor a nucleus, containing the DNA (deoxyribonucleic acid). The translation machinery however is located in the cytoplasm, separating transcription and translation events spatially. Export of the RNA must therefore be guaranteed via pores integrated into the so-called nuclear envelope. These pores also allow re-import of a protein into the nucleus, such as an RNA polymerase, that can then start a new cycle of DNA transcription.

One of the challenges in biology is now to understand how genetic information is organized in nuclear space. Spatial compartmentalization needs be studied to understand how and where information is accessible and actively used.

My PhD work has been organized into two parts: first, a molecular characterization of a transcriptional apparatus and second, a study on gene positioning within the nuclear volume. The goal of this dual study was to bridge the gap between our knowledge in molecular mechanisms of transcription and the functional distribution of genetic material within the nucleus. In the following part of my introduction I will review our

knowledge on transcription, especially focused on the synthesis of ribosomal components, followed by a detailed description of three dimensional (3D) genome organization.

1.1.2 Three forms of nuclear RNA polymerases

Within a eukaryotic nucleus one can find at least three RNA polymerases (see Table 1). Initially only one single polymerase had been characterized in extracts from different organisms varying from mammals to yeast (Frederick *et al.*, 1969; Weiss, 1960). Only about a decade later purification of *the* polymerase over an anion exchange column resulted in three different fractions, namely RNA polymerase (Pol) I, II and III (Roeder and Rutter, 1969). Recently, a forth nuclear Pol, Pol IV, has been postulated from sequence analyses, that seems to be specific to plants (Initiative, 2000). Originally described to be dedicated to siRNA amplification and involved in heterochromatin formation (Onodera *et al.*, 2005), it is now clear that Pol IV can be further sub-divided into Pol IVa and Pol IVb, dependent on the largest subunit of the enzyme. Furthermore, these two enzymes seem to be involved in different processes (such as RNA-directed DNA methylation which is independent of siRNA biogenesis) which haven't been elucidated by now (Pikaard *et al.*, 2008).

The three common polymerases consist of 12 (Pol II) to 17 (Pol III) subunits (see Table 1). They comprise a ten-subunit core, where five out of the ten subunits are commonly shared. In addition, Pol I and Pol III share two extra subunits (Cramer *et al.*, 2008). The mass of the holoenzymes is around 600 kDa. Almost half of the molecular weight comes from the two largest subunits in the polymerases that belong to the core but are specific for each polymerase. Further morphological and functional difference comes especially from Pol specific subunits, like three subunits (Rpc82, Rpc34 and Rpc31) forming a Pol III-specific subcomplex (Table 1, (Cramer *et al.*, 2008)).

The ribosomal RNA (rRNA) genes are transcribed by Pol I and -III. Pol I transcribes the large rRNAs, 25S, 18S and 5.8S as one large polycistronic precursor that is then processed in multiple successive steps into the mature rRNAs (Fatica and Tollervey, 2002).

The small rRNA, the 5S rRNA, is transcribed by Pol III. Pol III also transcribes tRNAs and other small, non-coding RNAs like U6 snRNA, some snoRNAs, RNaseP

RNA, RNase MRP RNA, microRNAs, but also virus-induced ncRNAs or viral RNAs themselves (as for Adenoviruses or Herpesviruses) (Dieci *et al.*, 2007).

RNA Pol II transcribes all protein coding genes into mRNAs, microRNAs, as well as snRNAs. With respect to ribosome biogenesis, Pol II generates mRNAs for ribosomal proteins and proteins required for the processing and modifying of rRNA and assembly of ribosomes. In addition to the protein-coding genes involved in ribosome biogenesis, Pol II also synthesizes ncRNAs, like snoRNAs that guide rRNA processing and –modifications (Fatica and Tollervey, 2002).

The three forms of polymerase are found in all eukaryotes. It is still unclear why this specialization of synthesis has been selected during evolution. One of the peculiar features of this specialization in RNA production is the association of the polymerase isoforms with different nuclear sub-domains. Each of the three Pols transcribes in a different nuclear sub-domain (see 2.2 and 2.3). During evolution a correlation is observe between the appearance of a dedicated compartment, the nucleus, with the emergence of three distinct Pols. This correlation may suggest that spatial segregation of the transcription apparatuses in the nucleus provides a selective advantage for eukaryotic cells.

The most complex interplay between all three forms of Pol is synthesis of ribosomes.

Ribosome production involves all three nuclear RNA polymerases. To assure that all ribosomal components are available in stoichiometric amounts, in levels that are well adapted to the proliferation state of a cell, the polymerases need to be tightly co-regulated (Warner, 1999). This co-regulation is a central aspect of my work and will be detailed in the following part of the introduction.

1.2 rRNA Synthesis

Each of the three nuclear Pols is required for the synthesis of ribosomal constituents. The production of 5S rRNA by Pol III will not be described here. I will start by the structure and function of Pol I, responsible for the synthesis of the three large rRNAs. I will then detail ribosomal protein gene transcription and regulation by Pol II. The main goal of this part is to provide a background on how cells could reach a stoichiometric production of ribosomal constituents, while using a distinct transcription machinery for each component.

1.2.1 Structure of RNA Polymerase I

Pol I consists of 14 subunits (Carles *et al.*, 1991), summarized in table 1. It shares five subunits with Pol II and seven subunits with Pol III (see table I). The other subunits possess highly conserved domains within paralogous catalytic subunits (Cramer, 2002; Cramer *et al.*, 2008). This allowed Kuhn *et al*. recently to fit the high resolution X-ray crystallographic structure of Pol II (Cramer *et al.*, 2001) into lower resolution cryo-electron microscopic density map obtained for Pol I (Kuhn *et al.*, 2007). The respective cores, including the active center fit nicely, demonstrating the conservation between RNA polymerases (Cramer, 2002). Globally, the polymerase has a globular structure that is incised by a cleft (see Figure 1). This cleft can be closed from one side, by a domain forming a mobile clamp. DNA is supposed to enter from the top of the cleft "jaws" into the polymerase, where it approaches the active site. Beyond the active site, the DNA-RNA hybrid hits a "wall" from where the DNA and newly synthesized RNA follow an angle of almost 90˚ with respect to the incoming DNA to exit the polymerase. Next to the clamp, a characteristic protrusion extends from the polymerase, termed the "stalk". Nucleotide triphosphates (NTPs) have access to the active center via the so-called "funnel" at the opposite side of upstream DNA exit site (Cramer, 2002; Cramer *et al.*, 2008; Kuhn *et al.*, 2007). Local differences between the Pol II structure and the Pol I fit have been encountered for the clamp and a domain called "foot" (next to the NTP entry funnel) (Kuhn *et al.*, 2007).

The biggest differences described however stem from two subunits that form a heterodimer and that do not belong to the core of the polymerase: Rpa14 and Rpa43 (Bischler *et al.*, 2002; Peyroche *et al.*, 2002). They show only weak homology to their Pol II counterparts Rpb4 and Rpb7. Due to their differences, these are the regions that are candidates for polymerase specific functions. Kuhn and co-workers crystallized the Rpa14/Rpa43 heterodimer from ectopically expressed proteins and fitted the obtained X-ray structure into the EM map. As for Pol II, the dimer forms the above mentioned "stalk" in the 3D structure of the polymerase (Bischler *et al.*, 2002; Kuhn *et al.*, 2007; Peyroche *et al.*, 2002). Thus, the interaction between Rpa43 and Rrn3

RNA polymerase	Pol I	Pol II	Pol III
Ten-subunit core	Rpa190	Rpb1	Rpc160
	Rpa135	Rpb2	Rpc128
	Rpc40	Rpb3	Rpc40
	Rpc19	Rpb11	Rpc19
	Rpa12.2	Rpb9	Rpc11
	Rpb5	Rpb5	Rpb5
	Rpb6	Rpb6	Rpb6
	Rpb8	Rpb8	Rpb8
	Rpb10	Rpb10	Rpb10
	Rpb12	Rpb12	Rpb12
Rpb4/7 subcomplex	Rpa14	Rpb4	Rpc17
	Rpa43	Rpb7	Rpc25
TFIIF-like subcomplex[a]	Rpa49	(Tfg1)	Rpc37
	Rpa34.5	(Tfg2)	Rpc53
Pol III-specific subcomplex	–	–	Rpc82
	–	–	Rpc34
	–	–	Rpc31
Number of subunits	14	12	17

Table 1. Yeast nuclear DNA dependent RNA polymerases.

In red: the commonly shared core subunits. In green: subunits shared by Pol I and Pol III.
a) The two subunits in Pol I and Pol III (forming a hetero-dimer in Pol I) are predicted to be functionally homologous to the Pol II initiation / elongation factor TFIIF.
Adapted from (Cramer *et al.*, 2008).

(Peyroche *et al.*, 2000), the activator of Pol I ((Milkereit and Tschochner, 1998), see 1.2.2), happens on the upstream surface with regard to the direction of transcription. The position of the Pol I specific subcomplex, Rpa49 and Rpa34, has been determined comparing EM structures of polymerases with and without the two subunits (Kuhn *et al.*, 2007). Recovering previously published results with increased resolution (Bischler *et al.*, 2002), the heterodimer seems to be situated diagonally opposed to the Rpa14/Rpa43 "stalk", near the "funnel" of the enzyme, towards the backside of the polymerase.

The smallest Pol I subunit, Rpa12, has been mapped at the back of the polymerase, on the "upper part, near the exit site of the RNA and coding strand (see Figure 1B). The two largest Pol I subunits, according to their molecular weight Rpa190 and Rpa135, form the center of polymerase. They lie opposite of the cleft (Bischler *et al.*, 2002) and harbor, evolutionary conserved the catalytic center (Cramer, 2002).

Rpa49 and Rpa34 are specific to Pol I, they are non-essential and without described counterparts in other polymerases (Kuhn *et al.*, 2007). The two proteins form a heterodimer (Kuhn *et al.*, 2007) and consistent with this, the Rpa49 subunit gets lost from the polymerase during purification procedure in an *rpa34*Δ background (Gadal *et al.*, 1997). The dimer can also be dissociated from the holoenzyme using high salt conditions (Huet *et al.*, 1975). The resulting 12 subunit Pol I, termed Pol I*, is transcriptionally less active in promoter-independent assays (Huet *et al.*, 1975). This finding has been confirmed recently, demonstrating that a Rpa49/Rpa34-deficient Pol I has an elongation defect (Kuhn *et al.*, 2007), suggesting that these two subunits act as an intrinsic elongation factor, whose function in the Pol II system could be covered by the general transcription factor TFIIF. This prediction is based on a similar localization of TFIIF on Pol II and a similar secondary structure. A genetic study could partially confirm this speculation as Rpa49 is required both for efficient initiation and for Rrn3 release during elongation. Rpa34 forms a dimer with Rpa49, and seems to be mostly required for stabilization of Rpa49. Association of Rpa34 with Pol I requires Rpa49 and *RPA34* specific defects are suppressed by *RPA49* over-expression (Beckouet *et al.*, 2008).

Interestingly, deletion of either *RPA49* or *RPA34* is lethal when combined with a deletion of *RPA14* which is in direct contact with the essential subunit Rpa43 (Gadal *et al.*, 1997; Kuhn *et al.*, 2007), two subunits that are critical for initiation events

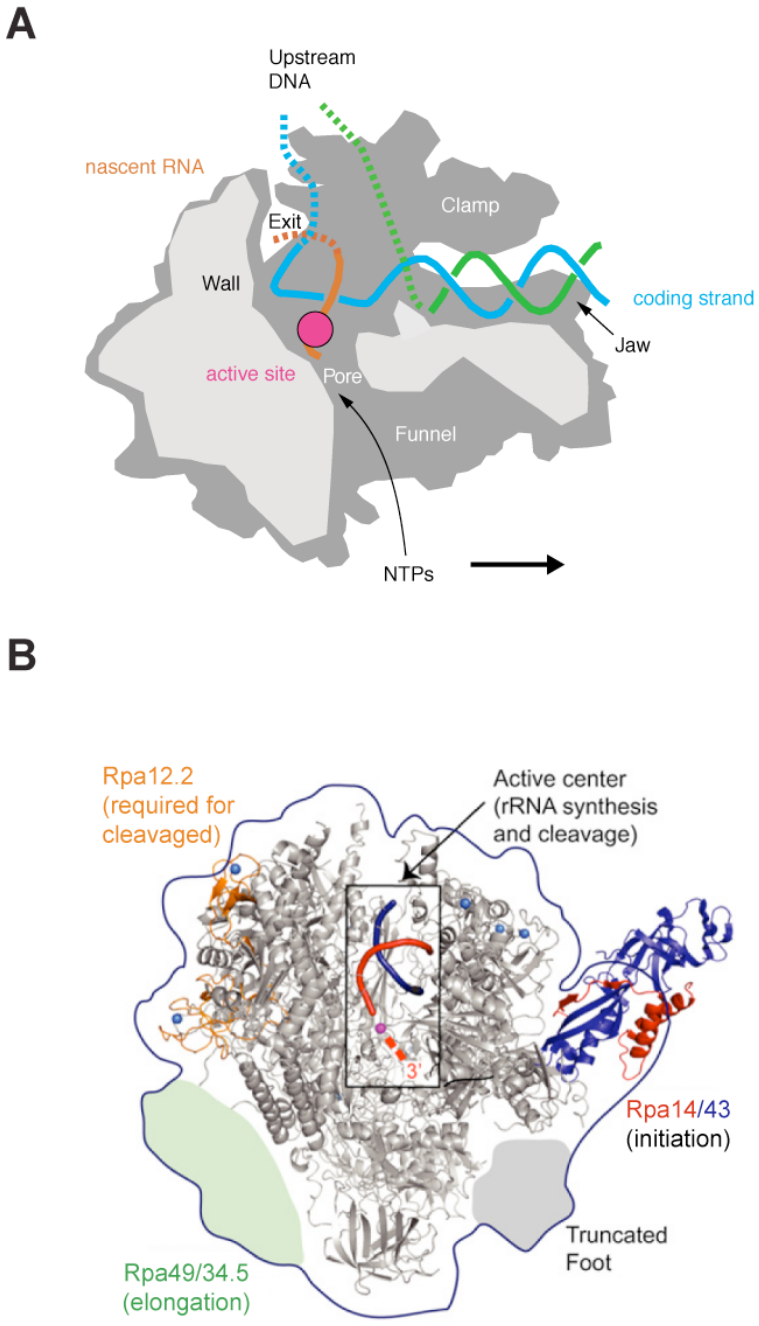

Figure 1. Structure of yeast RNA polymerase I.

A. Cutaway of a yeast Pol elongation complex. The transcribed DNA enters from the right hand side between the "jaw" and the "clamp" structures. DNA is melted open and reaches the enzymes active site just in front of the "wall", where it kinks in a right angle. NTPs reach the active site via the "funnel" and a pore structure. Adapted from (Cramer, 2002).

B. Pol I structure and functional allocation of some sub-units. From (Kuhn *et al.*, 2007).

(Peyroche *et al.*, 2002). It seems as if weakening elongation or initiation can still be tolerated, while negatively influencing both events results in lethality (Gadal *et al.*, 1997).

It has previously been described that the smallest Pol I subunit, Rpa12, is required for Pol I termination (Prescott *et al.*, 2004). It has now been demonstrated that the C-terminal domain of Rpa12 mediates a strong intrinsic RNA cleavage activity (Kuhn *et al.*, 2007). As for the other Pols, this intrinsic RNA cleavage activity of Pol I is stimulated in trans by an additional factor (Tschochner, 1996).

Kuhn and co-workers finally showed that, as for the homologous Rpc11 subunit of Pol III (Chedin *et al.*, 1998), the C-terminal part of Rpa12 is needed to perform this RNA cleavage (Kuhn *et al.*, 2007). This domain, that resembles the Pol II elongation factor TFIIS, could be required for efficient circumvention of "roadblocks" suggesting that RNA cleavage could be part of the Pol I termination process. However, C-terminal Rpa12 deletion mutants do not show any growth defect *in vivo* (Mullem *et al.*, 2002). Finally, as for Rpa49/Rpa34, *RPA12* deletion cannot be tolerated in an *rpa14*Δ background (Gadal *et al.*, 1997).

Apart from the complete enzyme, additional complexes and factors are required for specific Pol I initiation.

The fundamental difference of Pol I compared to the two other nuclear Pols is the high number of "non-essential" factors. For Pol III, all specific subunits are essential. This characteristic may reflect redundancy and could be linked to the robustness of rRNA synthesis. Pol I accounts for a large fraction of RNA synthesis and presumably, cells have evolved numerous, partly overlapping processes to enhance efficiency of rRNA synthesis. These properties may explain why novel, important but not essential Pol I factors are still identified to date.

1.2.2 RNA Polymerase I pre-initiation complex

Like for other eukaryotic RNA polymerases, Pol I requires additional transcription factors to transcribe its substrate. Pol I transcription factors are organized in two protein complexes that bind to two promoter sequences, the upstream element (UE) and the core element (CE) (illustrated in Figure 2A). The UE is situated from -146 to -51 bp with respect to the transcription start site (TSS) (Kulkens *et al.*, 1991; Musters *et al.*, 1989) and is bound by a protein complex termed UAF for upstream activating

factor (Keys *et al.*, 1996). Further proximal to and including the TSS locates the CE at position -28 to +8 bp (Kulkens *et al.*, 1991; Musters *et al.*, 1989), targeted by the core factor or CF (Keys *et al.*, 1994; Lalo *et al.*, 1996).

UAF is composed of six proteins, UAF30, Rrn5, Rrn9, Rrn10 and the two histones H3 and H4 (Siddiqi *et al.*, 2001). CF consists of three proteins, namely Rrn6, Rrn7 and Rrn11(Keys *et al.*, 1994; Lalo *et al.*, 1996).

Furthermore, the TATA-binding protein TBP participates in Pol I initiation, whilst not belonging directly to either the UAF, or the CF. TBP has however been demonstrated to interact in the pre-initiation complex (PIC) with both complexes (Steffan *et al.*, 1996), suggesting a bridging between UAF and CF.

In vitro, only the CF is essential for Pol I initiation as shown in transcription assays (Keys *et al.*, 1994; Lalo *et al.*, 1996; Lin *et al.*, 1996). In the same system, the UAF is not essential but is required for high levels of transcription (Keys *et al.*, 1996).

UAF binds stably to the UE, while CF interaction with the CE is not very pronounced and strengthened by auxiliary factors from crude yeast extracts (Keys *et al.*, 1994). Assembly studies draw the current picture as follows: UAF is first recruited to the UE. TBP binds to the UAF and recruits / stabilizes the CF onto the CE. UAF and CF form a stable complex. Besides the common interaction of TBP with the two, Rrn7 is also required for the stabilization. This assembly onto the yeast ribosomal DNA (rDNA) promoter forms the pre-initiation complex, to which Pol I is recruited.

However, only a fraction of Pol I can be recruited, namely Pol I that is associated with another Rrn gene product, initially identified in a genetic screen (Nogi *et al.*, 1991): Rrn3. This protein has first been characterized to interact with Pol I and its pre-initiation complex (Yamamoto *et al.*, 1996). It has further been demonstrated that: Rrn3 specifically interacts with Pol I subunit Rpa43 (Peyroche *et al.*, 2000), only less than 2 % of Pol I is associated with Rrn3 in whole cell extracts and that it is this fraction that is competent for initiation (Milkereit and Tschochner, 1998). The two Pol I subunits Rpa49 and Rpa34 also seem to influence interaction properties between Rrn3 and Pol I (Beckouet *et al.*, 2008).

Some question still remain, mostly on how Rrn3 is recycled back onto Pol I. *In vitro*, association of Rrn3 with Pol I is very inefficient and no activity has been identified

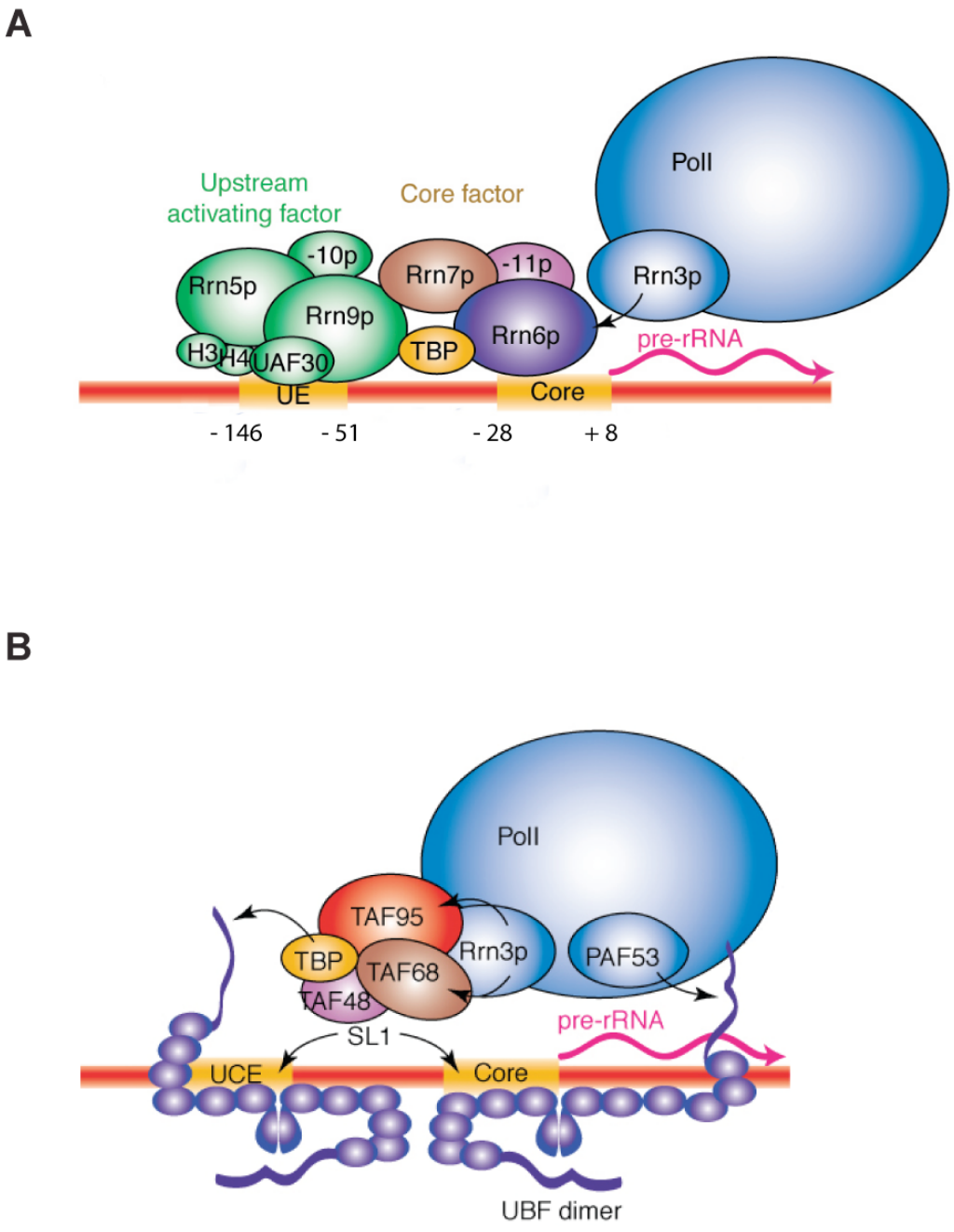

Figure 2. The Pol I pre-initiation complex (PIC).

A. Yeast PIC. The six sub-unit containing upstream activating factor (green) binds the upstream element (UE). The core factor (composed of Rrn6, Rrn7 and Rrn11) binds the core element ("core"). The two complexes are bridged via the TATA-binding protein, TBP.

B. Mammalian PIC. The upstream control element (UCE) is bound by UBF. The core element (core) is bound by the selectivity factor (SL1), composed of at least three transcription activating factors (TAFs) and TBP. UBF is also bound to the core element. Conversely, SL1 is also detected at UCE.

Adapted from (Moss, 2004).

that could strengthen this association. *In vivo*, no putative candidate is known for this activity so far.

The situation is different in mammals (for review see (Russell and Zomerdijk, 2005) and (Moss *et al.*, 2007), Figure 2B). The promoter is structured similarly, with an upstream element called upstream control element, UCE and the CE comprising the TSS. The proteins binding these DNA sequences however are different. Yeast CF is represented by the four or more subunit containing selectivity factor 1, SL1. It comprises at least three, but probably as recently suggested five, TAFs (two of which have a homology to Rrn7 and Rrn11 respectively) and TBP (Denissov *et al.*, 2007; Gorski *et al.*, 2007; Russell and Zomerdijk, 2005). Interestingly, SL1 is also detected on the UCE.

The UAF in mammals is replaced by a single protein, the upstream binding factor or UBF. UBF is an HMG-box protein that binds the rDNA promoter in form of a homodimer (cf. 3.2 and (Moss *et al.*, 2007)). It is detected at the UCE and also at the CE. This led to the model of the "enhancesome" (Stefanovsky *et al.*, 2001a): UBF forms a dimer, and binds to both promoter elements, inducing a helical turn of the promoter region, bringing UCE and CE into close spatial proximity. This allows SL1 to bind to both elements. However this model, in which UBF prepares the landing platform for SL1 is disputed, and it could well be the other way round. In either case, UBF and SL1 stabilize each other on the promoter, forming the PIC.

A novel, potentially complementary function of UBF has recently been suggested from *in vitro* studies (Panov *et al.*, 2006a). Zomerdijk and co-workers presented evidence that UBF activates Pol I transcription after PIC formation, namely during the process of promoter escape and clearance.

Recruitment of the polymerase onto the PIC on the other hand seems conserved from yeast to human, hRrn3 (or TIF-1A) associates with a fraction of Pol I rendering the polymerase competent for initiation (Bodem *et al.*, 2000).

Interestingly, Pol I subunits orthologous to *S. cerevisiae* Rpa34 and Rpa49, respectively PAF49 in mouse (or CAST in human) and PAF53 both interact with UBF (Hanada *et al.*, 1996; Panov *et al.*, 2006b) (see Figure 2B).

The human and the yeast Pol I transcription systems are characterized by different TFs, with little or no similarity. However, functional similarities are obvious, such as between the UAF and UBF, the CF and the SL1 or Rrn3 and TIF-IA, in yeast and humans respectively. Expression of human factors in the yeast system will allow to

evaluate the level of functional conservation and go beyond the level of similarity (see result section).

1.2.3 RNA Polymerase I elongation and termination

Little is known about the transition of Pol I from initiation to elongation. Rrn3 is released from the polymerase, shortly after Pol I has initiated (Bier *et al.*, 2004; Milkereit and Tschochner, 1998), much like the Pol II transcription factor TFIIF (Moss *et al.*, 2007). Dissociation of Rrn3 from Pol I does not seem to be vital, since a strain expressing an Rrn3–Rpa43 fusion protein replacing the endogenous Rrn3 and Rpa43 proteins does not show slow growth (Laferte *et al.*, 2006). Note that replacement of Rpa43 by the same Rrn3–Rpa43 fusion is lethal in an *rpa49*Δ mutant background (Beckouet *et al.*, 2008). Furthermore, this does not seem to hold true for mammals since a similar construct in mammalian cells (TIF-IA – Rpa43 fusion) followed by knock-out of the endogenous copies is lethal, suggesting that dissociation of the two proteins is required for transcription progression (Bierhoff *et al.*, 2008).
Furthermore it has been shown that Pol I – Rrn3 association requires phosphorylation of the polymerase (Fath *et al.*, 2001). Fcp1 phosphatase treatment of Pol I however did not increase *in vitro* elongation (Fath *et al.*, 2004). The influence of phosphorylation on transcription are currently under investigation, and recent analysis of Pol I phosphorylation site mutants showed that it is unlikely that one single phosphorylation site is responsible for the Rrn3-Pol I interaction (Gerber *et al.*, 2008). Further investigation of Pol I elongation defects by analyzing mutant growth on 6-azauracil (6AU) containing medium showed no clear picture either. However, the authors describe an Rpa190 mutant less sensitive to 6AU treatment, as well as less sensible to high temperature stress, interpreted as being potentially less adaptable to stress mediation (Gerber *et al.*, 2008).
Another factor, that has originally been described in the Pol II system, the elongation complex the Spt4/Spt5 (Mason and Struhl, 2005), has been demonstrated to also be involved in helping Pol I elongation (Schneider *et al.*, 2006). As in the case for Pol II the authors conclude that it is not the elongation rate (nucleotide addition per minute) that is increased by Spt4/Spt5, but rather the processivity of the polymerase (nucleotide addition per initiation event).

In animal cells, the influence of UBF on Pol I elongation is also been discussed. This possibility was studied after it had been discovered that UBF binding to the rDNA is not restricted to the promoter region, but spans the whole unit, i.e. the Pol I transcribed region (O'Sullivan *et al.*, 2002). Recent work from Tom Moss' laboratory suggests that UBF could act as a negative regulator of elongating Pol I, rendering the chromatin inaccessible for the transcribing enzyme (Stefanovsky *et al.*, 2006a). They further report this function to be regulated by phosphorylation of UBF ((Stefanovsky *et al.*, 2006b), see 1.4).

Our understanding about elongation and termination in the Pol I cycle is rather low compared to the regulation of initiation events. To date, some proteins described to be associated with Pol I could also act on elongation. To explore this possibility, additional experimental set-ups need to be exploited and / or developed (see results section).

1.3 Ribosomal protein gene transcription

Pol I is responsible for the synthesis of the precursor containing the large rRNAs. In this section, I will not describe the assembly of rRNAs into the mature ribosome, instead I will rather focus on a specific regulon of Pol II, the one of ribosomal protein genes, that follows dedicated regulative pathways. This fine tuned regulation is required for the stoichiometric production of the ribosomal constituents ribosomal proteins and rRNA.

1.3.1 Transcription factors of ribosomal protein genes

In yeast 138 genes code for the 79 ribosomal proteins (Link *et al.*, 1999; Warner, 1999). Almost all ribosomal protein gene (RPG) promoters are characterized by the presence of Rap1 binding sites (Nieuwint *et al.*, 1989; Rotenberg and Woolford, 1986); in the rare cases, where it does not bind, the Rap1 sites are often replaced by Abf1- (Hamil *et al.*, 1988) or Reb1 (Lascaris *et al.*, 1999) binding sites. Only very few RPG are predicted as not to be bound by any of these three proteins (Lascaris *et al.*, 1999). The Rap1 binding sites are required for strong RPG expression (Nieuwint *et al.*, 1989; Rotenberg and Woolford, 1986). However, Rap1 is a very versatile protein

and is found associated with different kinds of strong promoters, like glycolytic genes and genes coding for translation factors, at telomeres and at the *HMR* and *HML* silencers where it nucleates silencing (reviewed in (Piña *et al.*, 2003)). There are speculations that the different described consensus sequences specific to one gene class could model the surface of Rap1, allowing it to be recognized by transcription factors specific to these genes (Piña *et al.*, 2003). The initial task of Rap1 seems to be to clear nucleosomes from the stretch of DNA that it binds to, facilitating the binding of specific factors (Yu and Morse, 1999).

Fhl1 and Ifh1 are transcription factors specific to RPGs (Lee *et al.*, 2002; Schawalder *et al.*, 2004). Fhl1 seems to serve as a landing platform for the essential RPG transcription factor Ifh1 (Martin *et al.*, 2004; Rudra *et al.*, 2005) (see Figure 3). Another key protein is the transcription factor Crf1, that has been reported to act as a negative regulator, which competes with Ifh1 for binding to Fhl1 (Martin *et al.*, 2004).

An additional protein brought into play for RPG regulation is the activating transcription factor Sfp1 (Jorgensen *et al.*, 2004). It seems that, as for Crf1, shuttling of Sfh1 between the nucleus and the cytoplasm could be implicated in tuning RPG transcription (Marion *et al.*, 2004). It has been speculated that activation through this protein could mark another, Fhl1-Ifh1 independent mechanism (Zhao *et al.*, 2006a).

Other regulatory factors that have been studied in the context of the regulation of RPGs are chromatin modifying proteins. One of these is the multi-protein RSC (Remodel the Structure of Chromatin) complex (Angus-Hill *et al.*, 2001). Angus-Hill and co-workers showed in expression analyses that shifting a temperature sensitive (ts) mutant of the essential RSC component Rsc3 to its restrictive temperature results in a general RPG down-regulation compared to the “shifted” wild type (WT) strain. Deletion of the non-essential RSC constituent Rsc30 however causes a dramatic RPG up-regulation compared to the isogenic WT. Even though these contradictory results do not allow one to conclude about the role played by RSC, they clearly demonstrate its involvement in RPG expression control (Angus-Hill *et al.*, 2001).

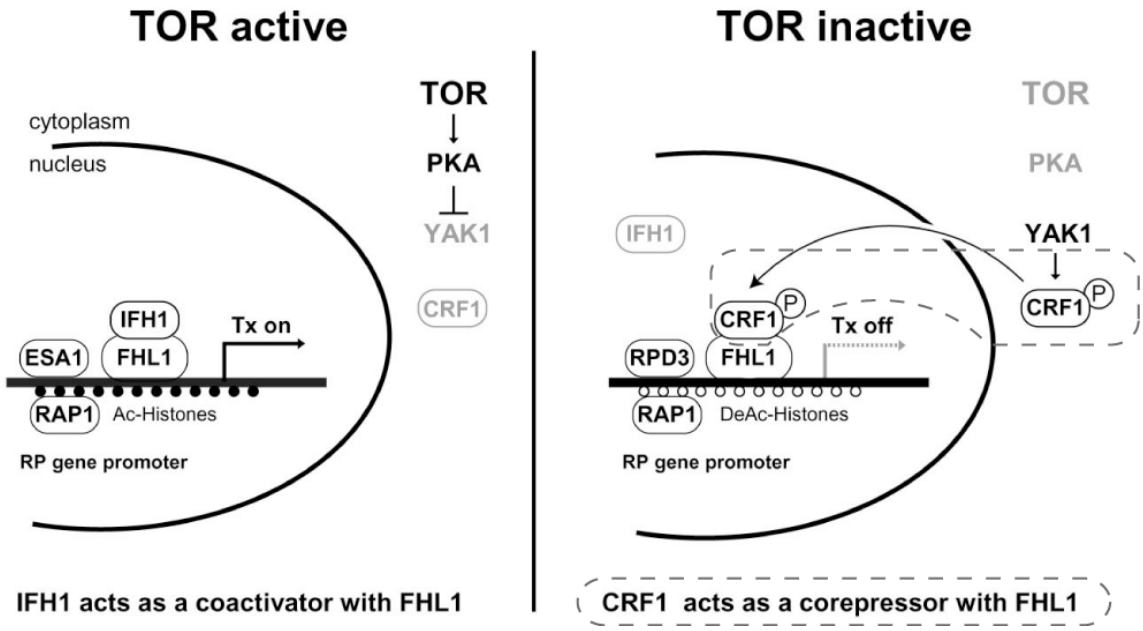

Figure 3. Model for ribosomal protein gene regulation.

Active ribosomal protein gene (RPG) promoter situation (left) and repressed RPG promoter situation (right). Rap1 binds most RPG promoters, though not all of them. Fhl1 and Ifh1 are RPG specific transcription factors. Esa1 has been mapped on active RPG, while it seems to be replaced by Rpd3 under repressive conditions. Histone acetylation is reflected by the presence of the histone acetylase and –deacetylase, respectively. Whether RPG repression requires a co-repressor (Crf1), is still a matter of debate (see text for details). Adapted from (Martin *et al.*, 2004).

Other chromatin modifying enzymes described in the context of RP genes are the histone acetylase Esa1 (Reid *et al.*, 2000) and its counterpart, the histone deacetylase Rpd3 (Rohde and Cardenas, 2003) (see Figure 3). Esa1 has been demonstrated to bind to a small subset of yeast gene promoters, including RPG promoters (Reid *et al.*, 2000). Esa1 recruitment to RPG promoters seems to require Rap1 or Abf1 binding. Starving cells of amino acids correlates with reduced Esa1 occupancy at RPG promoters and depletion of Esa1 leads to a specific decrease in RPG transcription (Reid *et al.*, 2000). Rpd3, apparently complexed in form of the Rpd3-Sin3 histone deacetylase complex, also binds RPG promoters and is reported to be important for RPG repression under starving conditions (Kurdistani *et al.*, 2002; Rohde and Cardenas, 2003).

Recent structure analysis of RPG promoters by Jonathan Warner's group however demonstrated that these elements are largely devoid of histone molecules, suggesting, if at all, very low nucleosome occupancy (Zhao *et al.*, 2006a). This finding raises the question whether Esa1 and Rpd3 are located at the RPG promoters to potentially acetylate and deacetylate proteins different from histones (Reid *et al.*, 2000; Zhao *et al.*, 2006a).

In summary, multiple pathways can control RPG expression. However, the question remains as to how a stoichiometric production of ribosomal proteins can be coordinated with the level of pre-rRNAs present in the cell.

1.3.2 Regulatory pathways controlling ribosomal protein gene expression in response to nutrient availability

Ribosomal protein expression is tightly regulated in the cell, i.e. a cell needs to adjust RP production with respect to extra-cellular signaling ((Warner, 1999), see 1.4).

Three pathways have originally been identified in regulation of RPG expression. The first mechanism is the so-called "stringent response": due to amino acid deprivation, uncharged tRNAs accumulate within the cell which leads (amongst other effects) to the repression of RPG transcription (Moehle and Hinnebusch, 1991). This could be directly mediated through a Gcn2-Gcn4 pathway that is ultimately activating many diverse genes, while repressing RPG expression (Natarajan *et al.*, 2001).

The second pathway described to be involved in adjusting RP levels to growth rate is the Ras-cAMP-PKA pathway (Klein and Struhl, 1994). Presumably Cdc25, a

guanosine exchange factor (GEF), senses nutrient availability and activates the small GTPase Ras. This activates adenylate cyclase resulting in the production of cyclic AMP (cAMP). cAMP then leads to dissociation of the catalytic subunit of protein kinase A (PKA), called Bcy1, that ultimately renders PKA active (Broach, 1991). The Ras-cAMP-PKA pathway is a constitutively active pathway, shutting it down results in cell cycle arrest (G1, G0), while over-expression prevents cell cycle arrest (Broach, 1991).

Finally, the target of rapamycin (TOR) pathway is involved in regulating RPG expression (Thomas and Hall, 1997). The first mechanistic analyses of the effects of inhibiting TOR using the antibiotic rapamycin were performed in mammalian cells. They pointed to a ribosomal protein regulation at the translational level, involving a ribosomal protein mRNA-specific pyrimidine-rich stretch at the 5' end of the mRNA referred to as 5'TOP (terminal oligopyrimidine) (reviewed in (Thomas and Hall, 1997)). This initiation dependent RP expression regulation also involves phosphorylation of ribosomal protein S6 (Thomas and Hall, 1997). However yeast ribosomal protein mRNAs do not possess a 5'TOP sequence, neither does the mutation of the S6 phosphorylation site has an effect on yeast growth (Johnson and Warner, 1987). It is therefore thought that regulation of RPG expression is mainly controlled at the transcriptional level (Martin *et al.*, 2006). Yeast studies have so far described the TOR pathway to act via PKA on Crf1 (Martin *et al.*, 2004) (see Figure 3). While Fhl1 associates with the RPG promoter under different growth conditions with or without rapamycin treatment, which inhibits TORC1 (Bar-Joseph *et al.*, 2003; Martin *et al.*, 2004; Rudra *et al.*, 2005), Ifh1 binds the promoter only under growth favorable conditions (Martin *et al.*, 2004; Rudra *et al.*, 2005; Schawalder *et al.*, 2004; Zhao *et al.*, 2006a). The laboratory of Michael Hall showed a binding of the protein Crf1 to Fhl1 as Ifh1 dissociates from it (Martin *et al.*, 2004). At the same time they observed a shuttling mechanism of the co-repressor Crf1 that is regulated by rapamycin treatment; Crf1 translocates into the nucleus upon rapamycin treatment. The phosphorylation of Crf1 by the kinase Yak1 is responsible for this translocation event (Martin *et al.*, 2004). Yak1 activation is mediated by the Ras-cAMP-PKA pathway (see above) (Martin *et al.*, 2004) as shown in *in vitro* and *in vivo* experiments. Upon rapamycin treatment, PKA, Yak1 and Crf1 translocate to the nucleus. Constitutive activation of the Ras-cAMP-PKA pathway suppresses several rapamycin-treatment induced phenotypes, including down-regulation of ribosome

biogenesis genes (RPG, Pol I and –III, (Schmelzle *et al.*, 2004)). The authors therefore suggest that the Ras-cAMP-PKA pathway is another branch of the TOR pathway. However, in another study, Crf1-mediated RPG repression has been described to be strain specific (Zhao *et al.*, 2006a).

The regulation of ribosomal protein production is still an area of intense research and some factors are probably still missing, specifically those linking Pol I to RPG expression (see below and result section).

1.4 Co-regulation processes controlling stoichiometry of ribosomal components

Ribosome biogenesis is a highly energy consuming process. Already only the transcription of the components involved in this process represents approximately 95 % of all transcription events in a proliferating cell (Martin and Hall, 2005). In terms of energy costs, this translates into about 80 % of a cells total energy expenses (Thomas, 2000). A ribosome is built from components in stoichiometric amounts, namely 79 ribosomal proteins and four rRNAs. Tight co-regulated expression and / or degradation of the constituents is required for the cell to not waste energy and to accumulate one (unused) constituent over another. However, expression of the mentioned ribosomal parts requires the action of all three nuclear DNA dependent RNA polymerases, complicating co-regulation between the transcriptional machineries.

At least in yeast, Pol I transcription seems primarily to be regulated at the level of initiation via the interaction of Pol I and Rrn3. Carles and co-workers constructed a fusion protein of the essential Pol I activating factor Rrn3 and the Pol I subunit Rpa43. This fusion protein can substitute the two essential endogenous proteins, and has a normal growth phenotype. However this strain termed *CARA* for *C*onstitutive *A*ssociation of *R*rn3 and Rp*RPA43*, was largely insensitive to rapamycin treatment / TORC1 (TOR complex 1) inhibition. Although the interpretation is disputed (the fusion protein needs to be over-expressed in the cell), this result suggests that the interaction Rrn3 with Pol I is sufficient to regulate Pol I mediated rRNA synthesis in response to nutrient availability. Rrn3 expression in the cell occurs only at catalytic

levels, suggesting that its availability is a good means to regulate Pol I transcription. Rapid Rrn3 degradation could therefore be a means to quickly adapt to changing growth conditions, a possibility speculated about in the mammalian (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990) as well as in the yeast (Claypool *et al.*, 2004) system. Recently, further evidence has been added to this speculation (Fátyol and Grummt), and more experiments will be needed to clarify the role of Rrn3 degradation in yeast.

Another study revealed that rRNA can also be directly degraded within the nucleolus (Dez *et al.*, 2006). This nuclear surveillance pathway is mediated via the TRAMP complex (Dez *et al.*, 2006).

The main pathway involved in regulating all three polymerase in response to nutrient and energy availability is as described above, the TOR pathway (see Figure 4).

More recent studies also describe a cross-talk between the polymerases: A Pol III mutant defective in tRNA expression is also defective in 35S production, suggesting a communication between the two polymerases (Briand *et al.*, 2001). Conversely, a Pol I mutant examined in the same work showed a deregulated tRNA phenotype (Briand *et al.*, 2001). More recently cross co-regulation has also been demonstrated between Pol I and RPGs transcribed by Pol II (Laferte *et al.*, 2006). The CARA strain mentioned above has besides its effect of a deregulated Pol I upon TORC1 inhibition another characteristic: Pol II transcribed RPGs as well as the Pol III transcribed 5S rRNA gene are as deregulated as the Pol I transcribed gene. This argues either for an upstream role of Pol I in the pathways regulating all polymerases, or that the signal(s) sent out by the active Pol I is / are stronger than the repressive signals received from the other polymerases individually.

In parallel, the group of Jonathan Warner described a complex of four proteins, casein kinase 2 , Utp22, Rrp7 and Ifh1, termed CURI that they could purify after glycerol gradient centrifugation (Rudra *et al.*, 2007). Since Ifh1 is an important RPG transcription factor and Utp22 and Rrp7 are involved in pre-rRNA processing events, the authors proposed a link between these two processes: while rRNA is active Utp22 and Rrnp7 would be busy, when Pol I transcription slows down, the two factors

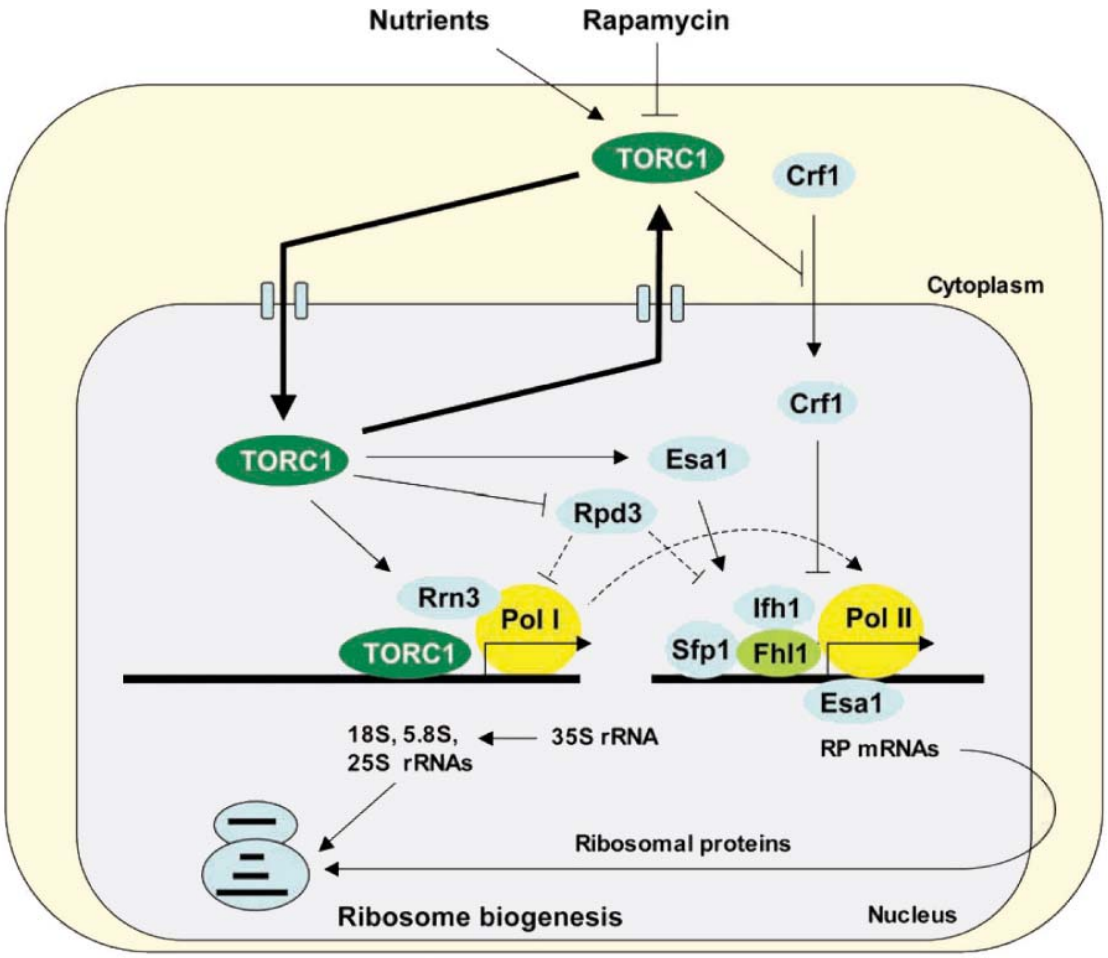

Figure 4. TOR Complex 1 transcriptional regulation of Pol I and –II expressed genes coding for ribosomal components.

Schematic illustration of regulatory interactions. Cross-regulation of Pol I with Pol II is indicated with dashed lines. Adapted from (Tsang and Zheng, 2007).

become available, bind casein kinase 2 and sequester Ifh1 reducing RPG transcription.

Further mechanistic details on how TOR acts on Pol I transcription has been brought forward by demonstrating that the catalytic subunit of TORC1, Tor1, shuttles between the nucleus and the cytoplasm in a starvation and / or rapamycin dependent manner (Li *et al.*, 2006). Furthermore, TORC1 associates with the rDNA locus (5S and 35S promoters) when nucleoplasmic and its association is required for rDNA transcription by Pol I (Li *et al.*, 2006). Rapamycin dependent rDNA binding and shuttling of Tor1 is not altered in a *tor1*-mutant that renders the cell rapamycin insensitive. This demonstrates the multiple levels of action mediated through TOR signaling.

More work is needed to clarify this link, and our work on Hmo1 (see below and results section) specifically addresses this point.

2. FUNCTIONAL COMPARTIMENTALIZATION OF THE NUCLEUS

Three enzymes are responsible for DNA dependent synthesis of RNA in the cell nucleus. How specificity of gene transcription is coupled with spatial positioning is still an open question. In the following chapter of the introduction, I will present a general overview on chromatin organization in cell nucleus and on the possible driving forces required for genome organization. I will conclude this chapter on the existing correlations between spatial positioning and transcriptional regulation.

2.1 Chromatin

2.1.1 Chromatin composition

2.1.1.1 Deoxyribonucleic acid (DNA)

Cellular deoxyribonucleic acid (DNA) forms two chains that are oriented anti-parallel against each other. The double strand forms a double helix. At least 3 three-dimensional structures of these chains are thought to be physiological: The so-called A-, the B- and the Z-DNA (Ghosh and Bansal, 2003). The B form is the most wide-spread and the one that has structurally been determined by Watson and Crick in 1953 (Watson and Crick, 1953) (Figure 5A). A- and B-DNA are organized as right-handed, mainly uniform helices; the Z-DNA in contrast is a wide left-handed helix where the backbone follows a zig-zag path (Figure 5B). The transition from B- to A-DNA or B- to Z-DNA structure is dynamic and transient, which is why the studies of these transient states is difficult (Sinden, 2005). While A-DNA conformation is favored by higher salt concentrations, the switch from B- to Z-DNA has been described with more realistic *in vivo* conditions (Ghosh and Bansal, 2003; Rich and Zhang, 2003). Transcription can be positively or negatively influenced since transcription factors do exhibit different binding affinities for B-DNA than for Z-DNA (Rich and Zhang, 2003). Along this line it has been reported that CpG-methylation of the DNA also shifts the equilibrium towards Z-DNA formation (Behe and Felsenfeld, 1981).

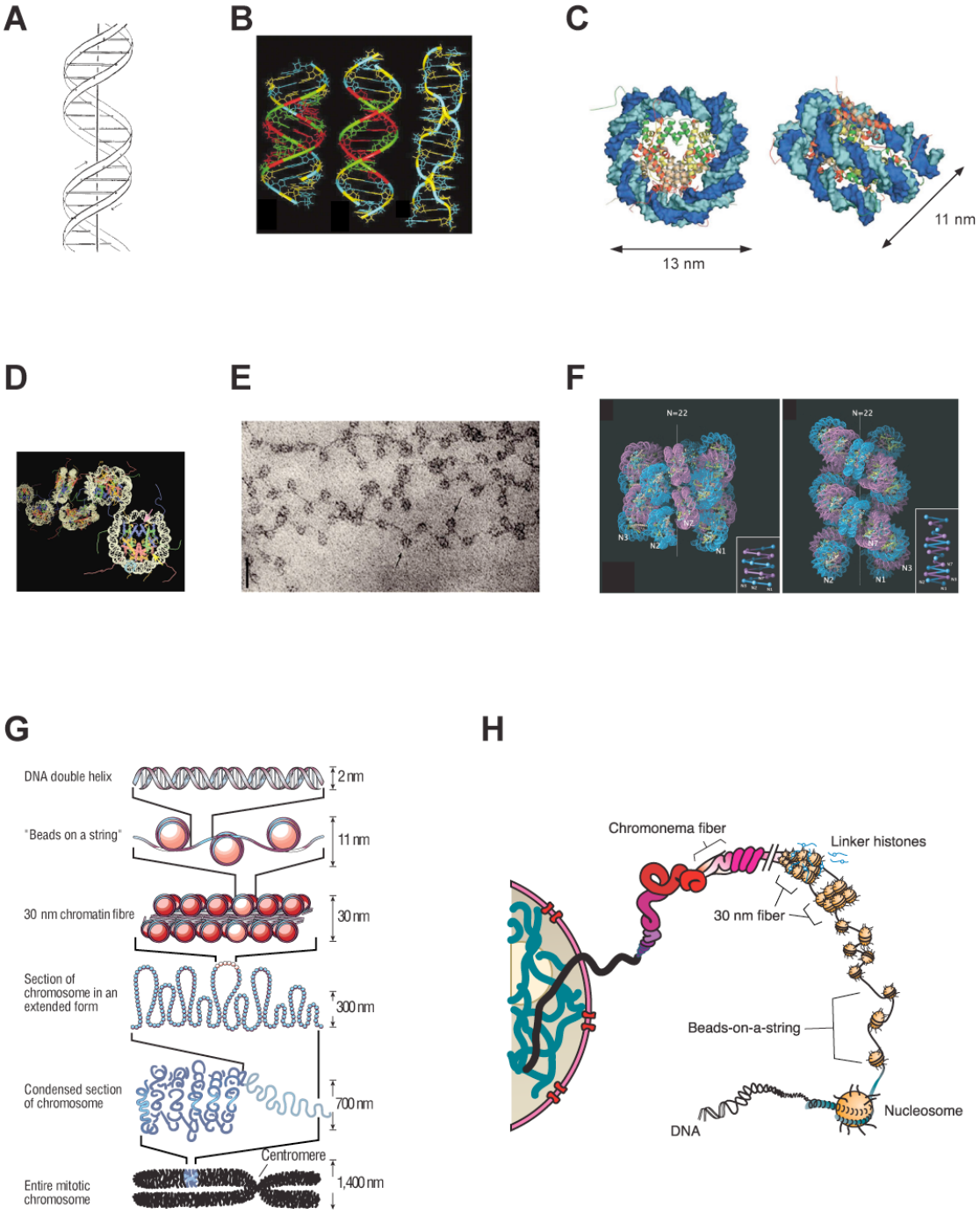

Figure 5. DNA and chromatin compaction.

A. DNA double helix structure as originally proposed by Watson and Crick in 1953. Adapted from (Watson and Crick, 1953).

B. Three possible helix structures: A-, B- and Z-structure models (from left to right). Adapted from (Ghosh and Bansal, 2003).

C. DNA wrapped around core histones (structure model), forming a nucleosome. Adapted from (Khorasanizadeh, 2004).

D. Structural model of nucleosomal DNA forming the 10 nm fiber. Adapted from (Luger, 2002).

E. Electron micrograph of a 10 nm fiber. “Beads-on-a-string”. Adapted from (Olins and Olins, 2003).

F. Structural predictions of a 30 nm fiber. “Solenoid helix” on the left and “zig-zag helix” on the right. Adapted from (Robinson *et al.*, 2006).

G. Textbook image of compacting DNA into a mitotic chromosome, assuming looping of the 30 nm fiber. Adapted from (Felsenfeld and Groudine, 2003).

H. Alternative model, predicting a further super-coiling of the 30 nm fiber leading into the so-called chromonema fiber. Adapted from (Horn and Peterson, 2002).

Like a telephone cord, additional twists can be added into the helix (positive super-coils), or the degree of twisting can be reduced (negative super-coils). In a cell, the DNA double helix is usually slightly negatively supercoiled (Giaever *et al.*, 1988). Topoisomerases are enzymes inserting these negative coils. Negative supercoiling has the physical effect to weaken base pairing interactions, facilitating process like transcription or replication (Vologodskii *et al.*, 1979).
A “relaxed“ (not supercoiled) B-DNA is 2,37 nm in diameter. One total twist is achieved with 10,4 basepairs or every 3,4 nm (Mandelkern *et al.*, 1981). With these values in hand a diploid human genome would sum up in ~ 2 m of DNA, but the nucleus containing this thread is only about 5 to 10 μm in diameter. This simple calculation demonstrates that further compaction of the DNA needed.

2.1.1.2 Histones

The next important constituent of chromatin is the nucleosome core particle. A complex assembled out of evolutionary highly conserved proteins called histones. Two copies of four different histones form this particle: H2A, H2B, H3 and H4 (Kornberg, 1974). The so called core histones are small, basic proteins with a common characteristic motif, called the histone fold consisting of three alpha helices in the C-terminal part are connected by short loops (Luger *et al.*, 1997). The histone fold is important for their ability to form dimers. Two H2A-H2B dimers and two H3-H4 dimers assemble into the histone octamer or the core particle. The nucleosome

core particle is forming a cylindrical structure with a diameter of 11 nm and a height of 5,5 nm ((McGhee and Felsenfeld, 1980), and references there in).

While the C-terminal parts containing the histone fold assemble in the inside of this particle, the N-terminal parts of the core histones orient in a manner fully accessible towards the exterior (Luger *et al.*, 1997). These so-called histone tails are especially rich in lysine and arginine, and are subjected to ongoing post-translational modifications (Kouzarides, 2007). Eight different types of modifications have been discovered so far. The best described modifications are the 3 small covalent modifications of acetylation, methylation and phosphorylation (Kouzarides, 2007). Especially the tails of H3 and H4 can be modified at multiple sites with important biophysical consequences on DNA-histone interactions or in changing binding properties of effector molecules like proteins. Importantly, one modification has an influence on another modification either in cis (on the same histone tail) or even in trans (influencing the modification on another histone tail within the same nucleosome core particle) (Jenuwein and Allis, 2001). Furthermore, regarding methylation of arginines or lysines, the residues can be either mono-, bi-, or tri-methylated (Kouzarides, 2007; Kubicek and Jenuwein, 2004). A modification by itself also at times has a consequence on itself, but mostly it depends in context to other modifications. Since this interplay is very complex a “histone code” has been postulated (Jenuwein and Allis, 2001). It implies that the modification of histone tails largely widens the information of the genetic code.

In summary, a nucleosome is clearly the first module of genome organization in eukaryotic cells. It is described up to the atomic level. We now need to use this structural description to establish the higher order organization of chromatin within the nucleus.

2.1.1.3 other components

histone variants

Although the core-histones are the major protein constituents of chromatin, there are a lot more proteins that are associated with it. Histone H1 is an another histone that helps to compact nucleosomes (Allan *et al.*, 1980). Additionally, there is a variety of histone variants that can replace the canonical core histones. While for H4 no variants

have been reported so far, There is one variant for H2B (H2Bv), two variants for H3 (H3.3, CENPA) and four variants for H2A (H2AX, H2AZ, macroH2A, H2ABBD) (Sarma and Reinberg, 2005). Specialized, function associated chromatin structures are established when a histone variant replaces a canonical histone. CENPA has a very distinct N-terminal region and has been shown to localize exclusively to centromeric chromatin having an important function in kinetochore assembly (Sullivan *et al.*, 1994). H3.3 is another example that has been associated with transcriptional activation, while macroH2A has been associated with transcriptional repression in X chromosome inactivation (Sarma and Reinberg, 2005) ,and references therein).

Histone variants are often associated with specific nuclear localization. This could clarify how compartimentalization is achieved in the nucleus. However, the correlation between histone variant association and sub-nuclear enrichment do not demonstrate a causal link between histone variants and spatial gene positioning.

HMG proteins

There is also a group of non-histone proteins associated with chromatin. They all belong to the HMG group discovered in the early 70s. They are named according to their mobility on polyacrylamid gels: After histone removal, chromatin preparations left two classes of proteins differing in their mobility on the gel, the “low mobility group” and the “high mobility group” proteins (Goodwin *et al.*, 1973). The HMG proteins turned out to be the second most abundant class of chromatin proteins after histones (Bianchi and Agresti, 2005).

The HMG family can be subdivided into 3 classes: HMGA-, HMGB- and HMGN proteins. The last letter indicates the characteristic of the proteins. HMG*A* proteins contain an *A*T-hook, a nine amino acid sequence that binds to the minor groove of AT-rich DNA stretches. HMG*B* proteins contain (at least one) HMG *B*ox, an 80 amino acid domain that also binds to the minor groove of DNA, but with very limited or no sequence specificity. HMG*N* proteins finally bind within *N*ucleosomes, between the DNA and the histone octamers (Bianchi and Agresti, 2005). They are the only non-histone proteins known so far to bind within the nucleosome.

HMGA and HMGN proteins are homogenously sequence unspecific. Instead, they recognize a discrete chromatin structure like bent, kinked and/or unwound DNA, generally described as “distorted DNA” (Hock *et al.*, 2007; Stros *et al.*, 2007). The

group of HMGB proteins can be split into two sub-classes: the one that is behaving exactly like HMGA and HMGN proteins with if at all, a very limited sequence specificity, while another sub-class is composed by specific transcriptions factors. These usually contain only one HMG-box and recognize their DNA-binding target with good sequence specificity (Stros *et al.*, 2007). Depending on the definition, since these transcription factors possess sequence specificity, they are sometimes classed as "HMG-box proteins", but not HMGB proteins. (Bianchi and Agresti, 2005).
In the last part of the introduction (see 3.), and in result section, a large aspect will be the protein Hmo1, an HMG-box protein. The function of HMG proteins are clearly linked to both, spatial positioning and gene regulation, and constitute a paradigm for understanding the causal link between gene position and transcriptional regulation (see discussion).

Other chromatin associated proteins

Other chromatin related proteins include protein families that are dedicated to the maintenance of a certain chromatin state, like a transcriptionally repressive or active state. Two famous families within this regard are Polycomb and Trithorax group proteins. While polycomb proteins are proteins that establish and maintain a repressed structure, trithorax group proteins do the inverse, establishing a transcriptionally active chromatin (Schuettengruber *et al.*, 2007).
Furthermore, any chromosome associated protein can be considered to be part of chromatin. Thus, depending on the definition, a general transcription factor can be judged as a chromatin component.

RNA

RNAs interacting with chromosomes have been first described in the mid-sixties (Huang and Bonner, 1965) and have shortly after been shown to interact with repetitive DNA sequences (Sivolap and Bonner, 1971). Today we know that these are non-coding, non-translated RNAs that bind sequence specific to their DNA, mainly to inactivate by their presence themselves or to further recruit an inactivating protein machinery. The first example studied thoroughly over the last years is the *XIST* RNA ("*X i*nactive *s*pecific *t*ranscript", (Brown *et al.*, 1991)). It is responsible for starting a

reaction known as “dosage compensation”, inactivating one of the 2 female X-chromosomes in mammals. At the end of this inactivation process, *XIST* covers the whole inactivated X-chromosome helping to render it inaccessible for the transcription machinery and therefore rendering it transcriptionally silent (Chow *et al.*, 2005).

That DNA accessibility is mediated by association between RNA and DNA closes an interesting regulatory loop: RNA polymerase synthesizes RNA, and the product can inhibit RNA synthesis. However, this speculative model based on a feedback regulatory principle remains to be established.

2.1.2 Chromatin compaction

2.1.2.1 nucleosomes and the 10 nm fiber

The first level of DNA compaction consists of DNA wrapped around a histone octamer (Figure 5C). Due to its negatively charged sugar phosphate backbone, DNA gets pulled onto the positively charged exterior of the histone octamer. This structure was first observed by electron microscopy (EM) in the mid 1970s (Olins and Olins, 1974) and termed nucleosome (Oudet *et al.*, 1975). Oudet and co-workers presented the electron microscopic and biochemical data of the repetitive structure of histones on DNA, discussed a year before by Roger D. Kornberg (Kornberg, 1974; Oudet *et al.*, 1975). The authors already describe nucleosomes as “spherical particles, about 125 Angström (Å) in diameter” and mark out the varying “internucleosomal distance (…) from about 10 to several hundred Å” (Oudet *et al.*, 1975). Nucleosome binding to DNA is largely sequence unspecific, although recent genome wide nucleosome positioning experiments in yeast and in drosophila report a vague consensus of preferred nucleosome binding (Mavrich *et al.*, 2008; Segal *et al.*, 2006; Yuan *et al.*, 2005). Today nucleosome organization is described in detail: 146 bp of DNA are organized in 1,65 flat, left-handed superhelical turns around a histone octamer. A slightly disc shaped nucleosome is 13 nm in diameter and 11 nm in height (Khorasanizadeh, 2004; Luger *et al.*, 1997; Oudet *et al.*, 1975) (Figure 5C). Nucleosome are separated by a DNA stretch of 0 to ~ 80 bp (Widom, 1992). This

"free" DNA is called *linker DNA*, opposed to the DNA in contact with the histone octamer, termed *nucleosomal DNA* ((Hansen, 2002) and references therein).
Due to the size of a nucleosome, this compaction level is also known as the *10-nm-fiber*, or, with respect to the nucleosomes positioned repetitively on the DNA thread, *beads-on-a-string* (see Figure 5D and 5E). It allows to compact DNA by about 6 to 7 fold. This level of compaction cannot account of the organization observed in the cell nucleus. A higher order of chromatin organization is required. However, each subsequent level of organization remains speculative and remains mostly at the level of models.

2.1.2.2 The "30-nm fiber"

The next level of compaction is achieved by wrapping the DNA even further around the histone octamer. Under these circumstances (where the number of turns reaches 2) the nucleosome is stabilized by the linker histones, predominantly H1 (Allan *et al.*, 1980) or its close relative H5 that has so far only been found in nucleated (avian) erythrocytes (Neelin *et al.*, 1964).
Linker histone binding induces the formation of the next level of chromatin compaction, the "30 nm fiber", as shown by numerous *in vitro* studies. This allows an additional compaction of a factor of around 6 (Hansen, 2002). The actual structure of 30 nm fiber has not been determined yet (the structure is too compact to be visualized), which is why several hypothetical conformations are discussed. The two most plausible models arising from the information known about the nucleosome structure are referred to as the "solenoid helix" or one-start helix, and the "zig-zag" or two start helix (see Figure 5F). While a low resolution crystal structure seemed already to contribute to the key argument for the zig-zag structure (Schalch *et al.*, 2005), another study provided good arguments for a third, interdigited structure by EM measurements (Robinson *et al.*, 2006). Last but not least, researchers have not succeeded yet in demonstrating that the 30 nm fiber really exists *in vivo*, too. All studies so far have been made only *in vitro* or on a purified fiber; despite big efforts the structure hasn't been observed in sections of whole nuclei (Tremethick, 2007).
Recent work in yeast has tried to contribute to the discussion using light microscopical techniques (Bystricky *et al.*, 2004). Bystricky and co-workers used fluorescent probes that hybridized on the same chromosome arm at various distances

to one another. The positions of the loci were determined with high precision and the calculated values correlated with the known physical distances in bp between them. The authors conclude that their values support a 30 nm like structure *in situ* (Bystricky *et al.*, 2004).

The 30 nm fiber compacts the DNA now to a factor of 30 – 40. Further compaction is still needed. This is achieved by arranging the 30 nm fiber in higher order structures, which remains to be understood.

2.1.2.3 Chromatin loops and / or the chromonema fiber

At this level most studies describing chromatin arrangement use biophysical modeling of the fiber. These models are supported by some experimental data but require more investigation to test their validity in nuclei of living cells. Accordingly, chromatin organization above the 30 nm fiber is disputed.

One can summarize three different models of higher order chromatin fiber structure:

(1) The radial loop model. About 10 radial loops of ~ 50 to 200 kbp each form a rosette like chromatin structure. One chromatin rosette would then be constituted of about 1 Mb (Münkel *et al.*, 1999). A simulation by Münkel *et al.* assuming this structure fits the results observed by fluorescence in situ hybridization, at a 10 and 100 Mb scale. Furthermore, according to the authors, the model is applicable over all cell cycle stages.

(2) The giant loop model. Again a loop structure is proposed. The important difference is that only one big loop composed of about 3 Mb of DNA is assumed (Sachs *et al.*, 1995). The flexibility within such a loop would be much higher, which is an explanation for the interpreted random walk of adjacent DNA sequences. Loci with various distances from each other had been labeled using FISH and their observed mean square distances plotted against their genomic positions (Yokota *et al.*, 1995).

Both of these models have the fact in common that they form loops around a certain structure. Often this structure is considered as a stiff nuclear skeleton which causes some controversy (see 2.2). Other explanations are less implicit, speaking rather of “attachment points” (Sachs *et al.*, 1995). With this less rigid definition, chromatin loops could for example be linked to each other by functional protein complexes. One such thought is that DNA replication is taking place at the bottom of the loops

(Gilbert *et al.*, 2004), where a very stable replication machinery localizes (Leonhardt *et al.*, 2000), implying that DNA is spooled through these complexes rather than the machinery around the DNA.

(3) the chromonema fiber. While most textbooks describe a looping model (see Figure 5G), others draw the picture without any looping but rather with additional helical coiling of the 30 nm fiber as the last form of chromosome condensation (Belmont, 2002) (see Figure 5H). In 1994, Andrew Belmont reported DNA structures with a diameter of either 60-80 nm or of a slightly thicker 100-130 nm (Belmont and Bruce, 1994). He proposed that the 30 nm fiber gets further coiled up as a helical structure, termed "chromonema fiber". More recent data from his lab seems to be consistent with a more complicated structure than a looped 30 nm fiber structure: they engineered different densities of scaffold attachment sequences onto a chromosome arm trying to force loop formation of a chromatin stretch. However, no loop formation could have been observed by followed ultra-structural *in vivo* analysis (Strukov *et al.*, 2003).

There is no clear answer to higher order chromatin structure, yet. Some observed phenomena fit better to one model and *vice versa*. Co-existence of the three structures a is possible scenario too. Such a co-existence has already been proposed for the two different loop models (Kosak and Groudine, 2004).

The next step to better understand genomic organization would be to gain some experimental measurements of genomic organization to test these biophysical predictions. Some of our work could contribute to give experimental data to confront these models to yeast genomic organization (see result and discussion sections).

2.1.3 Chromatin distribution

In the first part of the chromatin organization chapter, I tried to describe the existing structural data, and the biophysical model of chromatin fibers mostly extrapolated from this structural knowledge. Another approach is to observe nuclear organization with fluorescent or electron microscopes to describe the organization of the chromatin chains in the nucleus. In the following part, I will describe the organization of chromosomes in the nucleus, and the hypothetical model built from these observations.

2.1.3.1 Euchromatin and Heterochromatin

In the beginning of the 20th century, Emil Heitz could observe two types of chromatin that stained differently on treating cells with carmin acetic acid. Using this staining technique he could observe cells with a light microscope through the cell cycle. He termed the two staining patterns euchromatin (EC) and heterochromatin (HC, (Heitz, 1928)) (see Figure 6A). Euchromatin translates into "true" chromatin, since he observed that this part of chromatin decondenses during cell cycle progression from metaphase to interphase, while heterochromatin did not. He also postulated that heterochromatin was "genicly inactive" and "contain(ed) no or somehow passive genes" (Heitz, 1929).

Later the distinction between "facultative and constitutive HC" was made (Brown, 1966). Constitutive HC is mainly characterized by highly repetitive DNA structures, found especially at peri-centromeric regions (of metazoans) and telomeres (Grewal and Jia, 2007). Constitutive HC is permanent, stays in the HC-state during the whole lifespan of the cell. Facultative HC is tissue dependent, what is "heterochromatinized" in one cell type is found in the euchromatic compartment in others; the chromatin state can also change in response to cellular signals and gene activity (Grewal and Jia, 2007). We now start to understand that this change in state is a consequence of histone modifications (like of histone 3 lysine 9 methylation) followed by recruitment of chromodomain proteins (like HP1) (Grewal and Jia, 2007). Further research will be required to elucidate the interplay of non-coding RNAs,

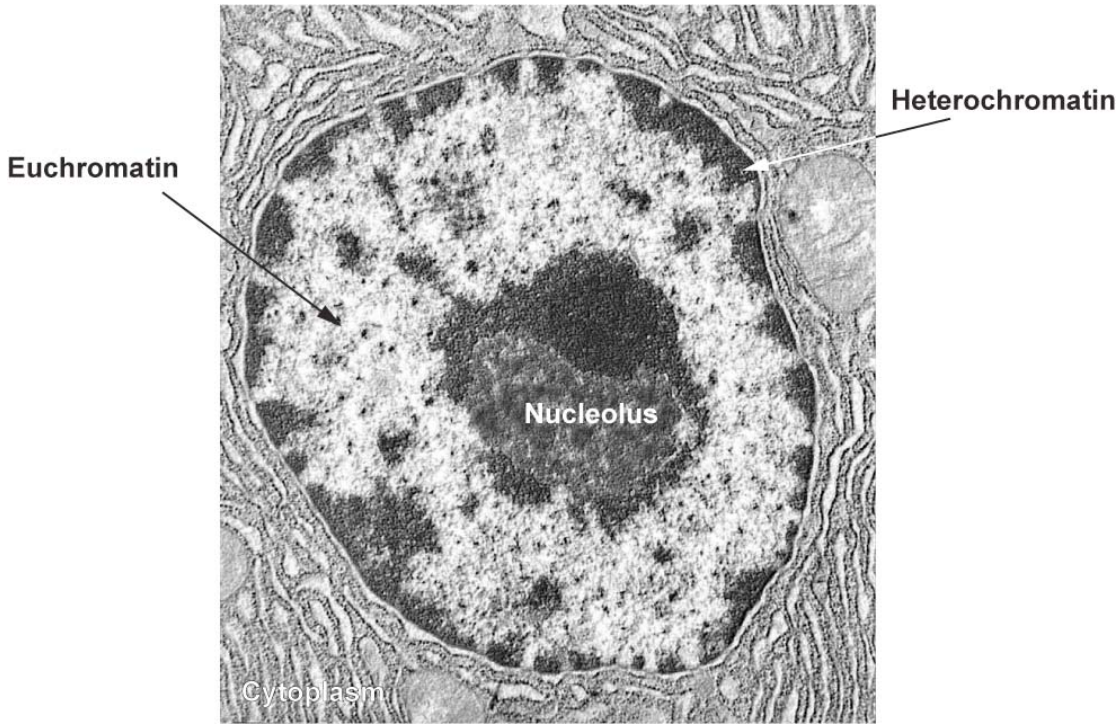

Figure 6. Hetero- and euchromatin seen in transmission electron microscopy.

Vertebrate cell nucleus. A nucleolus can be seen in the center, surrounded by electron-dense (darker) heterochromatin, which can also be found concentrated at the nuclear periphery. Euchromatin is more electron permeable (lighter) and is mainly situated in the more central nuclear domains. Courtesy of Kenneth M. Bart, Hamilton College, NY, USA.

protein recruitment, histone modifications and DNA methylation in the formation of HC.

2.1.3.2 Chromosome territories

Chromosome territories (CTs), the structured arrangement of chromosomes within the interphase nucleus, has been first postulated by Carl Rabl in 1885 (Rabl, 1885). The name "chromosome" did not exist at that time, yet he suggested that the structures he observed during mitosis in amphibian nuclei would stay as entities during interphase (when they turned invisible to his microscopic observation). The name "chromosome territory" was then given by Theodor Boveri in 1909 (Boveri, 1909), who added the notion, that chromatin bundles are pervaded by an interchromatin space (Boveri, 1909; Cremer and Cremer, 2006a).

Furthermore Rabl speculated that the orientation of the chromosome during mitosis, with the centromeres on one side and the orientation of the telomere towards the other, would to some extent, be conserved in interphase (Rabl, 1885).

After a time of disregard and disgrace, the CT were continued to be studied using new techniques in the late 1970s (for review see (Cremer and Cremer, 2006b)). Two big advances in modern CT studies should be pointed out: (1) the first fluorescent *in situ* hybridization (FISH) experiment "painting" a whole human chromosome in a (human) metaphase and interphase nucleus (Lichter *et al.*, 1988) and (2) the first *in vivo* labeling of a whole chromosome using fluorescently modified nucleotides. The nucleotides were injected into human fibroblasts. During S-phase the modified nucleotide incorporates into the newly synthesized DNA-strand. This way, a late replicating chromosome could be followed individually over some cell generations (Zink *et al.*, 1998).

These techniques used were major advances that, with others, clarified the existence of CTs and have revealed some major organizational features. While the sub-CT structure is still a matter of debate, discussing different loop-size models and a possible chromonema structure (see above). One question that has been addressed is how CTs are organized within the nuclear volume. Wendy Bickmore and co-workers suggested that gene poor chromosomes (chromosome 18) are localized towards the exterior, while gene rich chromosomes (chromosome 19) could be localized more

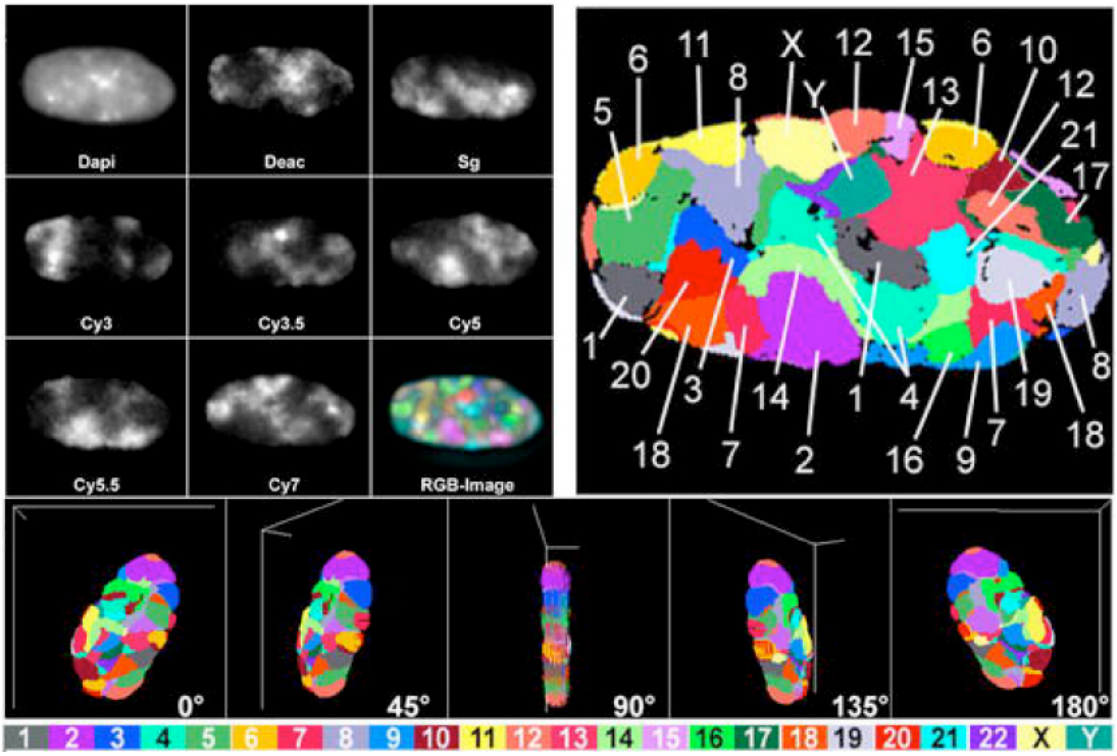

Figure 7. Chromosome territories and chromatin distribution.

Visualization of all 46 chromosomes in a human fibroblast using eight different dyes for fluorescence in situ hybridization. Strong territorial organization into chromosome territories can be observed. From (Bolzer *et al.*, 2005).

internally (Croft *et al.*, 1999). The studied chromosomes are about the same size, which is another aspect, recently proposed to play an important role on CT positioning: The laboratory of Thomas Cremer was able to "paint" all chromosomes in nuclei from different organisms (with different chromosome numbers) at once (Bolzer *et al.*, 2005; Habermann *et al.*, 2001) (see Figure 7). From their arrangement they first confirmed Bickmore's speculation about gene density having an impact on chromosome localization (Bolzer *et al.*, 2005). Second, they draw the conclusion that, irrespective of their gene content, smaller chromosomes tend to localize more central than bigger ones that seem to preferentially localize towards the exterior (Bolzer *et al.*, 2005).

Another important finding was that chromosome localization seems to change during differentiation. Chromosomes exhibit different localization patterns, when comparing different types of tissue (Parada *et al.*, 2004). Parada *et al.* also showed that chromosomes whose territories are in close proximity to each other are more likely to have translocation events occurring than those further away (Parada *et al.*, 2004). This demonstrates the functional relevance of chromosome territories.

Although CTs are considered as entities within the nuclear volume, this does not mean that they have sealed surfaces. The degree of intermingling of different chromosomes is still controversial. Three scenarios are discussed (Branco and Pombo, 2007; Heard and Bickmore, 2007):

(1) the original model with the highest degree of CT solitude, the interchromosome domain (ICD) or chromosome territory–interchromatin compartment (CT-IC) model, proposes a boundary and a space (the ICD) between adjacent CTs, containing the transcription machinery. Active genes here need to be situated at the outside of a CT, the transcription machinery cannot penetrate the CT (Zirbel *et al.*, 1993).

(2) opposed to this model stands a the "lattice model", proposing that chromatin fibers form a lattice with large CT accessibility, including the transcription machinery. It has been proposed after analyses using electron spectroscopic images of CTs (Dehghani *et al.*, 2005). On electron spectroscopic images no channel like structure of the ICD could be observed and the definition of ICD is the space between the 10 – and 30 nm fibers.

(3) An intermediate in terms of CT integrity is predicted by the interchromatin compartment (IC) or interchromosomal network (ICN) model. Here, chromatin can

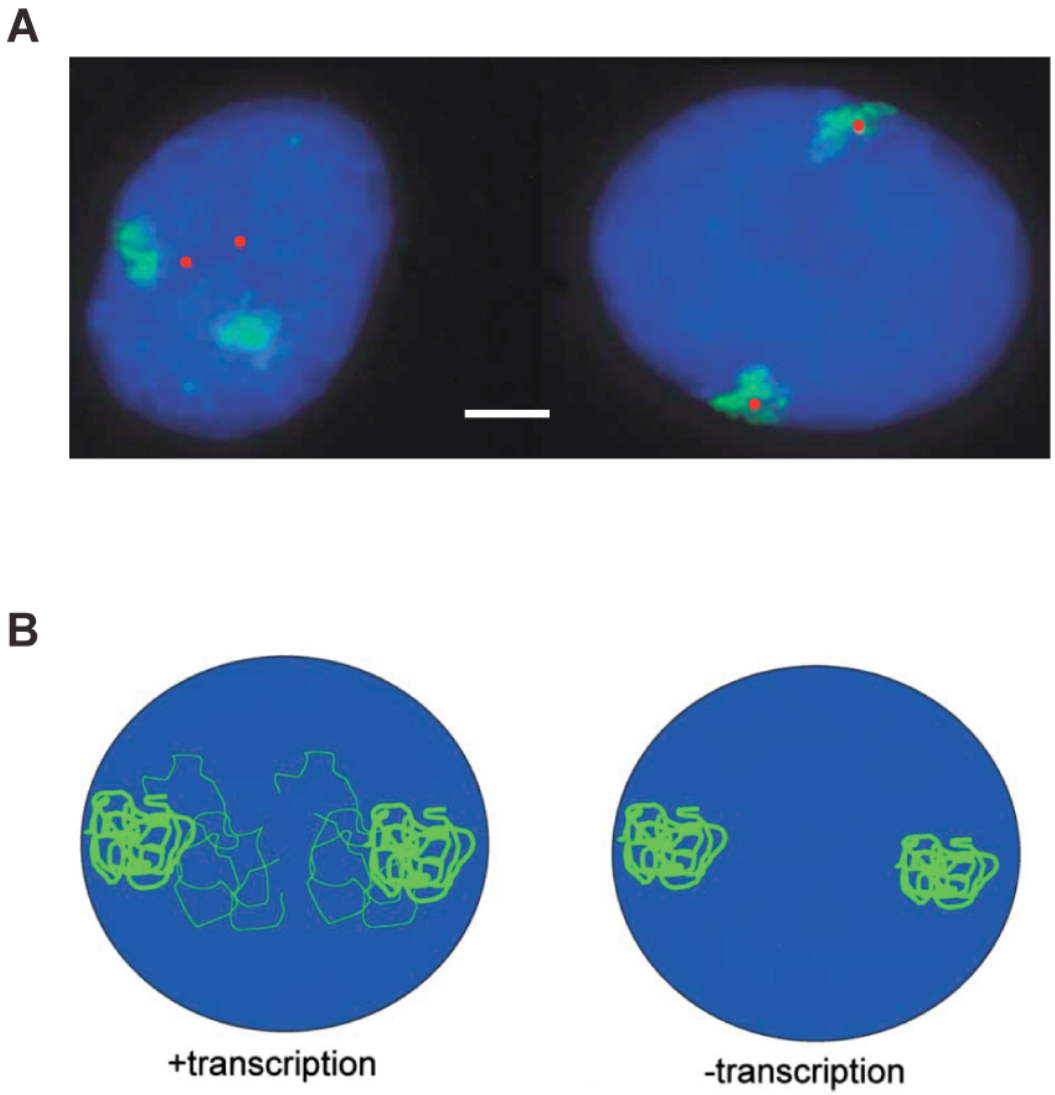

Figure 8. Transcription dependent gene localization with respect to the gene chromosome territory.

A. Loci (red, signal manually amplified) with very high transcriptional activity that are found outside their corresponding chromosome territory (CT) in human lymphoblasts. After transcription inhibition, loci are more frequently found within their CT. Scale bar is 5 µm.

B. Model, explaining the above described phenomenon with chromatin decompaction and –compaction.

Adapted from (Chubb and Bickmore, 2003).

loop out of its CT, way further and more frequently than in the ICD model, allowing high frequency contacts between loci situated on different chromosomes. Capturing chromosome conformation (3C) and its large scale variants support this model ((Dekker *et al.*, 2002; Simonis *et al.*, 2006) and see 2.3.4). This model also fits ultra-structural data obtained in a study using differently sized nano-gold particles for *in situ* hybridization followed by EM imaging (Branco and Pombo, 2006). Branco *et al*. also demonstrated that the intermingling is reduced when Pol II is inhibited.
The different models are not mutually exclusive. RNA Polymerase II exclusion has been demonstrated for the inactivated X chromosome in mouse cells (Chaumeil *et al.*, 2006), while activated loci can be observed looping out far from their CT (see (Heard and Bickmore, 2007) for review and references) (see Figure 8). Finally, activated genes can also be transcribed within their CT (Mahy *et al.*, 2002), as suggested by the lattice model.

This controversy illustrates the technical limitations of FISH, in which the existing tools limit the understanding of the structural organization. Further progress will be possible if we could integrate physical modeling of the chromatin fiber in our detection methods, or use newly designed super-resolution microscopy (such as PALM of STORM reviewed in (Heintzmann and Ficz, 2006)). We could likewise use a methodology using statistical descriptions to improve existing imaging techniques to determine gene positions (see result section).

2.1.4 Chromatin dynamics

There are three different understandings of “chromatin dynamics”. a) The chromatin constituents that are in constant exchange with the chromatin fiber (residence times). b) Chromatin constituents and especially nucleosomes that “slide” horizontally on the DNA, changing local DNA accessibility. c) Large scale chromatin relocalization / redistribution. The first two “small scale” definitions are most likely essential for “large scale” chromatin motion. I will therefore briefly mention these aspects.

2.1.4.1 Local chromatin dynamics / nucleosome dynamics

The early beads-on-a-string configuration description of DNA wrapped around nucleosomes is a rather static notion, but DNA must be accessible at many times during DNA metabolism. The published nucleosome localizations determined in numerous organisms (see 2.1.2.1) can therefore only be interpreted as statistical probabilities to find a nucleosome at a given position and a specific time. For some positions, the probability of nucleosome occupancy is high reflecting a stably positioned nucleosome while for others it is weak, indicating either the absence of nucleosomes or a higher mobility. The dynamics of the nucleosomes can be modeled by different means. As mentioned earlier, the protein composition can change dynamically by incorporation of histone variants (see 2.1.1.3). Furthermore, the post-translational modifications of histone tails within the nucleosome are important for all histone interactions (like histone-DNA, histone-histone and histone-non-histone protein interactions) and hence influence the stability of the nucleosome on the DNA. Finally, chromatin remodeling complexes, or "remodelers", can actively change nucleosome-DNA interactions, disrupt a nucleosome (partially, facilitating protein re-composition of the nucleosome, or complete eviction), or change its position on the DNA fiber ("nucleosome sliding"). Their co-ordination, recruitment to and functional consequence at a given locus is a field of extensive research at the moment and will not be further detailed in this introduction. Although the links between chromatin structure and biochemical chromatin modifications are not well established yet, all these changes, such as histone modifications, nucleosome composition or nucleosome occupancy have an important impact on the compaction level of the chromatin fiber and the DNA itself (Felsenfeld and Groudine, 2003; Heard and Bickmore, 2007). To prevent a gene's or a whole domain's cross-regulation with differently active neighboring genes, so-called insulators are present in the genome.

Insulators or boundary elements are DNA sequences that act as barriers to protect a gene or a domain against an activating influence from near enhancer elements associated with other genes or against encroachment of adjacent inactive, condensed chromatin (Burgess-Beusse *et al.*, 2002). Best characterized in humans and in Drosophila, insulators also have been described in yeast (Fourel *et al.*, 1999). This way, local activation of a chromatin domain could explain the looping of a domain out of its otherwise probably more compact chromosome territory (cf. 2.1.3.2).

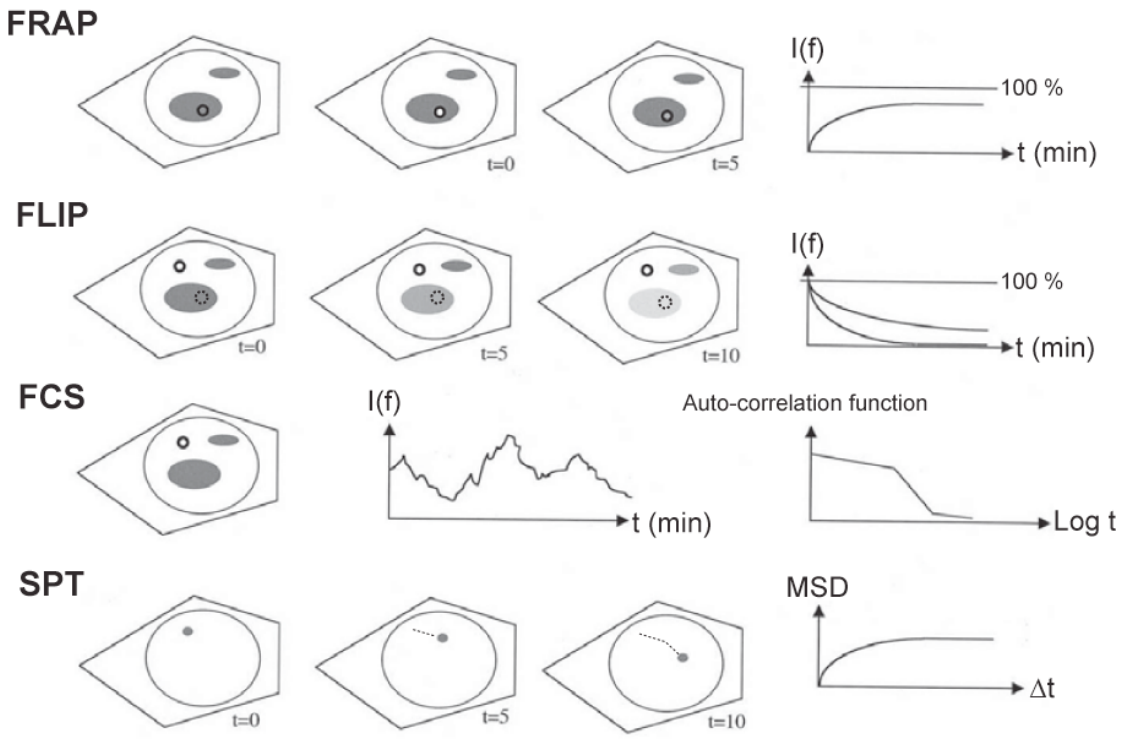

Figure 9. Different microscopic techniques to study the dynamics of fluorescent molecules.

FRAP. Fluorescence Recovery After Photobleaching. After having photo bleached a region of interest (circle), the signal recovery in this same area is measured over time.

FLIP. Fluorescent Loss In Photobleaching. A region of interest (circle) is photo bleached and loss of fluorescence in a different region is measured (dashed circle) afterwards.

FCS. Fluorescence Correlation Spectroscopy. A small volume (circle) is illuminated and the fluorescence intensity in this volume is measured over time with high temporal resolution and sensitivity. An auto correlation curve is then extracted from the measured values.

SPT. Single Particle Tracking. A single fluorescent particle is followed over time and the mean square displacements (MSD) are calculated and plotted against time intervals (Δt).

Adapted from (Boulon *et al.*, 2002).

2.1.4.2 Global chromatin dynamics / chromatin fiber dynamics

The dynamics of chromatin within nuclear space is another aspect of "chromatin dynamics". But here again a distinction has to be made between the mobility of chromatin constituents and a given locus itself. For the first, the "F-techniques" (FRAP, FLIP, FCS, see Figure 9 for details) have proven very useful. Expressing GFP-histone fusion proteins, several studies have demonstrated that residence times of histones are very high compared to other nuclear proteins (Cheutin *et al.*, 2003; Kimura and Cook, 2001; Phair *et al.*, 2004; Wachsmuth *et al.*, 2003). The authors could also make a distinction between a free pool of histone molecules and an apparently incorporated one.

The mobility of a locus on the chromatin fiber has so far essentially been studied using single particle tracking (see Figure 9) techniques. The most common approach to visualize a locus in a living cell is to insert repetitions of bacterial operator sequences into the genome (such as lacO or tetO) that are bound by their corresponding repressor protein (lacI or tetR, respectively), that has been genetically fused to a fluorescent protein (Marshall *et al.*, 1997; Robinett *et al.*, 1996; Straight *et al.*, 1996).

Marshall *et al*. were the first ones to determine the mobility coefficients and confinement of a given locus. They followed an array of tet-operators, integrated in the *LEU2* locus in S. *cerevisiae* for up to 10 minutes. First, they noted that the locus undergoes Brownian motion. In more detail, they observed a constrained diffusion with a diffusion coefficient of 5×10^{-4} μm^2/sec, and a radius of 300 nm. Treatment of the cells with nocodazole, which depolymerizes microtubules, increases the radius of the confined region to 700 nm. The *LEU2* locus is situated in chromosome III at only 22 kb of the centromere that is attached via microtubules to the SPB (see 2.2.4). This demonstrates the importance of this structure in organizing yeast nuclear architecture (see 2.2.4).

Further analyses of more loci confirmed the results obtained by Marshall and co-workers of a movement due to diffusion with a radius of confinement (Heun *et al.*, 2001). However, the originally measured diffusion coefficients in diploid yeast of 5×10^{-4} μm^2/sec (Marshall *et al.*, 1997) are one order of magnitude lower than the ones reported in all other studies (done since on haploid yeast) (Bystricky *et al.*, 2005; Cabal *et al.*, 2006; Gartenberg *et al.*, 2004; Hediger *et al.*, 2002; Heun *et al.*, 2001;

Taddei *et al.*, 2006). Furthermore, the results show, that the diffusion coefficients as well as the radii of confinement differ slightly, depending on the position of a locus on the chromosome or its transcriptional activity (see 2.3.2): telomeres anchored at the periphery seem to have a reduced mobility and area of confinement than loci located more centrally on the chromosome arm (Bystricky *et al.*, 2005; Cabal *et al.*, 2006; Gartenberg *et al.*, 2004); autonomously replicating sequences (ARS) tend to be more confined at the beginning of S-phase (Heun *et al.*, 2001) and activated genes that are tethered to the periphery also show a reduction in the volume that they can explore (Cabal *et al.*, 2006).

Cabal *et al*. also postulated that loci independent of activity do not undergo free diffusion (at least in small time scales up to one minute), as postulated by earlier reports. The authors rather observe an anomalous, sub-diffusive behavior for their locus of interest (Cabal *et al.*, 2006).

Occasional "jumps" of loci with an amplitude of more than 500 nm have been reported by Heun *et al*. (Heun *et al.*, 2001). These jumps were dependent on ATP.

Finally, is has been shown that a *LYS2* locus excised to form an episome shows increased mobility and is no longer restricted to confined regions within the nuclear volume (Gartenberg *et al.*, 2004).

Interestingly, observations in HeLa cells indicate that diffusion coefficients seem to be roughly similar in yeast and human cells (Chubb *et al.*, 2002; Heun *et al.*, 2001). The HeLa cell experiments also demonstrated that loci in the vicinity of the nuclear periphery and close to nucleoli are reduced in their mobility, in accordance with the idea of more condensed heterochromatin preferentially located in these places (Chubb *et al.*, 2002).

Recent work has now not only proposed an energy dependency for long range chromatin movement, but also postulated and demonstrated the involvement of nuclear actin and myosin (Chuang *et al.*, 2006; Dundr *et al.*, 2007; Nunez *et al.*, 2008). Ever since the discovery of nuclear actin 40 years ago (Lane, 1969), its role has been very controversial. The discovery of a nuclear non-polymerizing form of myosin I ("myosin Iβ") is another argument for a potential actin-myosin concerted action (Pestic-Dragovich *et al.*, 2000). Implications of nuclear actin have also been reported for all three nuclear RNA dependent polymerases (reviewed in (Percipalle and Visa, 2006)). Actin/myosin dependent long-range range chromatin movements now implicate a polymerized, filamentous form of actin, as tested with actin

polymerization / depolymerization specific drugs for example (Nunez *et al.*, 2008). This adds new fuel to an on-going discussion since influence of unpolymerized forms of these proteins had almost gotten accepted during the last years.

2.2 Determinants of nuclear compartimentalization

In this section of the introduction, I will present known evidence that the organization of the genome can contribute to the accessibility of defined genetic elements to the transcriptional machinery. How this specific organization is achieved becomes a central question in regulating nuclear process. I will start this section with opposed models that have been suggested to organize nuclear space: the nuclear matrix, and the self-organization. From these two diametrically opposed models, I will discuss recent evidence for local structural elements together with self-organization as driving force, which could help us to understand nuclear organization.

2.2.1 The "nuclear matrix"

The most intuitive idea of how a structure within the nucleus can be achieved and maintained is to imagine a stiff scaffold spanning the nuclear volume. To this lattice, all other structures could be attached via specific or unspecific interactions.
Such a structure is indeed described. In the 1940s a high salt extraction procedure has been shown to leave a residual structure within nuclei (Zbarskii and BDebov, 1948). In the mid 1970s the name "nuclear matrix" was given to this structure, using essentially the same preparation procedure (Berezney and Coffey, 1974). In metazoans, a thin network of type V intermediate filaments of about 10 nm in diameter, the nuclear lamina, is associated with the nuclear envelope (Fawcett, 1966). The lamina is composed of lamins and lamin-associated proteins (Gruenbaum *et al.*, 2005) (see also 2.2.3). Employing electron microscopy on an extracted nuclear matrix, one can observe the nuclear lamina surrounding a network of structured fibers (Nickerson, 2001) (see Figure 10) . These fibers have different diameters depending on the stringency of the extraction procedure. The "classical" matrix preparation

protocols include 3 steps (Nickerson, 2001). (1) non-ionic detergent extraction to remove membranes. (2) DNase digestion to fragment DNA. (3) hypertonic salt washes to remove the DNA. In the widest definition, all non chromatin structures found in the nucleus belong to the nuclear matrix (Fawcett, 1966; Nickerson, 2001). Stringent washes during matrix preparations are reported to leave nothing but a network composed of ribonucleoproteins or RNPs (Nickerson, 2001). Heterogeneous RNPs (hnRNPs) are therefore considered the most important backbone of the matrix (not considering the lamins). Other descriptions of additional matrix constituents are rather vague. A class of proteins termed the "matrins" in analogy to the lamins has been characterized to localize within the "fibrogranular matrix" and illustrates such an example (Hakes and Berezney, 1991). Not only RNA was retained in Matrix preparation but also short sequences of DNA. These DNA sequences remain in the matrix preparations after restriction enzyme digestion and stringent washes and are therefore supposed to be bound to the nuclear matrix (Boulikas, 1993). The sequences have been termed as matrix associated regions or MARs, or in the context of a mitotic scaffold SARs for scaffold associated region. The isolated sequences have been analyzed and consensuses have been determined. In some cases the sequences have been found to be related to active DNA elements, like transcriptional enhancer elements (Seo *et al.*, 2005) or close to a replication origin (Dijkwel and Hamlin, 1988; Hozak *et al.*, 1993).

The "nuclear matrix" itself is a highly disputed structure (for review see (Pederson, 2000)). The criticism arises from the high ionic strength extraction procedure, needed to visualize the filamentous network. Although two alternative procedures have been developed more recently to avoid this treatment (Jackson and Cook, 1988; Mirkovitch *et al.*, 1984), the main criticism remained: removing the DNA (and sometimes RNA) from the nuclei, the major anions that form electrostatic bonds with cationic groups of nuclear proteins is eliminated (Pederson, 1998). This can lead to protein rearrangement as seen in ribosomes whose rRNA is digested (see (Pederson, 1998) for references). Filamentous fibers have even been documented for hnRNPs when depleted from their RNAs (Lothstein *et al.*, 1985).

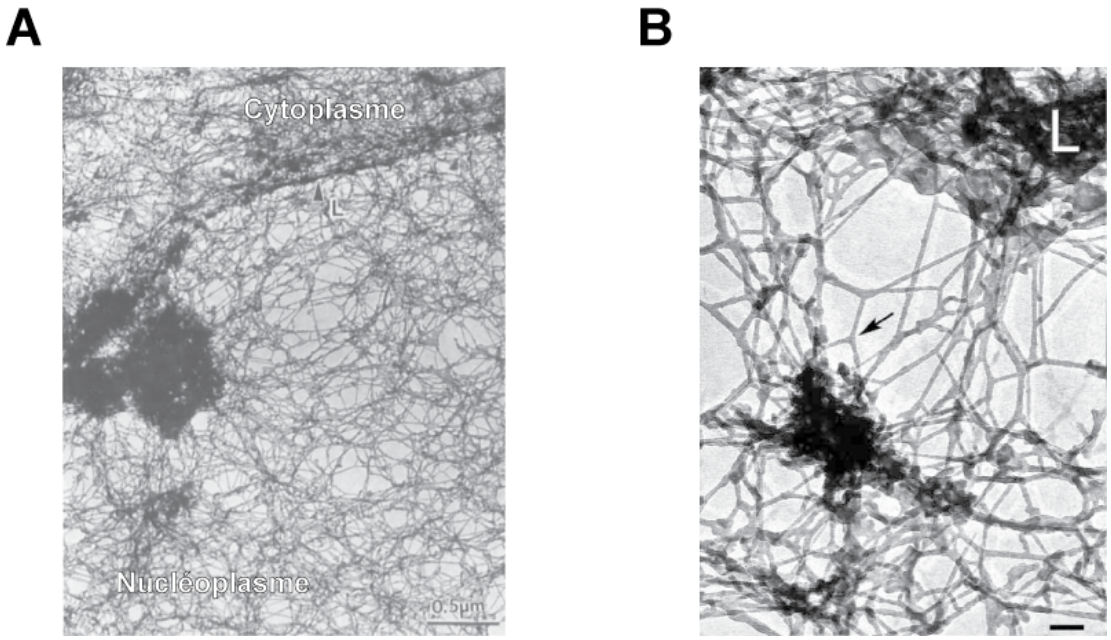

Figure 10. The nuclear matrix.

A. Electron micrograph of a HeLa cell extracted with 2M NaCl. The arrow head points to the lamina (L).

B. Geometric branching of 10 nm filaments (see arrow). Scale bar is 0.1 µm.

Adapted from (Nickerson, 2001).

Another criticism is that the "nuclear matrix" cannot be observed *in vivo* (using for example fluorescently tagged hnRNP proteins) or using standard RNP-highlighting methods in electron microscopy (Pederson, 2000). Since the matrix is expected to fill the interchromatin space, critics doubt the geometry of this space to be as straight and crisscrossing as the nuclear matrix appears after its isolation (Pederson, 2000). A last point that should be mentioned here from the critics' arguments is that in EM images of thin section matrix preparations trans-sections of the filamentous system such as cross, tangential or longitudinal sections would be expected, that have not been observed so far (Pederson, 1998).

Concluding, one can say that a global backbone, filling the intra-nuclear space is highly disputed and probably absent. However there are structural elements that have been demonstrated to be important for local organization of nuclear sub-domains, like the lamina or nuclear pore complexes (see 2.2.3).

2.2.2 The principles of self-organization

Opposed to the rigid nuclear scaffold idea, another more dynamic model of nuclear structure determination has been proposed by Tom Misteli (Misteli, 2001b).

The concept of "self-organization" predicts that it is the intrinsic properties of the components of a structure that allow organization, with physical interaction of molecules forming a steady-state structure (see Figure 11A). In terms of cell biology Misteli defines self-organization "as the capacity of a macromolecular complex or organelle to determine its own structure based on the functional interactions of its components" (Misteli, 2001b). The processes that occur within a self-organized structure themselves determine its own organization.

Self-organization had already been demonstrated for other cellular structures like microtubules, with tubulin subunits polymerizing in an equilibrium at the plus-pole of the fiber and disassembling subunits at its minus-end which forms different geometries (Nedelec *et al.*, 1997). Another cellular structure characterized by self-organization is the Golgi apparatus where constant flux of membrane through the compartment is needed for its characteristic structure (Glick, 2000). Recently, microtubule organizing center (MTOC) formation has been shown to be established

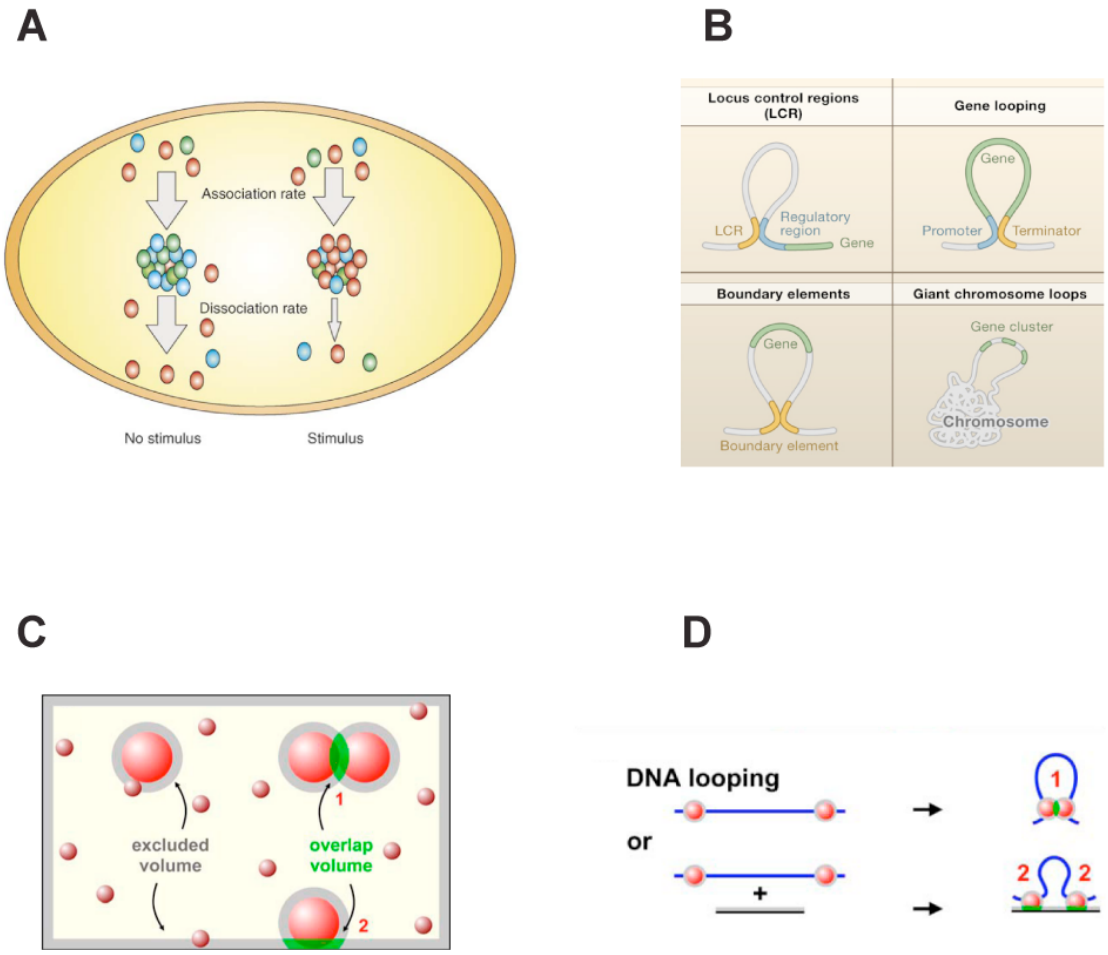

Figure 11. Alternative forces contributing to structure formation.

A. Schematic illustration of the auto organization concept. Nuclear body formation and regulation of its protein composition by an external stimuli, changing interaction properties of proteins. Adapted from (Gorski *et al.*, 2006).

B. Chromatin loop formation. Three examples of cis-interacting elements for transcriptional activation / repression (LCR - locus control region), coordination of initiation and termination and boundary function. Alternatively gene (cluster) expression can lead to giant loops protruding from the chromosome body. Adapted from (Misteli, 2007).

C. Principle of depletion attraction. The shaded regions represent a volume around the bigger spheres and the wall that is excluded to the mass centers of the small spheres. If two bigger spheres contact each other (1), or a bigger sphere contacts the wall (2), the total exclusion volume decreases, increasing the volume available for the small spheres, increasing their entropy. Adapted from (Marenduzzo *et al.*, 2006).

D. Depletion attraction in DNA loop formation. As in C), but this time, the bigger spheres (complexes) are bound to a fiber (DNA). An energetically more favorable situation is achieved, if the complexes aggregate or associate with a “wall” element. Adapted from (Marenduzzo *et al.*, 2006).

by sole self-organization in the absence of centrosomes in mouse oocytes (Schuh and Ellenberg, 2007). These examples can also be extended to nuclear sub-structures, like the nucleolus (see 2.2.5). Self-organization ensures structural stability while guaranteeing plasticity at the same time.

Misteli proposed the model of nuclear "self-organization" after current research had revealed that nuclear components, are highly dynamic, a prerequisite for this form of organization (Misteli, 2001b). Advances in light microscopic imaging techniques (see Figure 9), like FRAP have allowed better understanding of the dynamic properties of nuclear constituents (Misteli, 2001a). But also new analyses of organellar protein composition dynamics using mass-spectrometry in combination with stable isotope labeling allowed to study steady state and dynamics of nuclear sub-structures (Andersen *et al.*, 2005).

Self-interacting domains in proteins form an auxiliary forces driving self-organization in addition to the constituents common function (see Figure 11B). For some nuclear bodies at least one prominent protein has been described to have self-interaction domains ((Misteli, 2001b) and references therein). Another possibility is that a class of proteins involved in a common process has a special adherence to one and another, like specialized protein-protein interaction domains. This has been reported for serine/arginine-rich SR proteins involved in splicing (Amrein *et al.*, 1994). Nucleolar proteins are often highly charged potentially facilitating their self-integration (Misteli, 2001b).

Another force comes into play because the environment within a cell and especially its nucleus is crowded with 20 to 30% of the volume occupied by macromolecules (Minton, 2001). Under these circumstances macromolecules start to aggregate since this increases the entropy of a system (more volume is available to smaller molecules since the bigger ones aggregate, (Asakura and Oosawa, 1954), see Figure 11C and 11D), a phenomenon also known as "depletion attraction" (Marenduzzo *et al.*, 2006). This force could not only be an additional force in forming reactive macromolecules in the nucleus, but could also be the force driving the general nuclear organization including chromosome structuring and CT formation (Marenduzzo *et al.*, 2006). Recent data seems to provide evidence for this theory (Hancock, 2008; Richter *et al.*, 2007).

The appealing idea of the self-organization concept is the responsiveness of the structure due the fact that the interactions amongst its components are only transient.

The cell can thus adapt very rapidly, since signal cascades can induce post-translational modifications, followed by restructuring or dissolving of the macromolecule. An altered nucleolar structure after inhibition of RNA polymerase I transcription is such an example: the structure is dependent on its function (transcription, processing); as soon as the function is inhibited the nucleolar structure is disturbed ((Oakes *et al.*, 1993), see section 2.2.5).

Whether or not an internal nuclear scaffold exists *in vivo*, some structural elements in nuclei are well known. Therefore, the two apparently opposed models, matrix and self-organization, probably are each explaining partially the observed organization. For each structure, a co-existence of structural elements, providing a local backbone, together with self-organization could allow an accurate description of the total nuclear architecture.

2.2.3 The nuclear envelope

Eukaryotic genomes are separated from the rest of the cytoplasm by a double membrane. The so called nuclear envelope (NE) is constituted of two concentric lipid bilayers / membranes. The outer nuclear membrane (ONM) is in direct continuity with the rough endoplasmic reticulum at the cytoplasmic side of the NE. The protein composition of the ONM and the rough endoplasmic reticulum is therefore thought to be very similar, although ONM specific proteins enrichments exist (Lusk *et al.*, 2007). This means that ribosomes are usually also bound to the ONM. The ONM is also continuous with the inner nuclear membrane (INM). The space separating the two membranes is known as the peri-nuclear space, and it is continuous with the lumen of the endoplasmic reticulum. ONM and INM are joined at insertion sites of nuclear pore complexes (NPCs). The NPC serves as a barrier for diffusion of proteins in the two membranes, allowing specific ONM- and INM-protein composition (Lusk *et al.*, 2007). In metazoans, INM proteins are usually attached to a lattice of intermediate filaments about 10 nm diameter, the nuclear lamina which in turn interacts with chromosomes (Akhtar and Gasser, 2007).

NPCs allow the exchange of macromolecules between the nucleoplasm and the cytoplasm forming a channel. The NPC is an evolutionary well conserved huge macromolecule, although its molecular weight differs between yeast and vertebrates (~ 60 MDa and ~ 125 MDa, respectively, (Vasu and Forbes, 2001)). The number of

NPCs per cell nucleus varies greatly depending on the function of the cell; in a haploid yeast NE the number has been determined to be between 65 and 180 (Winey *et al.*, 1997). An NPC is constituted of ~ 30 distinct proteins, the so-called nucleoporins. Each of the nucleoporins is present as a multiple of eight (between 8 and 56 copies) and arranged in an eight-fold rotational symmetry around the pore-axis (Alber *et al.*, 2007). The diameter of the interior of the pore is around 9 nm allowing passive diffusion of small molecules (like metabolites). The size exclusion limit for passive diffusion for proteins is thought to be under 40 kDa (Gorlich and Kutay, 1999). But even for small proteins, like histones, this form of transport is very inefficient (Gorlich and Kutay, 1999). As is the case for larger particles, like hnRNPs and ribosome precursors, they are transported through the NPC with the help of active transport mechanisms, mediated by carrier molecules (Gorlich and Kutay, 1999). This allows for control of transport.

The cylindrical central part of the NPC is prolonged by cytoplasmic filaments towards the cytoplasmic face and a so-called "nuclear basket" towards the nucleoplasm. The nuclear basket and its constituents have been demonstrated to be an anchorage point for chromatin (Dilworth *et al.*, 2005; Galy *et al.*, 2000; Ishii *et al.*, 2002). Thus besides its role in nuclear – cytoplasmic exchange, the NPC seems at least in yeast to be implicated in genome organization (see 2.3.2). While the role of NPCs in anchoring telomeres in yeast is still a matter of debate, the anchoring of telomeres at the NE itself well established (Akhtar and Gasser, 2007). Great interest has especially been given to the proteins Tpr and Nup153, since these proteins form filaments at the extremities of the nuclear basket that reach up to 300 nm into the nucleoplasm (Cordes *et al.*, 1997; Pederson, 2000).

Another rather indirect indication for NPC-bound chromatin is a FRAP experiment on NPCs. It has been observed, that one fraction of the NPCs seems to be diffusing, while another shows an immobile behavior. This could suggest that these pores are in contact with chromatin and are thus "anchored" (Bystricky *et al.*, 2005).

2.2.4 The microtubule organizing center in *S.cerevisiae*

The yeast nucleus is, with regard to several aspects different from other nuclei (see Figure 12): The budding yeast genome does not code for lamins, they have exactly one nucleolus (see below), they undergo a "closed mitosis" (see below) with

persistence of transcription during mitosis and they have a specialized MTOC that orientates and organizes chromosomes also during interphase.

Yeast undergo a "closed mitosis", meaning that their NE, unlike to metazoan NEs, does not disassemble during mitosis (Health, 1980). Hence the MTOC is not only needed to pull the sister chromatids apart, but also to stretch the membrane to opposed cell ends. This is facilitated by integrating the MTOC into the NE. The functional equivalent to metazoan centrosomes in yeast is called "spindle pole body", or SPB. Its overall structure is not conserved compared to centrosomes, missing e.g. the major constituents of centrosomes, the "centrioles" (Jaspersen and Winey, 2004). The SPB is a gigantic structure of ~1-1.5 gigadalton, about 20 times larger in mass than a yeast NPC. This huge mass is however achieved with only ~20-30 proteins that are present in hundreds of copies (Bullitt *et al.*, 1997).

On its cytoplasmic side, the SPB organizes the microtubules of the cytoskeleton. Towards the nucleoplasm, the SPB is connected to the centromeres of the chromosomes via microtubules during the entire cell cycle (Jaspersen and Winey, 2004). Attachment is achieved via one single nuclear microtubule per centromere and is mediated by the kinetochore complex that binds to the 125 bp *CEN* region on a yeast chromosome (McAinsh *et al.*, 2003).This is an important peculiarity for nuclear organizing capabilities since this not only means that there are nuclear intermediate filaments, but it suggests at the same time a Rabl-like arrangement of yeast chromosomes, with centromeres all tethered at one side of the nucleus (see 2.1.3.2 and Figure 12B).

In G1-phase of the cell cycle, the SPB lies opposed to the nucleolus (see Figure 12B). Its position is maintained by the cytoplasmic and nuclear microtubules. During G1 the SPB starts to duplicate. Until the beginning of S-phase, the newly synthesized SPB continues to grow, to be fully functional when chromosome replication starts (Jaspersen and Winey, 2004). With further cell-cycle progression the SPBs now move, still within the NE, away from each other, to promote chromatid segregation.

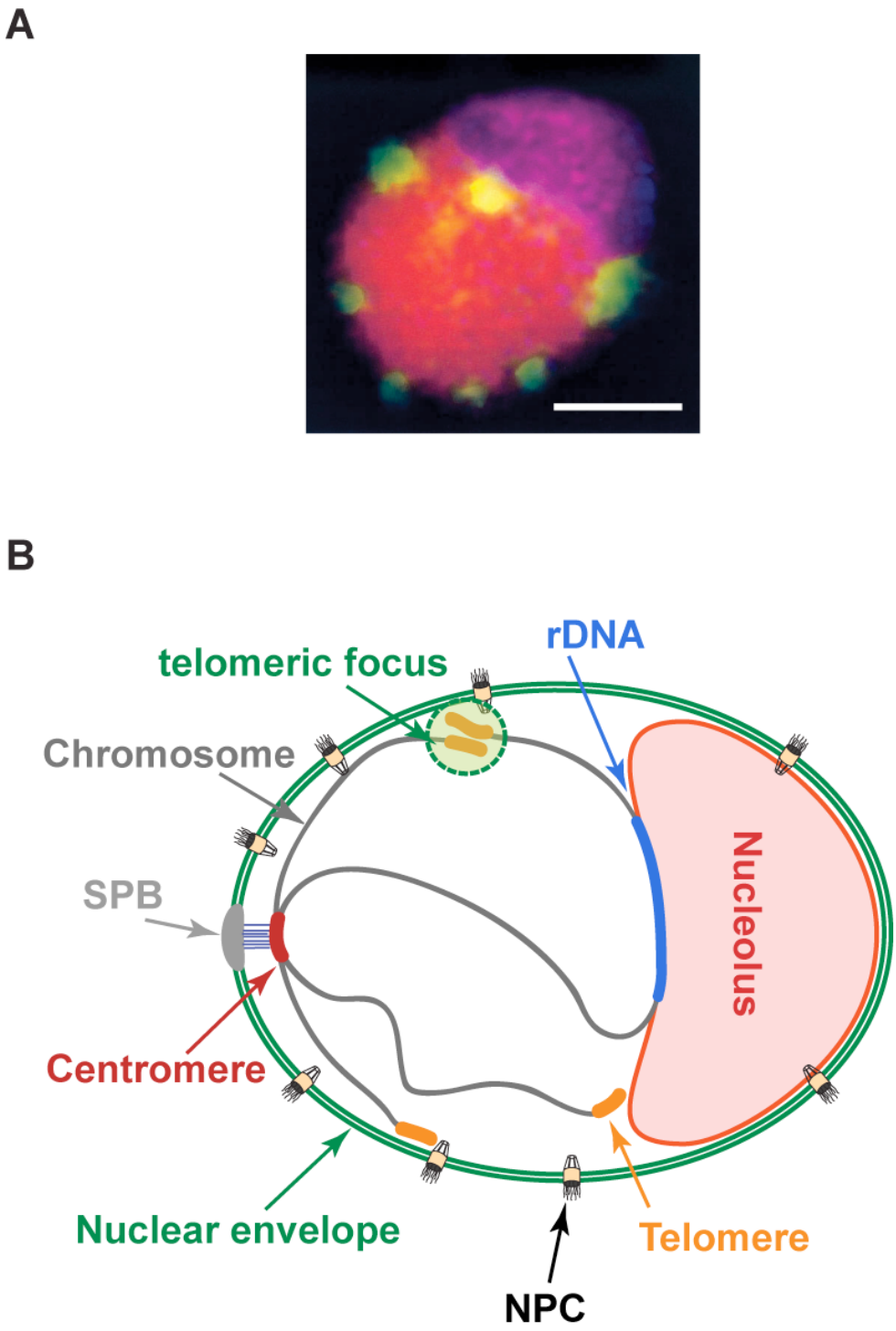

Figure 12. Yeast nuclear architecture.

A. Fluorescent image of a yeast nucleus. The nucleoplasm is labeled via ethidium bromide (red), the nucleolus is labeled by Nop1 (purple), and telomeric foci are stained with Rap1 (green). Scale bar is 1 µm. Adapted from (Gotta *et al.*, 1997).

B. Schematic illustration summarizing yeast nuclear architecture.

The SPB in yeast has a very special and distinct role in controlling genome organization throughout the cell cycle. The specific organization of the yeast nucleus furthermore imposes a cylindrical geometry, which is instrumental for our gene localization method (see result section).

2.2.5 The nucleolus

The nucleolus is the most prominent nuclear sub-structure (see Figures 6 & 13). This is reflected by its early discovery and description in the 18th century by Felice Fontana (Fontana, 1781). 50 years later, the body became was then termed "nucleolus" (Valentin, 1836). Another hundred years later, Emil Heitz discovered special (thin and fragile) chromatin segments that were visible in metaphase chromosomes stained with standard chromosome visualization techniques and importantly correlated these with nucleoli (Heitz, 1931). Barbara McClintock characterized the special chromatin segments and showed that nucleoli assembled *de novo* around these structures and hence termed them nucleolar organizing region (NOR, (McClintock, 1934)). With the development of electron microscopes more detailed observations could be made allowing morphological description.

2.2.5.1 Nucleolar morphology

In the nucleus of every actively growing cell is at least one nucleolus. While it is exactly one in yeast (see Figures 12 and 13A), the number of nucleoli per nucleus in other organisms varies and is also dependent of the cell cycle. Mammalian nuclei contain usually between one to five nucleoli. rRNA genes are characterized by their repetitive structure (Figure 14). While roughly 400 rDNA repeat copies are distributed over five chromosomes in humans, approximately 150 rDNA units in yeast are concentrated in one single cluster of tandem repeats on the right arm of chromosome XII. Interestingly, even at maximum rRNA expression levels only about half of these genes are actively transcribed (Dammann *et al.*, 1993). Active and inactive copies do not cluster but are rather randomly distributed within the rDNA (Dammann *et al.*, 1995; French *et al.*, 2008) (see Figure 14B).

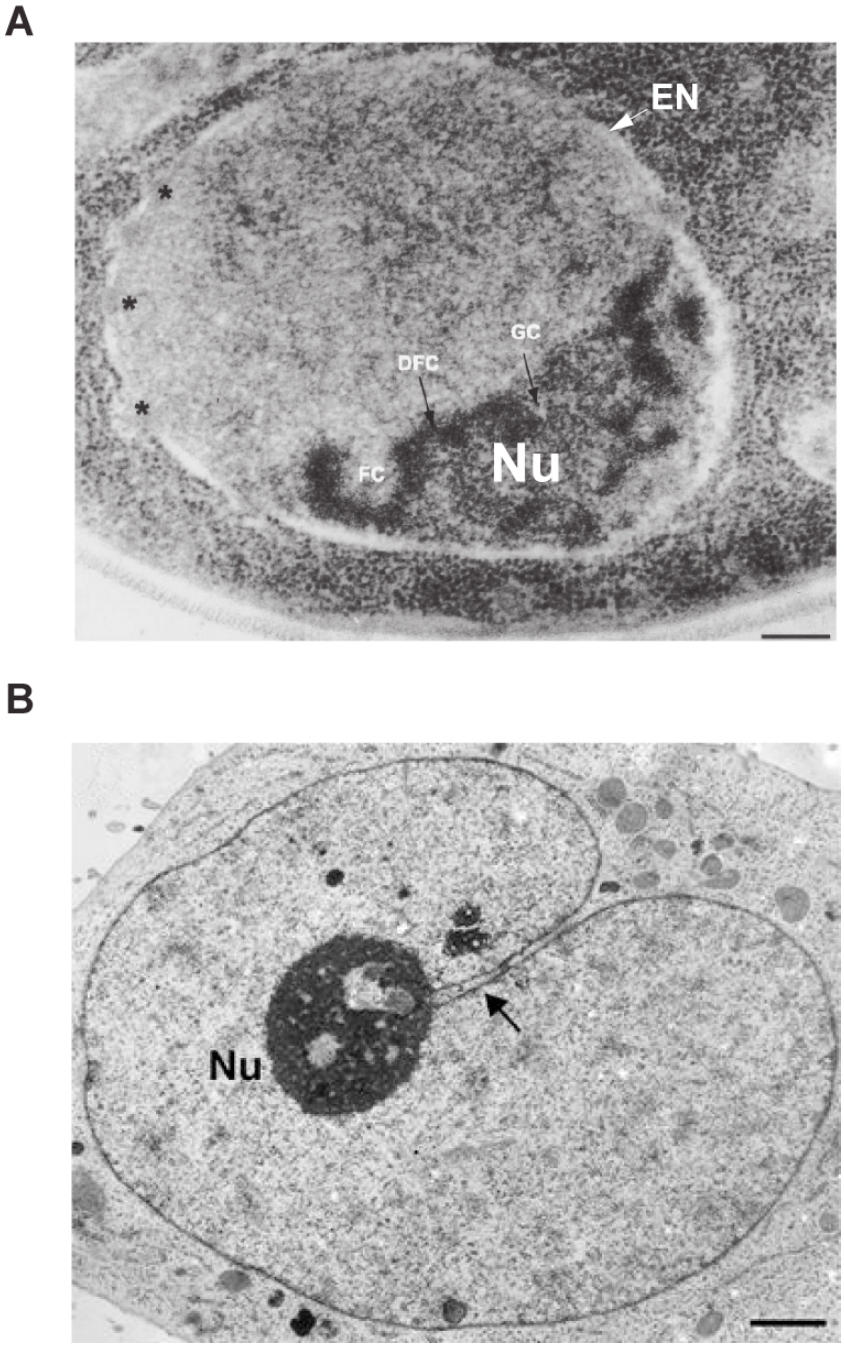

Figure 13. Nucleolar structure.

A. Electron micrograph of a yeast nucleus. Nuclear pore complexes are marked (*). The nucleolus can be seen as an electron dense structure next to the nuclear envelope (NE). The nucleolus is sub-structured into 3 sub-compartments visible in EM: the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC). Scale bar is 0.2 µm. Adapted from (Leger-Silvestre *et al.*, 1999).

B. EM image of a thin section of a HeLa cell nucleus showing an invagination of the NE (arrow) contacting the nucleolus (Nu). Scale bar is 1 µm. From (Hernandez-Verdun, 2006).

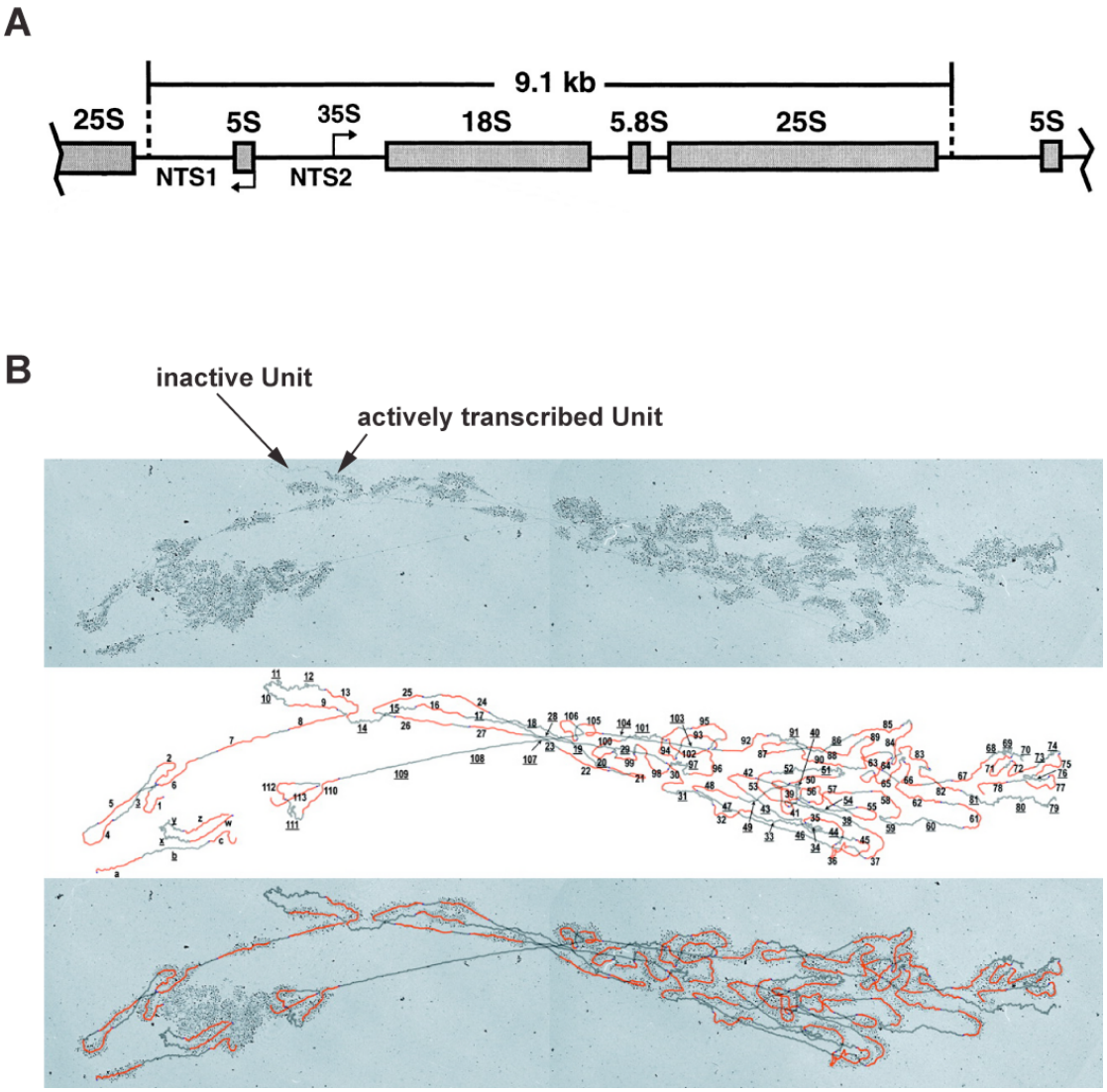

Figure 14. Ribosomal DNA.

A. Schematic illustration of a yeast ribosomal DNA (rDNA) unit. One unit is 9.1 kb in length. The large rRNAs (18S, 5.8S, 25S) are transcribed as a polycistronic rRNA precursor (35S) by RNA polymerase (Pol) I. The Pol III transcribed 5S rRNA gene is transcribed in the opposite direction. 35S and 5S gene are separated by two non-transcribed spacers (NTS1 and NTS2). Adapted from (Smith *et al.*, 1998).

B. Electron micrograph showing the entire rDNA. About 120 rDNA units are visible. Transcribed units are highlighted in red, silent copies in blue. From (French *et al.*, 2008).

Ultrastructurally, three different nucleolar sub-compartments can be distinguished: the fibrillary center (FC), the dense fibrillar component (DFC) and the granular component (GC) (see Figure 13).

FCs consist of fine fibrils, with a diameter between 4 and 8 nm that give a poor electron microscopic contrast (Schwarzacher and Wachtler, 1993). The numbers of FCs depend on the proliferating status of the cell. In non-proliferating cells only one globular FC can be observed, while a lot more FC (apparently up to 100) can be distinguished in actively dividing cells (Schwarzacher and Wachtler, 1993). These FCs are then much smaller in diameter and more irregular in shape. However, there are cell types (like Sertoli cells), where even in proliferating cells only one or very little or FC are visible (Schwarzacher and Wachtler, 1993). Recently it has been proposed that the structure of the FC has emerged during evolution and that in some cells, like yeast, a two component nucleolus, with a fibrillar and a granular component exists (Thiry and Lafontaine, 2005). This theory is still a matter of debate depending on the interpretation of EM images from these cells (Leger-Silvestre *et al.*, 1999; Thiry and Lafontaine, 2005).

Today we know that FCs are mainly composed of structural competent rDNA, Pol I and transcription factors, like upstream binding factor, or DNA Topoisomerase I (Schwarzacher and Wachtler, 1993). Some nascent pre-rRNAs can also be found in the cortical zone (Thiry and Lafontaine, 2005).

The FC are surrounded by the DFC, where the nascent rRNA transcripts accumulate (Cmarko *et al.*, 2000; Puvion-Dutilleul *et al.*, 1997). This arrangement of structural competent rDNA in the FCs and nascent transcript in the DFC suggests a model, where rRNA transcription from the rDNA occurs just at the interface between FC and DFC. The exact place of transcription is still a matter of debate. The two extreme models opposing each other either see rRNA transcription solely to happen within the FCs (Mais and Scheer, 2001), or exclusively in the DFC, with the FCs only serving as a dense storage compartment for inactively structured, non-transcribed rDNA (Koberna *et al.*, 2002). At the protein level, the DFC is characterized by rRNA modifying and processing enzymes like fibrillarin (Nop1 in yeast). This enzyme, a methyltransferase, has low sequence specificity and is directed onto the rRNAs by sequence specific snoRNAs (Reichow *et al.*, 2007). These, like U3 snoRNA, can also be found in the DFC (Koberna *et al.*, 2002).

Continuing the logic that the sub-structures are arranged to each other the same way as functional events proceed temporally (Cmarko *et al.*, 2000), the DFC is in contact with the GC, where pre-ribosome biogenesis continues. In the GC, the pre-RNA continues to mature, undergoing a series of endo- and exonucleolytic cleavages and further modifications (like pseudo-uridinylation). It is also in the GC that the rRNA is assembled with imported ribosomal proteins into the precursors of the large and small ribosomal sub-units, respectively the 60S and 40S pre-ribosomes (for review of these processes see (Fromont-Racine *et al.*, 2003)).

From the GC of the nucleolus, the pre-ribosomal particles are then exported to the cytoplasm via the NPC. In yeast a huge surface of the nucleolus is in close proximity with the NE and also in mammals nucleoli often associate with the NE (see Figure 13B, (Geraud *et al.*, 1989). When they are localized more centrally, NE invaginations touching the nucleolus have been observed that are dependent on nucleolar activity ((Hernandez-Verdun, 2006) and reference therein). This could facilitate the heavy nucleolar-cytoplasmic exchange required for ribosome biogenesis.

More recently an additional structure has specifically been discovered in yeast. The so-called “nucleolar body” is only visible in different mutant backgrounds and likely shows an accumulation of snoRNAs (Qiu *et al.*, 2008; Verheggen *et al.*, 2001). It is assumed that this nucleolar sub-compartment could functionally resemble the Cajal bodies (CB) in mammalian cells. In metazoans, these small, spherical nuclear bodies are often found associated to nucleoli (Gall, 2003). The interaction of CBs and nucleoli is mediated by the protein “coilin”. The structures are highly enriched in small nuclear- and small nucleolar RNAs that might there undergo some maturation steps (Sleeman and Lamond, 1999). Further functional references have been postulated regarding assembly and modifications of transcription factors (Gall, 2003). Hence, it is possible, that the nucleolar body from yeast is the functional homolog of animal Cajal bodies, which with evolution, have moved out of the nucleolus presenting discrete structures.

The latest sub-nucleolar structure described in yeast is the “No-body” (Dez *et al.*, 2006). This structure becomes visible in a cell that is defective in the protein Sda1 that is required for the export of the large pre-ribosomal subunit (Dez *et al.*, 2006). Tollervey and colleagues demonstrated that pre-rRNAs become degraded in this nucleolar sub-compartment by the nuclear exosome targeted via a TRAMP-mediated poly-adenylation.

All these reports give an impression, how many functions are organized within the small volume of the nucleolus. This further illustrates the difficulty to define precisely which model describes the observed organization most accurately: self-organization or structure based architecture.

2.2.5.2 Nucleolar dynamics

The nucleolus is highly dynamic. In metazoans, it disassembles during prophase and starts to rebuild with telophase in mitosis. Interestingly, although no nucleolar structure can be seen during mitosis, some nucleolar components (like the transcription machinery) stay in close proximity with rDNA during cell division (Roussel *et al.*, 1996). The nucleolus breaks down when Pol I shuts off and re-assembles when Pol I is getting reactivated (Sirri *et al.*, 2008). Although nucleolus reformation does not seem to solely depend on rDNA transcription ((Sirri *et al.*, 2008) and references therein), this timing underpins the hypothesis that structure follows function.

Another description of function dependent nucleolar morphology comes from the observation that inhibition of Pol I transcription using Actinomycin D leads to a segregation of the nucleolus into “nucleolar caps” around a “central body” (Sirri *et al.*, 2008). Similar results have been obtained in yeast, where Pol I mutants show heavily altered nucleolar structures (Oakes *et al.*, 1993).

Even in interphasic nucleoli, when the observed overall structure appears stable, the structure is still continuously in exchange with its environment. The picture of a static nucleolus from the 1960s and 70s has changed into a dynamic one, especially due to two novel techniques. One is quantitative mass-spectrometry approaches, such as stable isotope labeling of amino acids in cell culture (SILAC), where two phenotypes or experimental conditions can be compared, specifying the protein composition of extracted nucleoli at a given time point (Andersen *et al.*, 2005). The other is the use of modern fluorescence microscopy techniques, such as FRAP, determining the residence time and mobility of a protein (Phair and Misteli, 2000). The two techniques can be used in combination to eliminate technique-dependent artifacts (Lam *et al.*, 2007).

All these studies (and others) have shown the highly dynamic character of nucleolar proteins. There seems to be a constant flux and exchange of these proteins with the

nucleoplasm (Phair and Misteli, 2000). The mass spectrometric analyses also revealed hundreds of nuclear proteins that had so far not be identified as being required for nucleolar processes (Andersen *et al.*, 2005; Andersen *et al.*, 2002). The already characterized proteins have been described to be involved in all different kinds of nuclear processes, like DNA repair and replication and equally include splicing related proteins, kinases and phosphatases (Andersen *et al.*, 2005; Andersen *et al.*, 2002).

Experiments from the last decade have demonstrated that the nucleolus is not solely the place where ribosomes are produced but has many other functions. Examples are tRNA processing in yeast (Bertrand *et al.*, 1998b), sequestration of enzymes involved in cell cycle regulation (Shou *et al.*, 1999) or mediation of stress response signaling (Olson, 2004).

Once viewed as a static ribosome factory, the nucleolus is now considered a dynamic, multi-functional nuclear compartment.

2.3 Transcriptional regulation and nuclear space

It is clear that the three transcriptional apparatuses are not distributed randomly in nuclear space. The intra-nuclear position of a locus should be studied to characterize the local environment of a gene. In this part I will present our current understanding on where transcription actually occurs. I will then illustrate the correlation between gene positioning and transcriptional activity with some recent examples.

2.3.1 "Transcription factories"

To localize gene transcription activities, the labeling of nascent transcript using 5-bromo-uridine 5'-triphosphate (BrUTP) followed by immunofluorescent (IF) detection brought new advances (Cmarko *et al.*, 1999; Jackson *et al.*, 1993; Wansink *et al.*, 1993). The results revealed about one to several hundred discrete foci locally, depending on the cell line (see Figure 15). This number is lower than the expected approximately 10,000 pre-mRNAs estimated to be produced in vertebrate cells (Jackson *et al.*, 1993; Wansink *et al.*, 1993) and references therein). The original,

early interpretation of this discrepancy was difficult since it could not be excluded that only highly expressed genes were seen (Wansink *et al.*, 1993). But in analogy to clustered replication foci described earlier (Nakamura *et al.*, 1986), is has been proposed that transcription events could cluster equally (Jackson *et al.*, 1993; Wansink *et al.*, 1993).

Detailed analyses of the composition of these transcriptionally active foci demonstrated that they are also characterized by the presence of one of the three RNA polymerases, their transcription factors and some RNA processing factors (Grande *et al.*, 1997; Janicki *et al.*, 2004; Pombo *et al.*, 1999). As a nucleolus is a specialized Pol I transcription organelle, where Pol I, its necessary transcription factors and rRNA processing machinery accumulate, these foci seem to optimize the local environment in terms of transcription associated factors. Peter Cook termed these sites of enhanced active transcription "transcription factories" (Cook, 1999). He calculated the number of active polymerases within one "factory" to be between six and eight.

Other studies report accumulation of active genes at the surface of splicing speckles (Moen *et al.*, 2004; Shopland *et al.*, 2003) that are bigger in diameter than transcription factories (0.5 – 3 μm, (Shopland *et al.*, 2003)). Interestingly, a recent report describes SC35, a major constituent of splicing speckles, as a Pol II elongation factor (Lin *et al.*, 2008). This increases functional significance for a localization around these foci, besides more indirect co-transcriptional splicing events.

FRAP experiments proposed that Pol I and -II and transcription factors alternate between a chromatin bound, immobile and a diffusive behavior in the nucleoplasm (Darzacq *et al.*, 2007; Gorski *et al.*, 2006; Kimura *et al.*, 2002; Phair *et al.*, 2004). The authors also calculate a rapid exchange rate of transcription factors and polymerase bound to chromatin. These kinds of experiments do not exclude the possibility that factors already interact or bind to each other before reaching their target sequence. However, they provide an explanation for stochastic gene expression, since it has been reported that transcriptional activity of a specific gene at a given time point

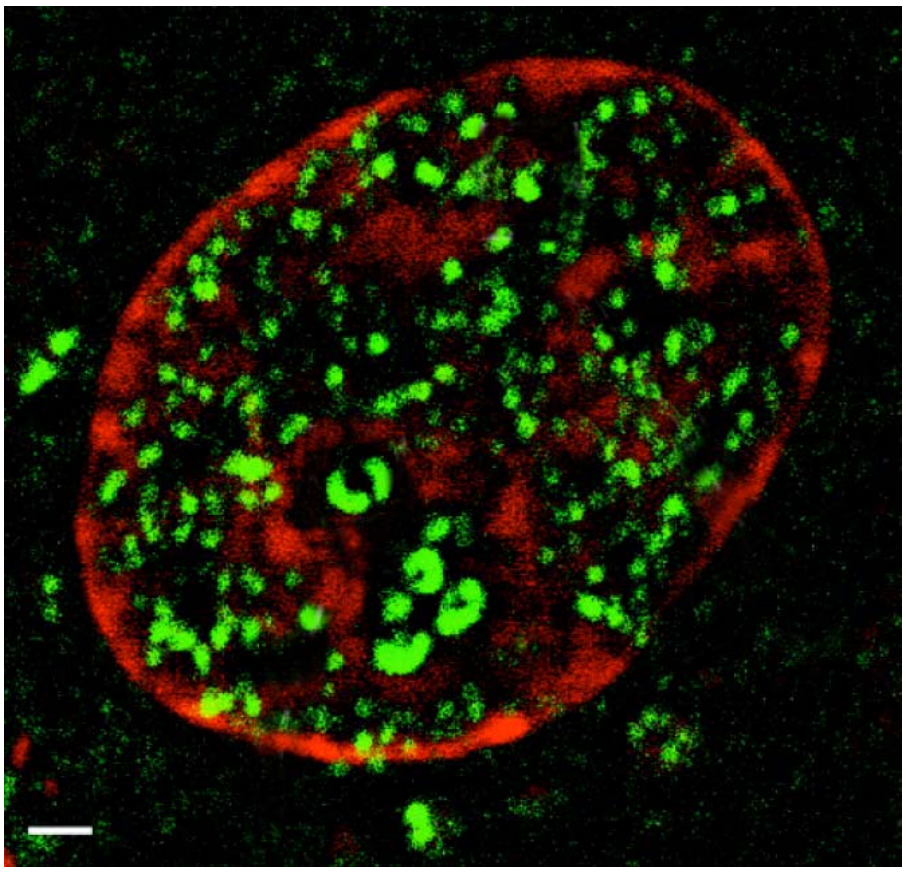

Figure 15. Visualizing "transcription factories".

Transcription foci in a HeLa cell. Nascent RNA are labeled with BrUTP and subsequently visualized by immunofluorescence (green). Total DNA is stained by propidium iodide (red). Scale bar is 1 μm. Adapted from (Cook, 1999).

varies largely between clonal cells (Blake *et al.*, 2003; Raj *et al.*, 2006). Transcription has consequently been proposed to rather happen in "bursts" than at constant lower levels, with important biological consequences like cell fate during differentiation (for review see (Raser and O'Shea, 2005). Thus, if a gene localizes in proximity to a local enrichment of transcription factors and polymerases, as a "transcription factory", its expression probability will be increased ("burst size") and *vice versa* (Raj *et al.*, 2006).

Concluding, one can say, that the localization of a gene with respect to "transcription factories" seems to be an important regulatory mechanism controlling gene expression.

2.3.2 The nuclear periphery

2.3.2.1 The repressive character of the nuclear periphery

The implication of nuclear architecture in gene regulation had first been proposed very early from EM images showing heterochromatin concentrated at the nuclear periphery and around nucleoli (Heitz, 1929). As mentioned above, Heitz postulated that heterochromatin would be gene-poor and only contain "passive" genes (translating into transcriptionally silent). The functional consequences of a peripheral localization have then been carried out especially in Drosophila, demonstrating the position-effect variegation of a gene: a gene localized within heterochromatic regions is silenced, if the same gene is located away from heterochromatin (e.g. by a translocation) it becomes transcriptionally active (for review see (Wilson *et al.*, 1990)). In yeast, Gottschling *et al.* demonstrated that a reporter gene placed next to a telomere could be either repressed or activated, first describing the yeast telomere positioning effect (Gottschling *et al.*, 1990). Gotta *et al*. then showed that yeast telomeres cluster at the nuclear periphery involving, besides other proteins, "silent information regulators" (SIRs) (Gotta *et al.*, 1996). The functional consequence of these peripheral Sir-protein containing clusters has then been demonstrated two years later: a non-functional *HM* locus that is not silent can be re-silenced by targeting it to the NE (Andrulis *et al.*, 1998). Further FISH experiments showed that a reporter gene inserted into a truncated telomere associates with telomeric foci when repressed, while it does not when it is transcriptionally active (Feuerbach *et al.*, 2002).

Recently, Drosophila and human cells have been studied for their genomic interactions with the lamina (Guelen *et al.*, 2008; Pickersgill *et al.*, 2006). This was done using a method termed DamID. *E.coli* DNA adenine methyltransferase (Dam) was fused to lamin B generating a chimeric protein. Expression of this fusion protein in metazoan cells leads to strong adenine methylation of DNA in contact with the lamina. Adenine methylation patterns are then analyzed using different techniques. The results revealed that lamin B is mostly in contact with intergenic regions. In Drosophila, 500 analyzed lamin-interacting genes are transcriptional silent and late replicating (Pickersgill *et al.*, 2006). In the same study, the authors also demonstrated a clustering of these genes. The clustered genes are furthermore described to be coordinately expressed during development. Looking at a bigger scope in humans, van Steensel an co-workers characterized regions of lamina-associated domains (Guelen *et al.*, 2008). The described domains are usually between 0.1 and 10 Mb in size and are associated with poorly expressed genes. The domains possess sharp borders towards transcriptionally active regions.

Artificial tethering of loci to the nuclear lamina in metazoan cells recently led to different conclusions: Kumaran and Spector showed that a gene artificially tethered to the nuclear lamina can still be induced (Kumaran and Spector, 2008). Another study showed that a reporter gene next to the lamina attachment site gets silenced upon tethering to the lamina (Reddy *et al.*, 2008). Further expression analysis showed that genes in a region of about 200 kb flanking the tethered site become transcriptionally silenced. A study from the Bickmore lab shifted a whole chromosome towards the periphery using the inner membrane protein Lap2β (Finlan *et al.*, 2008). Their expression analysis showed that some genes in the vicinity of the Lap2β binding sites of the chromosome are repressed, while others are not. Only a small number of genes located more distally are subjected to repression. Taken together, the results from the three studies suggest, that, in mammals, most genes can resist to the repressive effects at the nuclear periphery when they are forced to localize there.

2.3.2.2 The activating character of the nuclear periphery

More recently evolved the idea of the nuclear periphery as a transcriptionally activating environment. Activation at the NE was first associated with the nuclear pore and postulated by Günther Blobel in 1985 (Blobel, 1985). He hypothesized about

a link of transcribed genes with the NPC to allow directed mRNA export out of the nucleus and used the term "gene gating" for this mechanism.

The first experimental evidence for activating properties of the nuclear periphery was presented in 2002, and to date every report on gene activation at the periphery has always been associated with the NPC, supporting Blobel's initial hypothesis. Most of the experiments in this context have so far been done in yeast. The lab of Ulrich Laemmli was the first to experimentally assign transcriptional activity with the pores. With his co-workers, he showed that NPC could act as boundary elements (cf. 2.1.4.1), separating heterochromatin-like repressive chromatin structure from adjacent active loci (Ishii *et al.*, 2002). The natural association of endogenous loci with NPC proteins has subsequently been demonstrated using chromatin immunoprecipitation (ChIP) experiments (Casolari *et al.*, 2004). *In vivo* gene localization studies showed the preferential position of easily inducible yeast genes (*GAL* genes, *HXK1, INO1* or *HSP104*) at the nuclear periphery when activated (Brickner and Walter, 2004; Cabal *et al.*, 2006; Casolari *et al.*, 2004; Dieppois *et al.*, 2006; Taddei *et al.*, 2006). The studies showed that genes located at the periphery are transcribed (Cabal *et al.*, 2006) and that NPC-anchoring is required for optimal expression levels (Taddei *et al.*, 2006). In budding yeast, EC and HC have never been characterized. Certain regions are defined as "functional heterochromatin", but are probably too small (about 1 kb) to be detected by electron microscopy. However, when looking at EM images of metazoan cells one can observe that peripheral HC seems to be interspersed with EC adjacent to NPCs (see Figure 6). This observation has recently been confirmed and extended by high-resolution fluorescent microscopy showing that the previously seen, more or less homogenous DAPI staining of total DNA is actually structured with voids in front of NPCs (Schermelleh *et al.*, 2008) (see Figure 16A and 16B). In Drosophila the MSL complex, a histone acetyltransferase complex required specifically for the 2-fold upregulation of the male X chromosome for dosage compensation, physically interacts with the NPC components Mtor/TPR and Nup153 (Mendjan *et al.*, 2006). The authors demonstrated that depletion of these two proteins

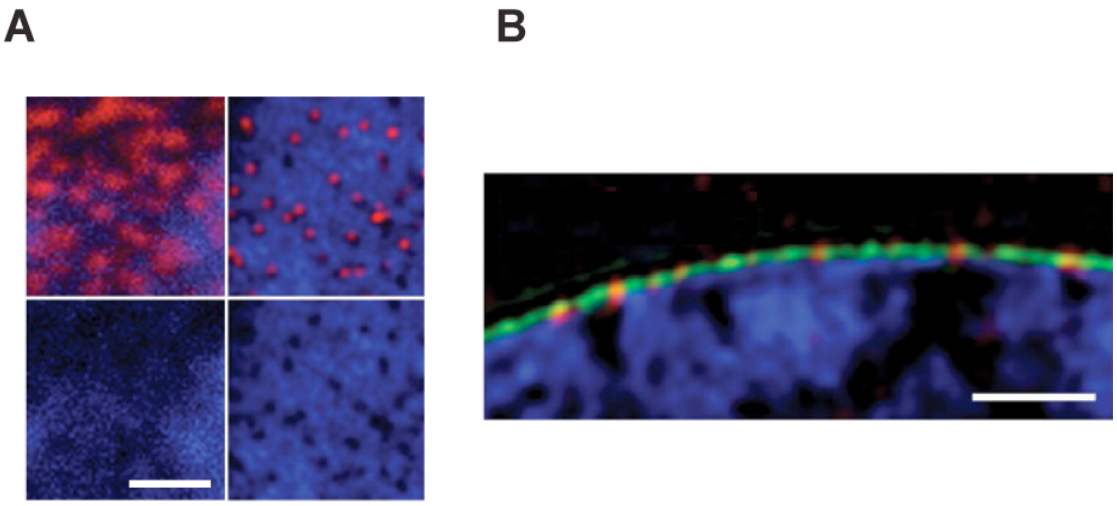

Figure 16. Chromatin structure around the pores.

A. Projection of four apical sections (corresponds to 0.5 µm) through a mouse myoblast. NPCs are stained in red, total DNA in blue (DAPI). On the left a conventional confocal scan micrograph, on the right a super-resolution reconstructed image. The technique reveals a high degree of structure within the total chromatin stain, e.g. chromatin voids become visible at NPCs.

B. An internal nucleoporin (NUP153) is colored in red, the lamina (Lamin B) in green and total DNA in blue (DAPI). As in B), but mid-sections are shown. Chromatin voids especially next to NPCs can be observed.

Scale bars are 1 µm. From (Schermelleh *et al.*, 2008).

using RNAi largely impaired dosage compensation. Furthermore, the same induction dependent localization of heat-shock protein-coding genes has recently also been demonstrated in Drosophila (Kurshakova *et al.*, 2007). In humans however, ChIP-on-chip experiments with NPC-interacting DNA in HeLa cells failed to recover previous yeast results demonstrating the preferential interaction with active genes compared to silent ones (Brown *et al.*, 2008).

The link with the NPC has been shown to involve the histone acetyltransferase complex SAGA (Cabal *et al.*, 2006) and the mRNA export factors Mex67 (Dieppois *et al.*, 2006). However, it is not clear whether the gene gets relocated to the nuclear periphery before activation, or whether is it the co-transcriptionally processed mRNA that is pulling the locus towards the NPC. Most experiments seem to favor the so-called reverse recruitment mechanism where a gene becomes activated and immobilized after it has moved to a specific site as suggested by the fact that the Mex67 chromatin-interaction is not mediated by RNA (Dieppois *et al.*, 2006). However, the idea that it is the processed and exported transcripts that are at least helping to haul the locus towards the exterior cannot be fully excluded. Assays like a very recently presented optical screen for mutants that do not show a relocalization of activated *GAL* loci (Vodala *et al.*, 2008) help to find new candidates required for a peripheral localization and probably to elucidate the recruiting mechanism.

The documented association of the *GAL* gene clusters with the periphery represents the first example of gene-localization associated activation studied at the molecular level. We still need to know how general this re-localization mechanism of activated genes is.

2.3.3 Peri-nucleolar localization

As for the nuclear periphery, peri-nucleolar localization has been associated with both transcriptionally repressive and activating characteristics. However the difference might in this case lie in the type of RNA polymerase.

As for localization close to the NE, nucleoli are often surrounded by heterochromatin (see Figure 6). As already described above, heterochromatin has since its discovery been associated with gene poor or inactive gene regions. The interesting question is whether the heterochromatin surrounding the NE is functionally different from peri-nucleolar heterochromatin. In mammals, inactivated X-chromosomes (Xi) are often

localized to larger heterochromatic regions, favoring a localization close to the NE or in proximity to the nucleolus (Bourgeois *et al.*, 1985). Furthermore it is known that the Xi is also very late replicating (Priest *et al.*, 1967). Recently it has been demonstrated that the Xi oscillates from peri-nucleolar regions and NE-close domains during S-phase (Zhang *et al.*, 2007). The authors showed that up to 90% of Xi chromosomes visits the nucleolus in mid- to late S-phase in female mouse ES cells. This dynamic localization was dependent on the *Xist* transcript. If this non-coding RNA gene is absent, peri-nucleolar targeting is lost and the X chromosome inactivation is no longer maintained. Transferring the *Xist* locus onto an autosome, targets the chromosome as the Xi towards the nucleolus. This suggests a mechanism by which specialized chromatin states can be replicated by spatial and temporal separation. This involves Xist RNA expression and assembly around the inactivated chromosome. The study could not resolve whether Xist RNA is expressed near the nucleolus, or whether it is transcribed elsewhere in the nucleus and needs the nucleolus-close localization for folding or assembly. As for gene recruitment to the nuclear periphery, this still leaves an important open question.

Transcriptional activity near the nucleolus is also well documented. In yeast Pol III transcribed 5S gene transcription naturally needs to occur within nucleoli due to the linear arrangement in an rDNA unit (see Figure 14A). In other organisms the 5S coding gene is usually separated from the Pol I transcribed 35S gene, though multiple 5S gene copies cluster preferentially (for review see (Haeusler and Engelke, 2006). However, spatially, these 5S genes seem to preferentially localize close to the nucleolus from plants to humans (Haeusler and Engelke, 2006). 5S RNA and other Pol III transcripts can be detected in a peri-nucleolar compartment (PNC) (Matera *et al.*, 1995) in human cells. A very high transcriptional activity has been assigned to PNCs, as documented by BrUTP incorporation times within 5 minutes (Huang *et al.*, 1998). This incorporation is not affected when inhibiting Pol I, indicating that Pol II and/or -III are responsible for the transcriptional events. Taking these results together, it seems as if the peri-nucleolar zone is transcriptional active concerning Pol III transcription, like 5S and other Pol III transcripts as tRNA.

In 2003, the laboratory of David Engelke showed in yeast that even though the 274 tRNA genes (tDNA) are dispersed over the 16 chromosomes, strong clustering of

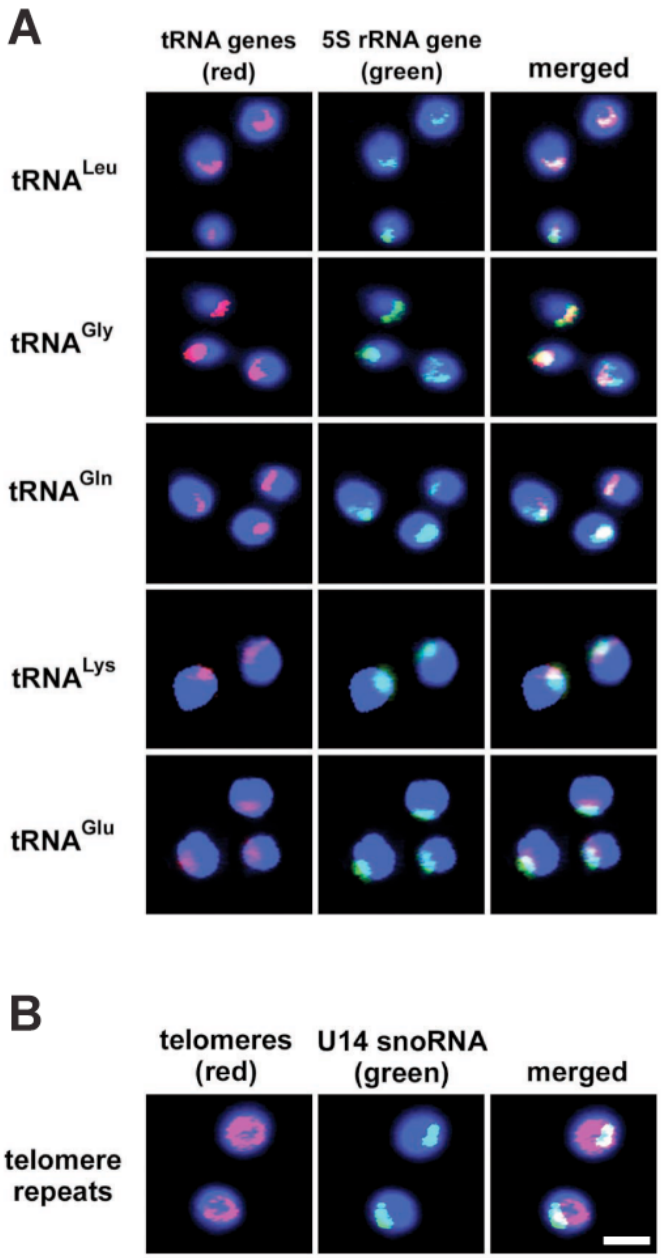

Figure 17. Nucleolar clustering of tRNA genes.

A. Different classes of tRNA coding genes (tDNA) have been labeled using fluorescence *in situ* hybridization (upper 5 panels, red). At the same time the nucleolus has been labeled using FISH against the 5S coding gene (upper 5 panels, green), showing a clustering of tDNA in proximity to the nucleolus.

B. As a control, FISH labeled telomeric repeats have been co-stained with a probe against the nucleolar snoRNA U14. Scale bar is 2 μm.

From (Thompson *et al.*, 2003).

tDNA near or in the nucleolus can be observed by FISH (Thompson *et al.*, 2003). The clustering is dependent on an active Pol I, since *rpa49*Δ mutants abolish peri-nucleolar tDNA localization (Thompson *et al.*, 2003; Wang *et al.*, 2005). A recent report from the same group further indicates that condensin is required for tRNA gene clustering, though not for their nucleolus-close positioning (Haeusler *et al.*, 2008). The positioning however gets disrupted when using microtubule depolymerizing drugs (Haeusler *et al.*, 2008). A functional link to this spatial distribution seems to exist, since an actively transcribed tRNA gene silences an adjacent Pol II transcribed gene, a phenomenon known as tRNA gene-mediated (tgm) silencing (Hull *et al.*, 1994). Indeed, Engelke's lab could show that mutants affecting tgm silencing result in modifications of tDNA FISH signal (Thompson *et al.*, 2003; Wang *et al.*, 2005). They further report that disrupting this localization by either mutating the promoter of a tRNA gene (*SUP53*), or using a Pol I mutant background affects not only the positioning, but also the silencing of an adjacent Pol II reporter gene. They conclude that tRNAs are transcribed in a place where Pol II transcription is repressed. However, localization analysis of the same locus did not reveal nucleolar localization in a different study (Bystricky *et al.*, 2005). Furthermore, global analysis of the expression rate of Pol II genes adjacent to Pol III genes in Pol III ts-mutants, did not reveal a role for Pol III activity for close Pol II expression sites (Conesa *et al.*, 2005).
Unfortunately, the resolution of the FISH images and the sampling number is too low to see, what "close to the nucleolus" actually means (see Figure 17). Does it mean, that tRNAs are in fact transcribed within the nucleolar structure or, given the size of the yeast nucleus, very close from it, such as within the resolution limit of a fluorescent microscope (200 in x-y, and 700 nm in z)?
Nevertheless, these results do have a very important impact on (1) chromatin organization and (2) transcriptional regulation in nuclear space. If 274 genes dispersed over all 16 yeast chromosomes are all (or even just a majority) localized at the nucleolus, this adds a critical parameter for spatial chromatin organization as drawn in Figure 18A. However, unlike for telomeres or centromeres this localization is transcription dependent. Second, these results are very interesting since they are strengthening the spatial partitioning of genes in nuclear space, if specific Pol II transcribed genes are indeed repressed when tRNA genes are transcribed, this could suggest that these Pol II gene products might be required in non-proliferating cells.

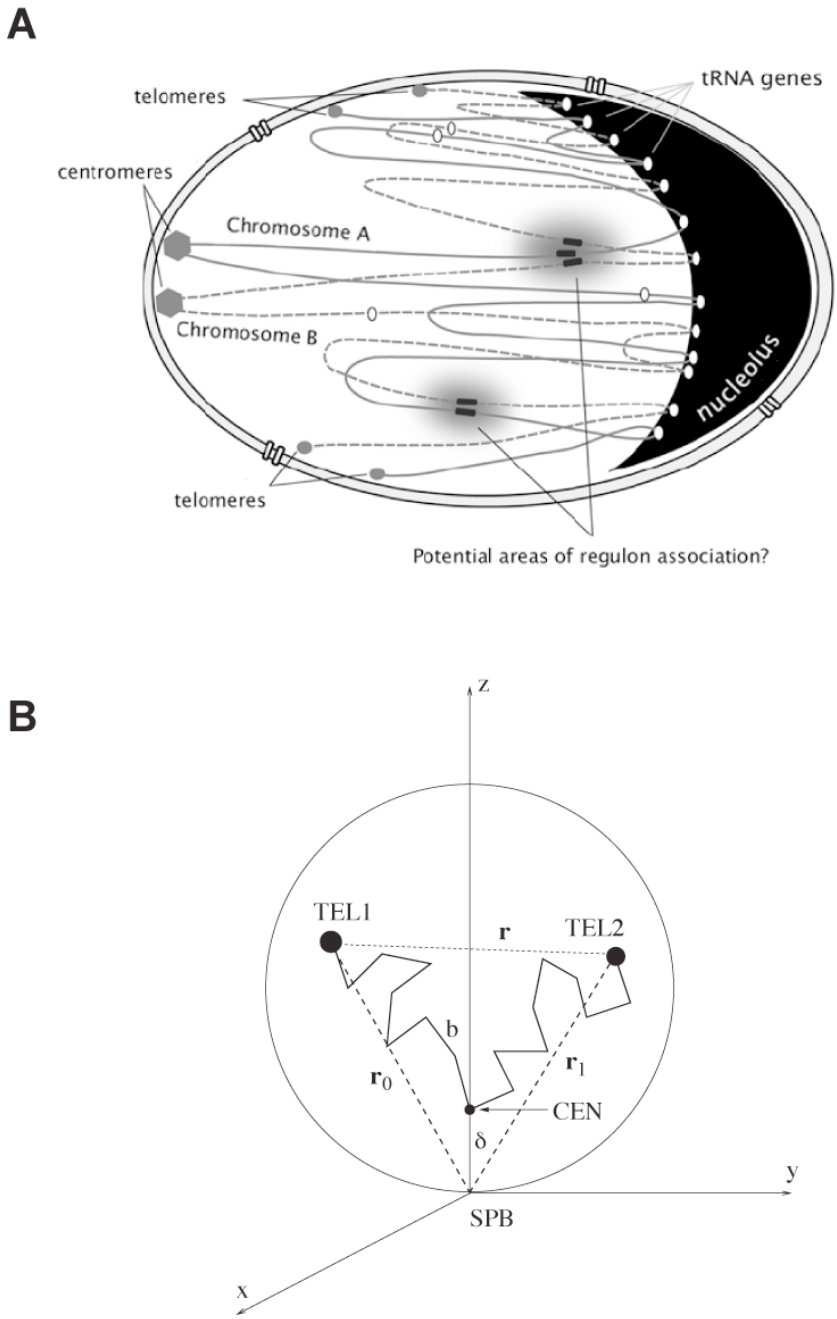

Figure 18. Models for chromosome arrangement in the yeast nucleus.

A. Model proposed by D. Engelkes group. Most of the tRNA genes localized at the surface of the nucleolus. With centromeres being attached to the spindle pole body (SPB, left hand side, not illustrated) and the telomeres at the periphery, this model requires intensive looping of the chromosome arms, to allow the tDNA to localize to the nucleolus. Adapted from (Haeusler and Engelke, 2004).

B. Rabl-like model. As in the previous model, all centromeres are attached with the help of microtubules to the SPB. However, the telomeres move away from the centromere towards the periphery and the nucleolus (at upper part of the illustration, not shown) in a much more direct way, allowing only minor looping of the chromosome arm. Adapted from (Gehlen *et al.*, 2006).

Finally, since tRNA genes only localize close to the nucleolus when transcriptionally active, this could suggest a co-regulating mechanism with genes coding for other parts of the "translation machinery", further discussed in the next paragraph.
Nevertheless, with the contradictory results reported above, it needs to be kept in mind that this is just a model. Other, more Rabl-like chromosome conformations are an alternative model, which is supported by *in vivo* data (Bystricky *et al.*, 2005; Gehlen *et al.*, 2006) (see Figure 18B). Further experiments are required to validate one of the two.

2.3.4 Co-Regulation and Spatial Co-Positioning

In yeast the most prominent and obvious co-regulation that is also spatially co-positioned is the Pol I dependent 35S gene transcription and Pol III transcribed 5S gene transcription. Both genes lie adjacent to each other within an rDNA unit (see Figure 14). This implies that an important fraction of the Pol III transcription machinery is concentrated on the rDNA in yeast nucleoli. Localizing different Pol III transcribed genes, whose gene products are also required to assure translation of mRNAs into proteins in spatial proximity seems to be another example for transcriptional coordination within nuclear space, given the results of Engelke's group (Thompson *et al.*, 2003; Wang *et al.*, 2005).
In the case of Pol II transcribed genes, all results of intra- or interchromosomal interactions come from vertebrate cells. Two techniques were particularly useful to analyze spatial chromatin arrangement. (1) FISH and (2) 3C and its variants. 3C is based on the following procedure: Cells are fixed and digested with a restriction enzyme subsequently followed by a ligation step. During this ligation, DNA fragments in spatial proximity are religated independently of their linear localization along the chromatin fiber. Afterwards, cross-linking is reversed, and the ligation products are analyzed. This allows to measure the interaction frequency of two loci with one another. While analysis was originally done looking for anticipated ligation products (Dekker *et al.*, 2002), a variety of large scale sub-methods now exist using microarray hybridization or large scale sequencing to identify the products (Simonis *et al.*, 2006; Würtele and Chartrand, 2006; Zhao *et al.*, 2006b). A drawback of the method is that it is a snapshot over a cell population, dynamic and transient events are not "captured".

The 3C techniques generally revealed that most interactions with a locus are in *cis*, with sequences form the same chromosome, in accordance with the chromosome territory picture (Würtele and Chartrand, 2006; Zhao *et al.*, 2006b). These intrachromosomal interactions can be increased for co-activated genes up- or downstream of the analyzed locus as demonstrated using DNA-, RNA FISH and 3C methods to analyze the β-globin locus (*Hbb-b1*) in differentiating mouse erythrocytes (Osborne *et al.*, 2004). Analyzing different genes on the same chromosome at a distance between 24 and 39 Mb, the authors could show that during differentiation when the loci of interest become activated, the genes frequently co-localized with *Hbb-b1*. Increased distance to the locus does not correlate with reduced interaction frequency, interactions rather tend to be more frequent for more distant loci. The use of immuno-FISH revealed that all loci share the same transcription spot / -factory. The same study also detected interchromosomal interactions. These interactions are about 6 to 8 fold less frequent than intrachromosomal interactions. The results have been confirmed and extended by other teams (Brown *et al.*, 2006; Simonis *et al.*, 2006).

A lot of other examples of co-localized, co-expressed loci have been reported that were recently summarized in a review by Peter Fraser and Wendy Bickmore (Fraser and Bickmore, 2007). I want to focus on three more interesting examples.

An analysis of the *Hoxd* locus during mouse development revealed that gene activity dependent “looping” of the locus out of its chromosome territory depends on the tissue, while it seems to be required in some cell types it could not be observed in others (Morey *et al.*, 2007). This led the authors to suggest that activation of the gene can be achieved in multiple ways and that looping depends on how a gene gets activated.

A negative regulative interaction has been reported in 2005. It has been demonstrated that the locus control region of TH2, so far described as being responsible for cis activation of cytokines on the same chromosome (chr. 11), also interacts with the gene coding for interferon-γ, *Ifng*, in naive T lymphocytes (Spilianakis *et al.*, 2005). During differentiation of the lymphocyte, the two regions become separated and *Ifng* gets expressed.

Another recent example is the co-localization of the proto-oncogene *Myc* (chromosome 15) with the highly transcribed gene *Igh* (chromosome 12) (Osborne *et*

al., 2007). The authors studied the two loci since a translocation between the two is the most frequently observed in plasmacytoma and Burkitt lymphoma (see (Osborne *et al.*, 2007) for references). *Igh* is a B cell specific, constitutively active gene, *Myc* becomes activated during the immediate early gene response that can be achieved by stimulating the B cell receptor pathway. Interestingly, activated *Myc* becomes relocalized to the same transcription factory that is already occupied by its frequent translocation partner *Igh*. This juxtaposition during transcription can explain this high translocation frequency of two otherwise separated loci.

As recently demonstrated and mentioned above, condensin seems to provide a mechanism for tRNA clustering in yeast. Similarly, a candidate for inter- and intra chromosomal gene association in human cells has been found to be the insulator (see 2.1.4.1) protein CTCF. It has been demonstrated that the interaction between specific loci is abolished after flanking CTCF binding sites had been mutated or after knock-down of the protein itself (Ling *et al.*, 2006; Zhao *et al.*, 2006b). Further experiments addressing the dynamics and determinants of co-localization events in these and other specific cases will be required to elucidate the nature of the interactions.

3. THE PROTEIN HMO1

When we designed the experimental part of my PhD, we decided to focus on the protein Hmo1. Hmo1 appeared a good candidate to be implicated in both controlling ribosomal components synthesis and organizing genes in nuclear space. This speculation was based on the nature of Hmo1. Hmo1 bears HMG-boxes, which could be involved in fine tuning transcriptional activity as also in modulating chromatin structure.

3.1 Molecular properties of Hmo1

Hmo1 is a nuclear protein that resides at the interface between the nucleoplasm and the nucleolus where it co-localizes with Fob1, a protein marker for the rDNA (Gadal *et al.*, 2002) (see Figure 19). Hmo1 is one of seven HMG-box proteins (see 2.1.1.3) described in *S. cerevisiae* that was first identified in 1996 (Lu *et al.*, 1996). The 246 amino acid protein has a molecular weight of 27.5 KDa (Lu *et al.*, 1996). The protein is moderately abundant with an estimated 1.9 x 10^4 molecules per cell (Ghaemmaghami *et al.*, 2003). It comprises a lysine-rich, basic, hydrophilic C-terminus and two HMG-boxes (Kamau *et al.*, 2004; Lu *et al.*, 1996). While the second one ("HMG-box B") resembles the HMG-box consensus at a good level, the first box, "HMG-box A", has only little sequence homology (Gadal *et al.*, 2002; Kamau *et al.*, 2004; Lu *et al.*, 1996). Hmo1 binds DNA with high affinity (Freeman *et al.*, 2000; Mitsouras *et al.*, 2002), and exerts strong DNA bending properties, requiring interaction of its N- and the C-terminus (Bauerle *et al.*, 2006). As mentioned in section 2.1.1.3., HMG-box proteins bind DNA with either little or no sequence specificity. For Hmo1 it has been reported that the protein has a sequence preference for long CAG tracts (26 to 126 bp tested) (Kim and Livingston, 2006).

While deletion of the characteristic C-terminus does not result in a growth phenotype, an *hmo1*Δ mutant has a severe growth defect (Lu *et al.*, 1996). Deletion of *HMO1* leads to comprised plasmid maintenance and hyper-sensitivity to micrococcal

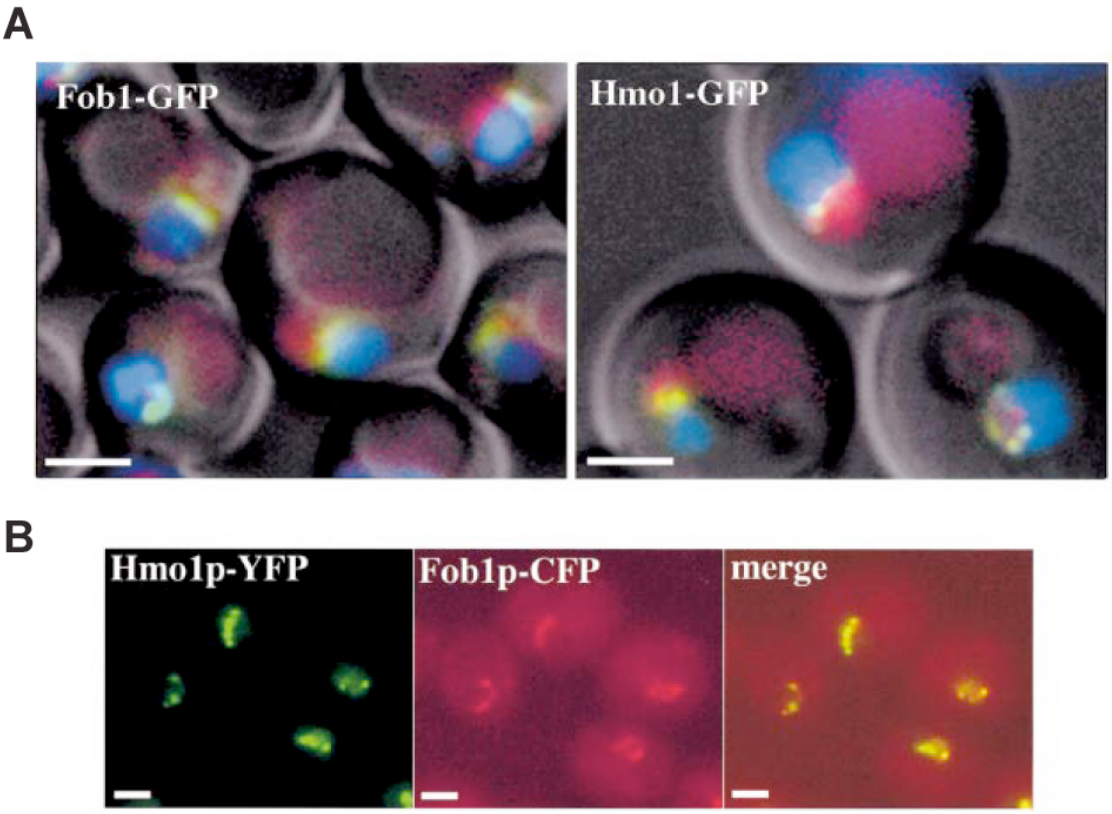

Figure 19. *In vivo* localization of Hmo1.

A. Fob1, an rDNA binding protein, and Hmo1 localize at the interphase beween the nucleolus and the nucleoplasm. Fob1-GFP and Hmo1-GFP are expressed in strains also encoding DsRed-Nop1 and stained with Hoechst 33352. Scale bar is 2 μm.

B. Hmo1 co-localizes with the rDNA marker protein Fob1. Hmo1-YFP and Fob1-CFP are co-expressed in a diploid strain. Scale bar is 2 μm.

From (Gadal *et al.*, 2002).

nuclease digestion of the chromatin, suggesting that Hmo1 may play a role in chromatin stability (Lu *et al.*, 1996).

Deletion of *HMO1* is lethal when combined with a mutation in *FPR1* (Dolinski and Heitman, 1999). Fpr1 is a peptidyl-prolyl cis-trans isomerase (PPIase) that interacts with the two structurally similar drugs FK506 and rapamycin (Heitman *et al.*, 1991; Koltin *et al.*, 1991). An *fpr1*Δ strain is insensitive to rapamycin (Koltin *et al.*, 1991). In yeast cells, Fpr1 complexed with rapamycin specifically inhibits one of the two TOR complexes, TORC1 (Loewith *et al.*, 2002). The genetic interaction is reinforced by a physical interaction between the two gene products, as shown by co-immunoprecipitation experiments (Dolinski and Heitman, 1999).

Another study suggests an involvement of Hmo1 in mutagenesis control (Alekseev *et al.*, 2002), but no mechanistic data has been provided to date.

3.2 Hmo1 and rRNA production

3.2.1 Genetic interactions of Hmo1 with RNA polymerase I

On top to the reported synthetic lethal (SL) interaction of *HMO1* and *FPR1*, earlier work demonstrated an SL interaction of *HMO1* with the two RNA polymerase I subunits, namely *RPA49* and *RPA34*, as well as the gene coding for the type I Topoisomerase Top3 (Gadal *et al.*, 2002). The initial link of Hmo1 to the Pol I transcription machinery had been discovered during a screen, looking for multi-copy suppressors restoring viability of a *rpa49*Δ mutant at 25 ˚C (Gadal *et al.*, 2002). Except from *RPA49* itself, the only suppressor that could be isolated from two independent screens was *HMO1*.

To test the direct involvement of a protein in Pol I activity, Nogi and co-workers established a very elegant system (Nogi *et al.*, 1991). Using an inducible Pol II promoter they could bypass lethality of mutants inactivating Pol I. This genetic tool has been used to isolate all essential proteins specifically required for Pol I activity (Nogi *et al.*, 1991), but it can also be used to test if synthetic lethal gene deletions are caused by a Pol I activity defect *in vivo*. The lethality of the *rpa49*Δ/*hmo1*Δ double mutant is specific to Pol I and rDNA transcription, since the mutation is viable when the large rRNAs are expressed by Pol II (Gadal *et al.*, 2002).

Further investigating the connection between Hmo1 and the Pol I machinery revealed *RPA34* (coding for the non-essential sub-unit Rpa34), *RPA12* and a viable mutant form of *RPA43* (*rpa43-24*) respectively as additional synthetic slow growth or SL interaction partners with *HMO1*. These results link Hmo1 to Pol I initiation (*rpa43-24*, (Gadal *et al.*, 2002)) and elongation since, as mentioned above (1.2.1), Rpa49 and Rpa34 form a hetero-dimer acting as an intrinsic elongation factor with a predicted function homologous to TFIIF (Kuhn *et al.*, 2007). Recently, Thuriaux and co-workers proposed that the Rpa49/Rpa34 heterodimer plays a role in Rrn3 recruitment to the polymerase and in the following Rrn3 dissociation from the elongating polymerase, acting in a dual role for initiation and the switch from initiation to elongation (Beckouet *et al.*, 2008). Finally, Rpa12 has been reported to act as a termination factor (Kuhn *et al.*, 2007; Prescott *et al.*, 2004), linking Hmo1, at least genetically, also to the last steps of the Pol I transcription cycle.

3.2.2 rRNA production in an *hmo1*Δ background

Steady state rRNA levels, as judged by total RNA extraction an quantification on an ethidium bromide stained agarose gel, are reduced in *hmo1*Δ cells after normalization to tRNAs and compared to wild type cells (Gadal *et al.*, 2002). This phenotype could also be a rather unspecific phenotype due to the slow growth of *hmo1*Δ cells (Waldron and Lacroute, 1975), however, a similar growth defect resulting from a Pol II mutation (*rpb9*Δ) did not reveal this phenotype (Gadal *et al.*, 2002).
Labeling experiments show a reduced *de novo* rRNA synthesis as compared to wild type cells, though the effect is not as drastic as for a *rpa49*Δ mutant analyzed in parallel (Gadal *et al.*, 2002). No rRNA processing intermediates could be detected as judged from autoradiographies of tritium-uracil pulsed cells, arguing against an involvement of Hmo1 in rRNA maturation (Gadal *et al.*, 2002).

In the course of my PhD, numerous reports on Hmo1 have been published (Hall *et al.*, 2006; Kasahara *et al.*, 2008; Kasahara *et al.*, 2007; Merz *et al.*, 2008) that will be discussed in detail in the results / discussion section below.

3.3 Hmo1, a homolog of human UBF?

Hmo1 is one of only seven HMG box proteins in *Saccharomyces cerevisiae*. It is the only one identified so far to interact with the RNA polymerase I transcription machinery. This led to the speculation that Hmo1 might be the homolog of mammalian UBF (see 1.2.2) in the evolutionary very well conserved Pol I transcription system (Gadal *et al.*, 2002). UBF exists in two proteins, produced by alternative splicing, human UBF1 (hUBF1) and hUBF2 of respectively 764 and 727 amino acids (Bell *et al.*, 1988; Chan *et al.*, 1991; Jantzen *et al.*, 1990). hUBF1 contains presumably six DNA-binding HMG boxes and the smaller hUBF2 variant lacks the second HMG box (Jantzen *et al.*, 1990). Even though Hmo1 contains only two potential HMG boxes and does not share other UBF-characteristics like the serine-rich, acidic C-terminal tail, a functional homology has been proposed since they are both associated with Pol I (Gadal *et al.*, 2002).

RESULTS

1. INFLUENCE OF HMO1 ON rDNA TRANSCRIPTION

Hmo1 is a nucleolar protein associated with the rDNA and acts synergistically with the conserved Rpa49/PAF53 subunit of Pol I during rDNA transcription (Gadal *et al.*, 2002). The published genetic link between Hmo1 and the Pol I apparatus strongly suggests an *in vivo* involvement of Hmo1 in rRNA production. To better characterize the function of Hmo1, I first focused on the putative functional conservation with hUBF and then assayed the effect of Hmo1 in *in vitro* transcription systems.

1.1 Conservation of Hmo1 and hUBF

Hmo1 and UBF have no significant sequence conservation, but both contain HMG domains. HMG domains have strong DNA-binding and -bending properties (Stros *et al.*, 2007). Proteins harboring HMG domains may act as architectural factors facilitating the recruitment of transcription factors by co-operative interactions (Stros *et al.*, 2007). Due to this domain conservation, it was previously proposed that Hmo1 is functionally related to animal UBF acting on Pol I-dependent transcription (Gadal *et al.*, 2002; Moss *et al.*, 2007).

Besides budding yeast Hmo1 is only conserved in closely related yeast species such as *S. kluyveri* (Neuvéglise *et al.*, 2000) but not in *S. pombe*. UBF has been described in mammals and amphibians (*Xenopus laevis*) but is probably restricted to vertebrates. UBF, much like Hmo1, is important but not essential for Pol I mediated transcription (Smith *et al.*, 1993). It has been demonstrated that over-expression of UBF greatly stimulates rDNA transcription in cardiomyocytes (Hannan *et al.*, 1996), reminding of the suppression phenotype of Hmo1. The described SL interaction of *HMO1* with the topoisomerases coding genes *TOP1* and *TOP3* has been correlated with an effect of HMG proteins on DNA topology mediated by strong DNA bending (Gadal *et al.*, 2002). Bending the rDNA, Hmo1 and UBF could organize a similar target structure from two very distinct promoter-binding complexes.

To test this hypothesis, we decided to express UBF in yeast.

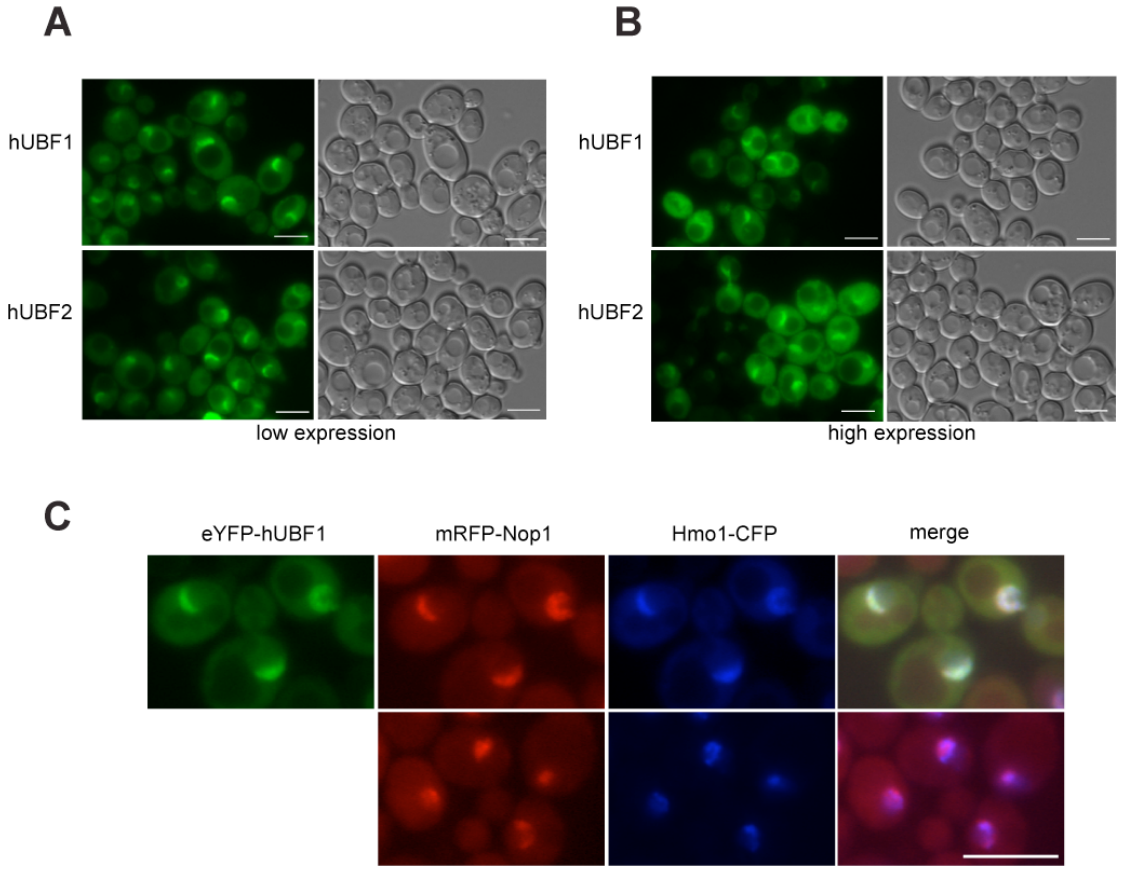

Figure 20. hUBF1 and hUBF2 localize in the yeast nucleolus.

A. hUBF1 and hUBF2 introduced into yeast cells on low copy expression vectors (plasmids pVV204-eYFP-C1-UBF1 and pVV204-eYFP-C1-UBF2, respectively) localize in the nucleolus. Cells were grown in SDC-Trp and transferred into YPD for 4 hours at 30°C prior to image acquisition. Exposure-time was 3 s. Scale bar is 5 µm.

B. As in A, but hUBF1 and hUBF2 were expressed from high copy vectors (plasmids pVV200-eYFP-C1-UBF1 and pVV200-eYFP-C1-UBF2, respectively). Exposure time was 1.5 s. Scale bar is 5 µm.

C. hUBF1 co-localizes with Nop1 and Hmo1 in the yeast nucleolus. YFP-hUBF1 fusion protein (green), expressed from the low copy expression vector using a regulatable promoter (pVV204-eYFP-C1-UBF1) was transformed into strain OGP069-1a, expressing an Hmo1-CFP fusion protein (blue) and an mRFP-Nop1 fusion protein (red) (pUN100-mRFP-NOP1). In the presence of hUBF1, Hmo1 and Nop1 fully co-localize (upper panel) whereas they do not in the absence of hUBF1 (lower panel). Scale bar is 5 µm.

1.1.1 *Localization of hUBF1 and hUBF2 in yeast*

To determine whether Hmo1 and UBF localize similarly *in vivo*, we expressed hUBF1 and hUBF2, in yeast. Both proteins were localized predominantly in a crescent shape structure flanking the nuclear envelope, reminiscent of the nucleolar structure (Figure 20A and -B).

The yeast nucleolus, as in all eukaryotes, can be divided into three distinct sub domains: the fibrillar centers (FCs), the dense fibrillar component (DFC) and the granular component (GC). FCs contain most of the rDNA. DFC corresponds to early ribosomal precursors, with a high concentration of Nop1, the yeast ortholog of mammalian fibrillarin and GC corresponds to the late ribosomal precursors (Leger-Silvestre *et al.*, 1999). A recent report questions the existence of distinct FCs in yeast, where FC and DFC would be one single fibrillar structure (Thiry and Lafontaine, 2005). The non-overlapping localization of Nop1 (DFC) and Hmo1 (rDNA) supports the existence of a third sub-domain, containing rDNA, distinct from the DFC (Gadal *et al.*, 2002; Leger-Silvestre *et al.*, 1999), described as nucleolar interstices in this recent report (Thiry and Lafontaine, 2005). In humans, UBF is found both in the DFC and the FC (Raska *et al.*, 1995). YFP-hUBF1 was expressed in a yeast strain bearing two fusion proteins, mRFP-NOP1 to map the DFC, and an integrated Hmo1-CFP to map the rDNA. YFP-hUBF1, Hmo1-CFP and mRFP-Nop1 appear fully co-localized (Figure 20C). In the absence of YFP-hUBF1 we observe a smaller nucleolar size and two sub-domains with Nop1 and Hmo1 only partially overlap (Figure 20C). Therefore, fluorescently labeled hUBF1 localizes in the yeast nucleolus and recruits the rDNA into the Nop1 containing region resulting in a changed nucleolar morphology. A further characterization of UBF in yeast is now needed to be able to functionally interpret these structural alterations of the nucleolar organization.

1.1.2 *Expression of hUBF1 and hUBF2 in yeast mutants*

To test their ability to rescue the growth defect associated with *HMO1* deletion, we expressed hUBF1 and hUBF2 in yeast. We first expressed YFP-UBF1 and YFP-UBF2 but could not observe any functional complementation (data not shown). Tagged UBF proteins were often found non-functional in *in vitro* transcription assays

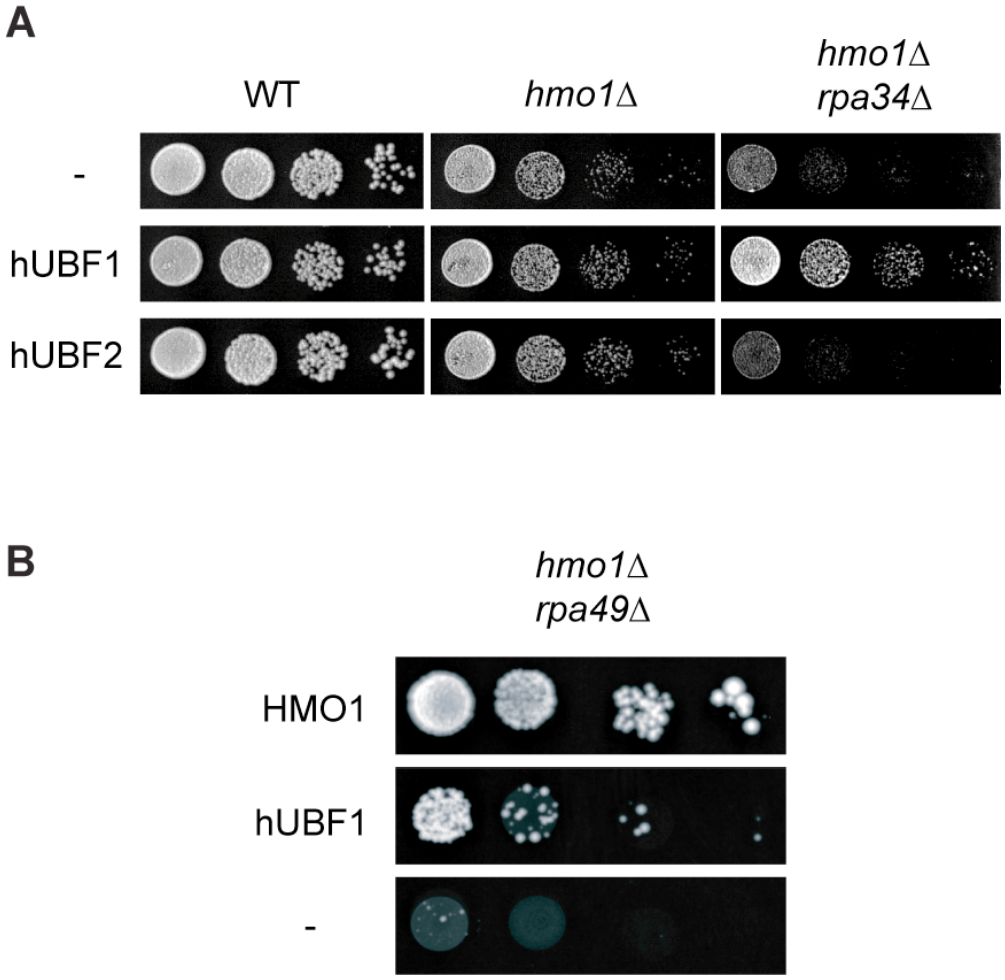

Figure 21. hUBF1 and Hmo1 function partially overlap.

A. hUBF1 but not hUBF2 partially complements the growth defect of *rpa34*Δ *hmo1*Δ strain. Strain BY4741 (WT), Y16969 (*hmo1*Δ) and YAB11-1a (*hmo1*Δ *rpa34*Δ) were transformed with pVV214 (-), pVV214-UBF1 (hUBF1) or pVV214-UBF2 (hUBF2). Tenfold serial dilution-series were spotted on SDC-U and growth was scored after 3 days at 25°C.

B. hUBF1 restores viability of the *rpa49*Δ *hmo1*Δ double mutant. Strain YAB9-1a (*hmo1*Δ *rpa49*Δ + pGID-HMO1) was transformed with pFL36-HMO1 (*HMO1*), pRS425-pPGK-UBF1 (hUBF1) or pRS425 (-). Growth was scored by a tenfold serial dilution-series on 5-FOA containing medium after 4 days at 30°C.

(Brian Mc Stay, personal communication). We next expressed untagged human UBF1 and UBF2 cDNAs in an *hmo1*Δ mutant background. As for the tagged versions, no significant rescue of the growth defect of *hmo1*Δ could be observed, showing that neither hUBF1 nor hUBF2 can substitute for Hmo1 *in vivo* (Figure 21A). We then combined *hmo1*Δ and Pol I specific subunit deletions. In the double mutant *hmo1*Δ *rpa34*Δ*,* untagged hUBF1 partially rescues the growth defect (Figure 21A). Expression of hUBF1 can also rescue the lethality of an *hmo1*Δ *rpa49*Δ (the ortholog of human PAF53) double mutant (Figure 21B). Thus, although hUBF1 could not fully rescue Hmo1 function, its expression can restore growth of Pol I mutants in the absence of Hmo1.

1.1.3 *Distribution of hUBF on the yeast rDNA unit*

In a next step we wanted to compare the localization of hUBF on the yeast rDNA to its "natural" localization on the human rDNA (O'Sullivan *et al.*, 2002) and to the binding sites of Hmo1 (see manuscript 2). Using hUBF-specific Antibodies (kind gift of Brain McStay), we performed chromatin immunoprecipitation analysis as described earlier (Galy *et al.*, 2004), followed by the stringent washing and DNA extraction procedures described previously (Bier *et al.*, 2004). Quantification of the precipitated DNA was achieved by quantitative real time PCR (qPCR), analyzing 20 amplicons covering the whole rDNA unit (Figure 22A, manuscript 2).

First, we observed a weak enrichment of rDNA when comparing UBF expressing strains to a control strain. While hUBF1 was found to be distributed all over the rDNA unit at low levels, no association of hUBF2 with the rDNA could be reproducibly observed (Figure 22B). As in vertebrates, we observe UBF localization all over the rDNA (O'Sullivan *et al.*, 2002). However, we do not detect any preferred UBF binding sites, while in vertebrates a preferential binding of UBF to the Pol I promoter region has been demonstrated (O'Sullivan *et al.*, 2002). Deletion of Hmo1 does not significantly affect distribution of hUBF1 within the rDNA (data not shown). If our detected low enrichment is significant, we conclude that hUBF1, but not hUBF2, is distributed all over the rDNA in yeast.

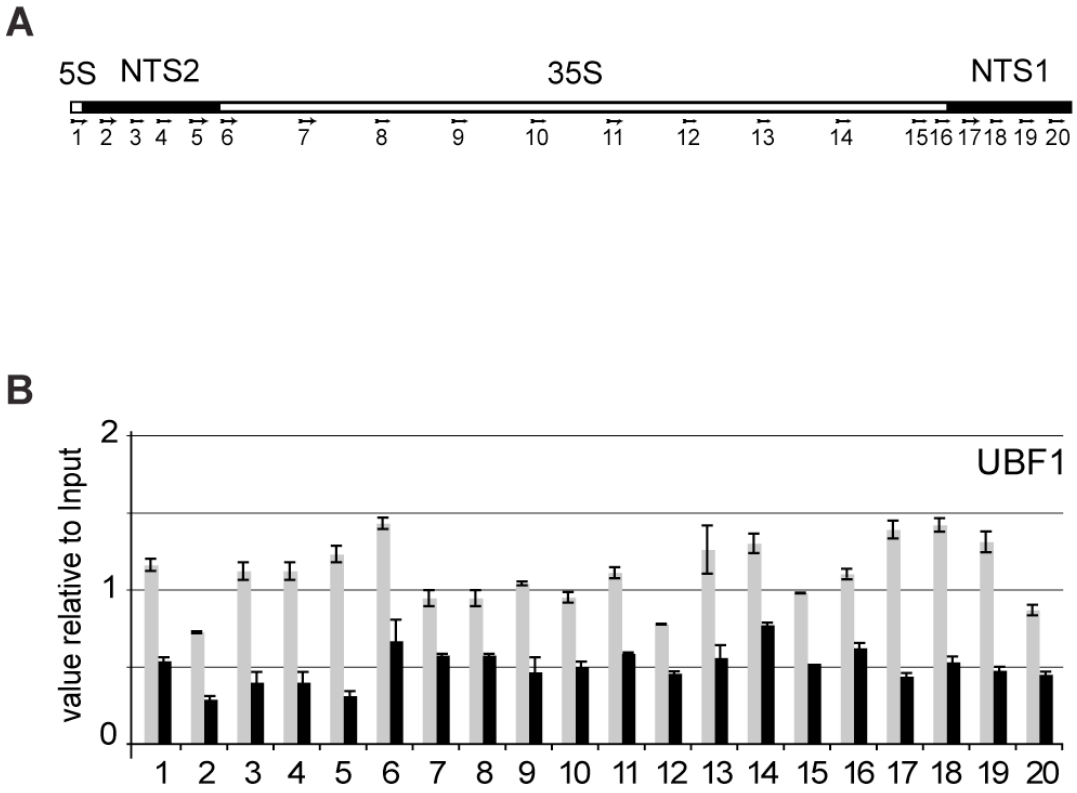

Figure 22. hUBF1 binds all over the yeast rDNA.

A. Schematic representation of an rDNA unit. The amplicons within the rDNA (NTS1 & -2: non-transcribed spacer 1 & -2) used to analyze the immuno-precipitated DNA by quantitative PCR are indicated by arrows (1 to 20).

B. hUBF1 chromatin immuno-precipitation. Relative values of immunoprecipitated DNA compared to DNA in the whole cell extract (Input) using UBF antibodies. Values obtained for strain BY4741 containing pVV214-UBF1 are shown in grey, results for the BY4741 control strain in black. Standard deviations from 3 different measurements are indicated.

1.2 Influence of Hmo1 on Pol I transcription

Genetic evidence linking Hmo1 to Pol I is very clear. Over-expressing Hmo1 strongly suppresses the cold sensitive defect of rpa49Δ mutants lacking the conserved Pol I-specific Rpa49/PAF53 subunit. *HMO1* null mutants are lethal in *rpa49*Δ cells. This defect is rescued by Pol II-dependent transcription of the rDNA. However, we lack the molecular mechanism describing the function of Hmo1 in the Pol I transcription cycle. Trying to characterize this effect, I will now present preliminary results from *in vitro* transcription assays. Although some of the results await confirmations by more extensive experiments some of the preliminary result are extremely encouraging.

1.2.1 Promoter-dependent transcription assays

Two protocols allowing partial purification of Pol I for *in vitro* transcription assays have been established (Keys *et al.*, 1994; Tschochner, 1996). I followed the method described by Herbert Tschochner which maximizes *in vitro* transcriptional efficiency. While a wild type fraction was already available (gift from M. Felle), I purified Pol I from two yeast mutant strains, *hmo1*Δ and *rpa34*Δ, to allow multiple testing. Each of these fractions allows to study a different functional aspect of Hmo1. Using the WT extract, complemented with recombinant Hmo1 (gift from H. Goetze), we could mimic *in vivo* Hmo1 over-expression. Using the *hmo1*Δ fraction, we could analyze transcription in the absence of Hmo1. Finally, Pol I purified from an *rpa34*Δ strain, in which not only Rpa34 is absent but also Rpa49 is largely destabilized *in vitro*, allowed analysis of Pol I lacking the subunits genetically interacting with Hmo1 *in vivo*.

1.2.1.1 Purifying Pol I transcription competent fractions

Pol I initiation dependent assays from its native promoter require besides the core enzyme itself a variety of co-factors some of which have already been described (see introduction, 1.2.2 and 1.2.3). Therefore, Pol I should be purified from total extract in a way that preserves associated factors required for initiation. Such a purification

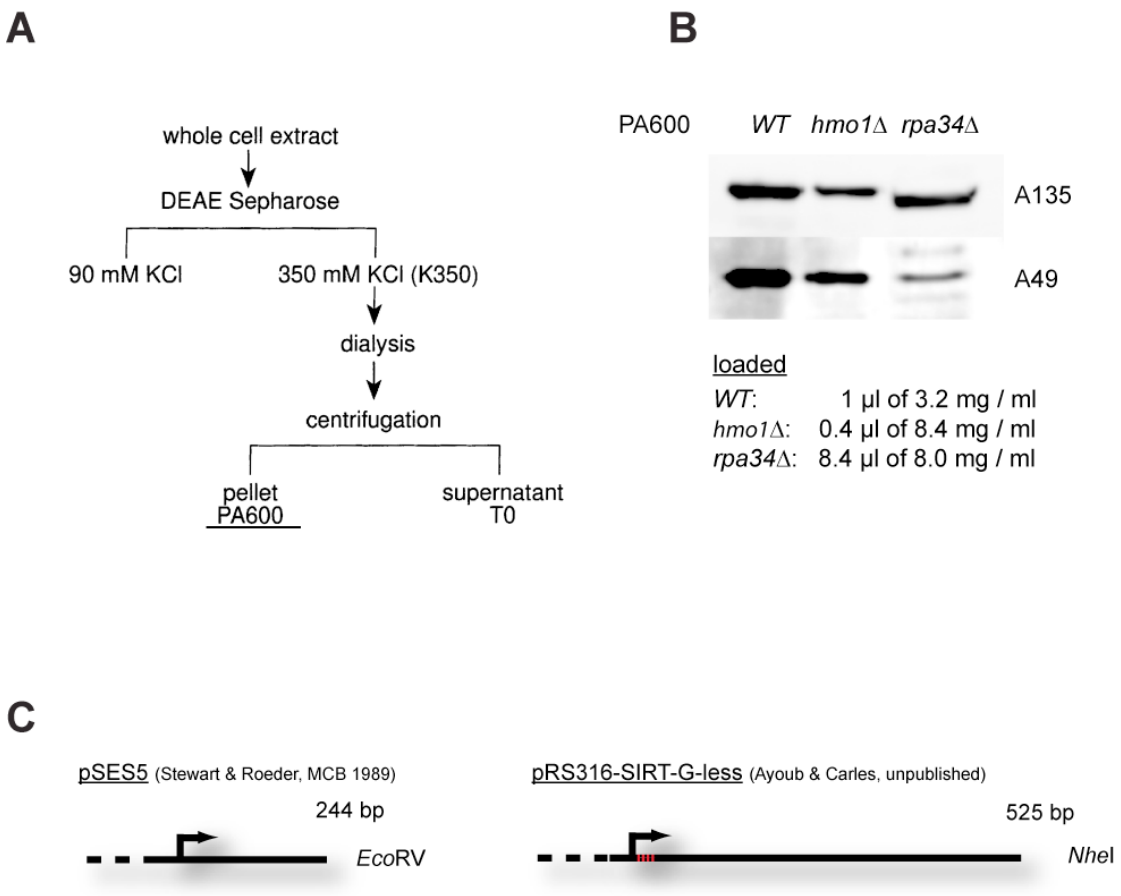

Figure 23. Components for *in vitro* transcription assays.

A. Representation of the purification scheme followed to obtain the Pol I containing fraction PA600. Adapted from (Milkereit and Tschochner, 1998).

B. Quantification of Pol I in PA600. Purifications from three different genetic backgrounds are analyzed: WT (BJ926), *hmo1*Δ (Y16969) and *rpa34*Δ (Y1127). Presence of Pol I subunits Rpa135 and Rpa49 was tested with specific antibodies. Note the slightly higher exposure of the blot probed with antibodies against Rpa49, making Rpa49 subunit loss in the *rpa34*Δ background more important. Total protein concentrations yielded and the volume loaded onto the gel are indicated below.

C. Templates used during the study. Plasmid pSES5 was digested *Eco*RV and plasmid pRS316-SIRT-G-less *Nhe*I, yielding templates of 244 bp and 525 bp in length respectively.

scheme has been established in the laboratory of H. Tschochner ((Tschochner, 1996), see Figure 23A) and used successfully thereafter (Milkereit *et al.*, 1997; Milkereit and Tschochner, 1998; Tschochner and Milkereit, 1997). In the course of this work we made use of this protocol to partially purify Pol I from wild type (fraction "PA600 WT"), a Pol I fraction from an *hmo1*Δ strain ("PA600 *hmo1*Δ") and a Pol I fraction from an *rpa34*Δ strain ("PA600 *rpa34*Δ"). While the purification of the "PA600 *hmo1*Δ" fraction yielded similar amounts of Pol I as the earlier purified WT fraction (see Figure 23B), the "PA600 *rpa34*Δ" fraction was greatly reduced in Pol I, presumably due to a changed charge of the mutant enzyme, making purification over the anion-exchange column (see Figure 23A) less efficient. Note that two antibodies were used to verify Pol I presence, one directed against the Rpa135 subunit and one against the Rpa49 subunit. Even though it has been reported that Pol I purified from an Rpa34 subunit deficient strain also lacks the Rpa49 subunit (Gadal *et al.*, 1997), our fraction purified from *rpa34*Δ cells still contained Rpa49, albeit in sub-stoichiometric amounts. Two plasmids were used as templates. The first one contains a WT portion of the yeast rDNA and has been used in *in vitro* studies since the late 1980ies (Stewart and Roeder, 1989). The second one is a mutated version of the first: the first six guanosines had been replaced by a cytosine or an adenosine to yield a guanosine-free 22-mer, followed by a engineered stretch of three guanosines (kind gift of N. Ayoub and C. Carles). This last plasmid could allow to distinguish initiation from elongation events by first providing only an NTP-mix lacking guanosine triphosphate (GTP) (initiation, promoter escape) while addition of (GTP) then triggers the elongation reaction. To define the transcript and the specificity of the templates, different restriction enzymes have been used define the length of the transcript. The chosen, cut plasmids yield transcripts of 244 bases (pSES5, *Eco*RV) and 525 bases (pRS316-SIRT-G-less, *Nhe*I), respectively (see Figure 23C) if the initiation events occur at the Pol I promoter. Any other product size could result from Pol I promoter independent initiation events (see below).

The three different Pol I fractions have been tested for their ability to transcribe the templates by titrating their concentration within the reaction volume following the protocol established by H. Tschochner's group (Milkereit *et al.*, 1997). Figure 24 shows that the 244 bases transcript is already clearly visible when 0.5 µl of wild type PA600 was added to the reaction (lane 3) and increases drastically when twice as

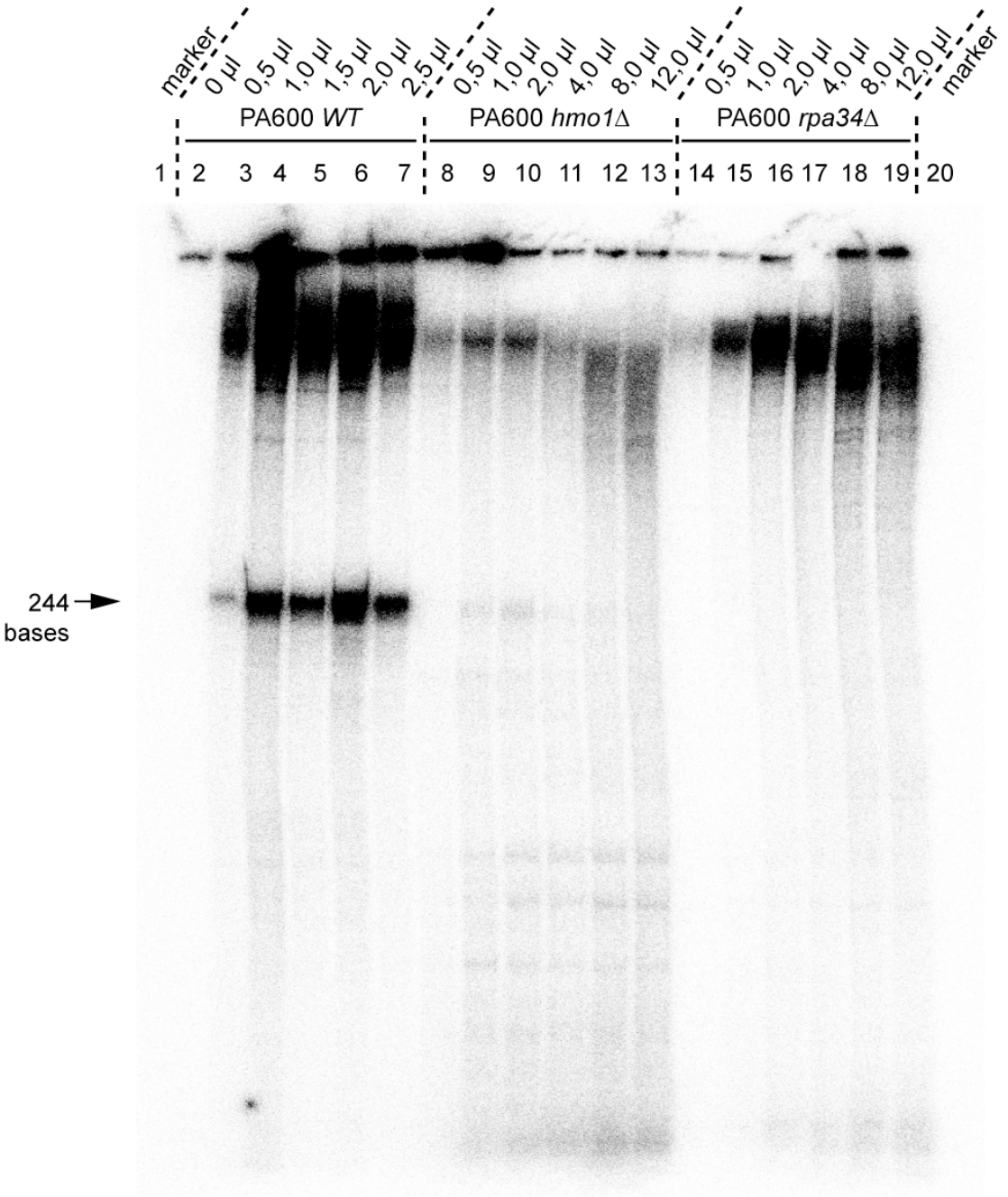

Figure 24. Titration of Pol I containing fractions for transcription assays.

Autoradiography of a dried polyacrylamid gel loaded with transcription reactions. 80 ng of template pSES5 were incubated with the indicated PA600 fractions and volumes in the presence of NTPs and ^{32}P-labeled GTP for 30 minutes at room temperature. The expected, promoter-specific transcript is indicated.

much extract was used in the reaction (lane 4). Increasing the amount of Pol I fraction above this level does not seem to increase the amount of the transcript (lanes 5 to 7). Note also that higher molecular weight transcripts, which probably initiate in a promoter independent manner, are detected. The template has been linearized allowing unspecific transcription events to occur since the polymerase can transcribe from the free ends of the plasmid. The situation is different for fractions "PA600 *hmo1*Δ" and "PA600 *rpa34*Δ". No specific transcript at all can be detected for the *rpa34*Δ fraction, no matter how big the amount of the fraction is (lanes 14 to 19). The "PA600 *hmo1*Δ" fraction does not yield a detectable specific template at the lowest amount of extract tested (lane 8), however, a faint signal becomes apparent if this amount is doubled or quadruplicated (lanes 9 and 10). Further increasing the amount of *hmo1*Δ fraction leads to disappearance of the specific transcript and the concomitant appearance of smaller transcripts (lanes 11 to 13). The amount of these last products increases with the amount of PA600 *hmo1*Δ fraction and seems to be specific, since they are not observed in the WT and *rpa34*Δ fraction containing reactions. These shorter transcripts may represent aborted transcripts suggesting a lack of processivity of the polymerase. We also observed transcription products much bigger than the expected 244 base product. For the *rpa34*Δ fraction, these signals clearly increase with increased PA600 *rpa34*Δ fraction amount (lanes 14 to 19). Prior to the assay, the template has been linearized allowing unspecific transcription events to occur since the polymerase can transcribe from the free ends of the plasmid. One can only imagine that the bigger products are unspecific transcripts from such "initiation" events. Increasing product amounts with increasing *rpa34*Δ polymerase fraction could suggest that the elongation capabilities of the mutant fraction are still present. We could confirm this speculation by initiation independent assays, as described in result section 1.2.3.

In this first characterization, we could observe that Hmo1 is probably dispensable for *in vitro* transcription since a specific transcript is observed in *hmo1*Δ fraction containing reactions. However, the strong reduction in transcription product could suggest an involvement of Hmo1 in this *in vitro* transcription assay. If this finding results solely from the direct absence of Hmo1, providing back Hmo1 in the assay should restore full activity. The result of the *rpa34*Δ fraction reactions not showing any specific transcription product is hard to interpret. It seems as if the potentially

unspecific, large transcripts increase linearly with increasing amounts of this Pol I fraction. At this stage we can only vaguely speculate that the fraction has a defect in initiating at the Pol I promoter.

1.2.1.2 Complementing the transcription assays with ectopically purified Hmo1

The previous assay could suggest a direct involvement of Hmo1 in the transcription reaction. To test for such a direct implication, we next tried to rescue the highly reduced (*hmo1*Δ) or not detectable (*rpa34*Δ) transcription potential of the two mutant fractions by addition of recombinant Hmo1.

The results are shown in Figure 25. For WT PA600, an amount had been chosen that would still allow to judge for activity increases (0.5 µl, compare Figure 24, lane 3). Addition of recombinant Hmo1 resulted in substantial product increase (Figure 25, lanes 1 to 6) equivalent to increased transcriptional activity detected when more WT extract is used (compare Figure 24 lanes 2 to 7).

In the case of the mutant extracts addition of Hmo1 has no positive effect on transcriptional activity (lanes 7 to 20). The lack of complementation is difficult to interpret. One possible explanation in case of the *hmo1*Δ-mutant extract is that other polymerase associated factors are getting lost during the purification procedure. In case of the *rpa34*Δ-mutant fraction that addition of Hmo1 cannot rescue the absence of Rpa34 and the reduced presence of at least one other protein namely Rpa49. Furthermore in the latter fraction it is of course possible too that other factors usually stabilized via Rpa34/Rpa49 are depleted during the purification.

In this experiment we cannot draw a conclusion for the mutant extracts. Further assays depicting the specific defect of the extracts are required. One such assay are promoter independent assays (see below). One can also try to complement transcription reactions with WT fraction that is strongly enriched in Pol I associated proteins but lacks Pol I transcriptional activity (fraction “B600”, (Milkereit and Tschochner, 1998)).

The stimulatory effect of Hmo1 on WT extract is extremely interesting. This is a good starting point allowing the molecular and functional characterization of Hmo1.

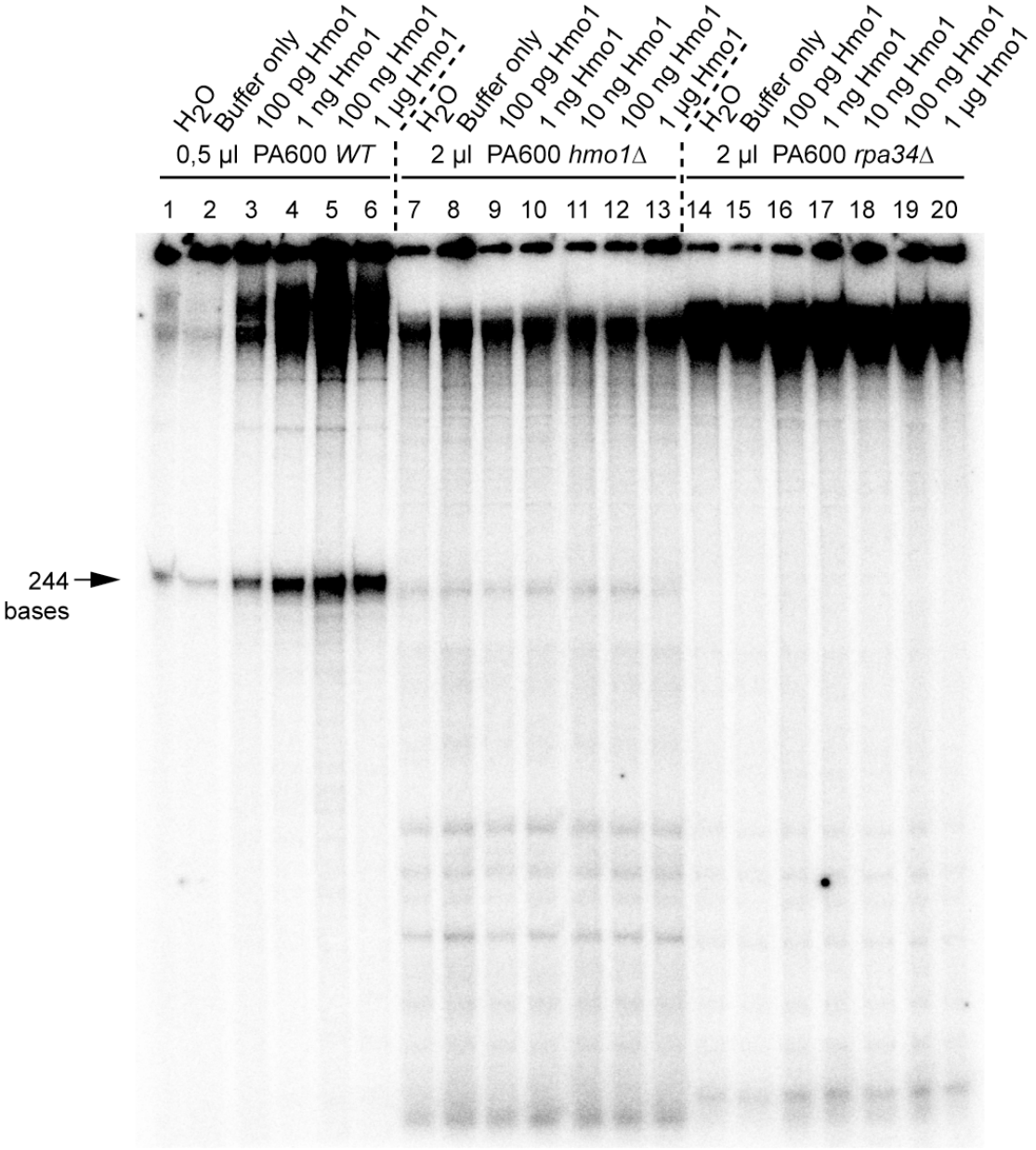

Figure 25. Effect of Hmo1 on promoter specific Pol I transcription.

Same assay as outlined in Figure 24. Ectopically purified Hmo1 was titrated into the reactions at indicated amounts (final reaction volume 25 µl). In the reaction "buffer only" NaCl and imidazol have been added to reach the same final concentrations as in a reaction complemented with 1 µg of purified Hmo1 (10 mM NaCl, 2 mM imidazol final).

1.2.1.3 Transcription from nucleosomal templates

Hmo1 is an HMG-box protein. As described in the introduction, HMG proteins are integral components of chromatin (see introduction, 2.1.1.3.). The rDNA chromatin structure is now well described, with differences between transcriptionally competent or active and inactive rDNA genes as revealed by psoralen cross-linking experiments (Dammann *et al.*, 1993). A recent study now adds the notion that the fraction of rDNA "largely devoid of histone molecules" coincides with actively transcribed genes (Merz *et al.*, 2008). However, histones have been mapped in actively transcribed genes suggesting a rather dynamic nucleosomal template (Jones *et al.*, 2007), supported by studies on *P. polycephalum* showing a rapid replication-independent exchange of histones at transcribed rDNA genes (Thiriet and Hayes, 2005). Other studies have demonstrated that histones are required for Pol I transcription (Tongaonkar *et al.*, 2005), besides their presence as part of the UAF (Keener *et al.*, 1997). While UBF has been found to associate with both, transcriptionally active and inactive loci in human cells (Sullivan *et al.*, 2001), a study published this year by Griesenbeck and co-workers describes Hmo1 to be specifically associated with actively transcribed rDNA genes (Merz *et al.*, 2008) in a reciprocal behavior to histone molecules (s. above).

To test whether Hmo1 is required for or facilitates transcription from a nucleosomal template, the templates described in Figure 23C have been assembled into chromatin. Using the salt gradient technique (Rhodes and Laskey, 1989), histones purified from Drosophila extracts have been assembled as previously described (Längst *et al.*, 1999). The concentration ratio of template (*Nhe*I digested pRS316-SIRT-G-less) versus histones during the assembly process has been titrated and judged appropriate at 1 to 1.2 as shown by micrococcal nuclease (MNase) digestion assay (Figure 26A).

Using this template assembled into nucleosomes in the aforementioned transcription assay did not yield a detectable transcription product for the WT PA600 nor for PA600 *hmo1*Δ (Figure 26B, lanes 6 and 16, respectively). Furthermore, addition of Hmo1 protein could not compensate for the apparent inaccessibility of the template.

In conclusion, this experiment did not provide functional evidence that Hmo1 alone is able to out-compete nucleosomes bound to the rDNA. However, it is still possible that

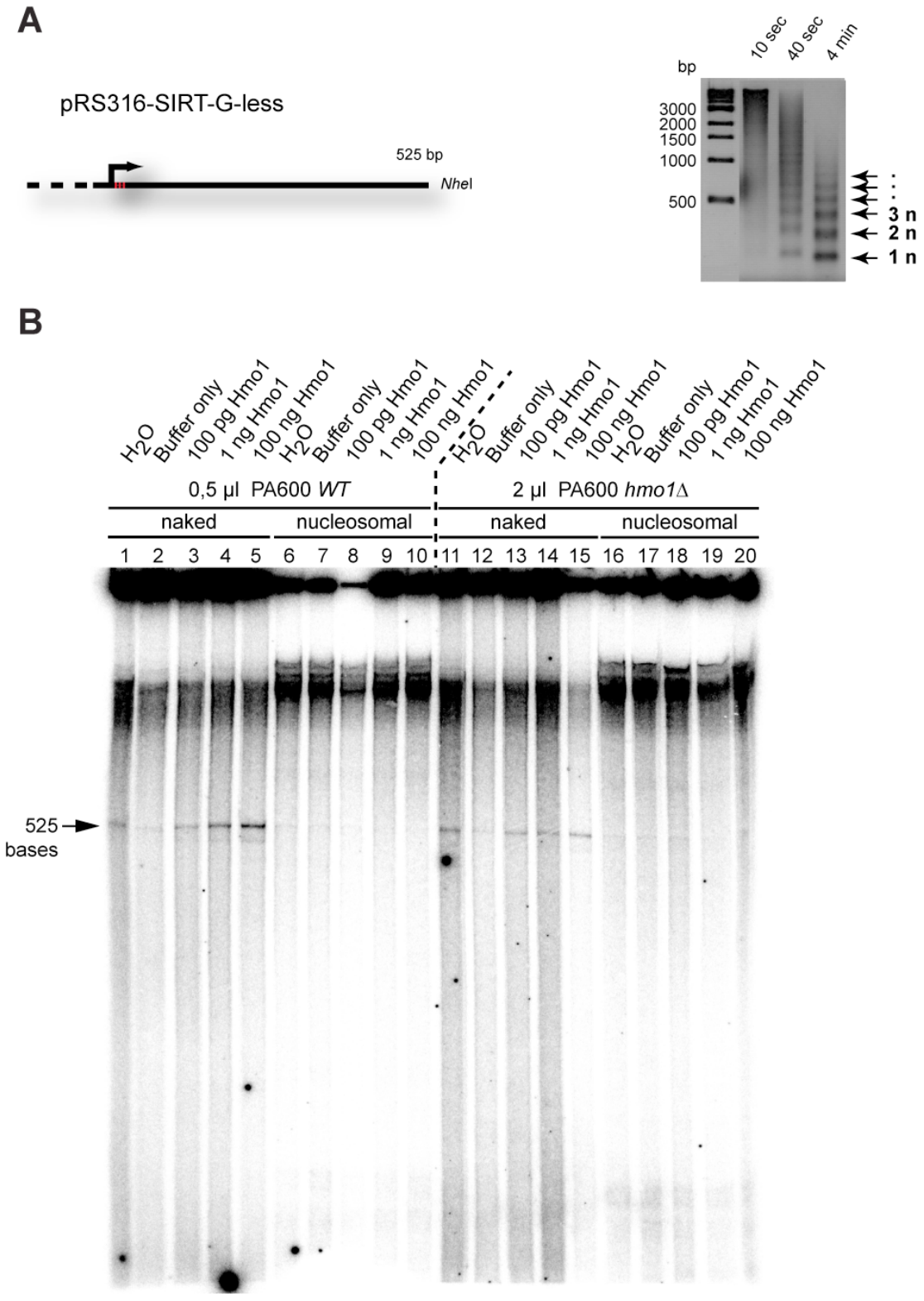

Figure 26. Hmo1 in transcription assays using a nucleosomal template.

A. Template pRS316-SIRT-G-less was assembled into nucleosomes using salt dialysis assembly of *Drosophila* histones as described (Längst *et al.*, 1999). Packaging was found to be most efficient at a mass ratio of template to histones of 1 to 1.2, as verified by MNase digestion (n depicts the number of nucleosomes).

B. Same assay as in Figure 23. WT and *hmo1*Δ PA600 fractions supplemented by varying amounts of Hmo1 have been assayed for their ability to transcribe from naked and nucleosomal templates. In the reaction "buffer only" NaCl and imidazol have been added to reach the same final concentrations as in a reaction complemented with 100 ng of purified Hmo1.

remodeling factors displace nucleosomes prior to Hmo1 binding. Hmo1 once bound could then stimulate transcription and prevent nucleosome re-assembly. This hypothesis could also be tested *in vitro*, pre-incubating the nucleosomal template with different remodeling factors available in the Tschochner lab.

1.2.2 Promoter-independent transcription assays

The first assays demonstrated a strong stimulation of Hmo1 on *in vitro* Pol I transcription depending on the Pol I promoter, a weak activity of the *hmo1*Δ extract and a no detectable specific activity for the *rpa34*Δ fraction in the same system. The presence of Pol I enzyme in all three extract has been demonstrated, but we wanted to test the non-specific activity of Pol I present in the three PA600 fractions.

1.2.2.1 Elongation competence of Pol I purified from WT-, *hmo1*Δ- and *rpa34*Δ cells

General polymerase activity can be tested since the polymerase has the ability to produce RNA, independently of promoter-bound factors.

The template for promoter independent transcription assays is partially digested DNA. We used calf thymus DNA partially digested with DNase I (Aposhian and Kornberg, 1962). In the presence of Mg^{2+} ions, DNase I leads to cleavage of each strand individually in a random fashion (Sambrook and Russell, 2001). This allows polymerases to access the template very efficiently at single strand nicks directly without the need of initiation events.

The assay makes use of tritium-labeled UTP that gets incorporated into transcripts; the transcription reaction is then filtered; filters are washed and tritium incorporation

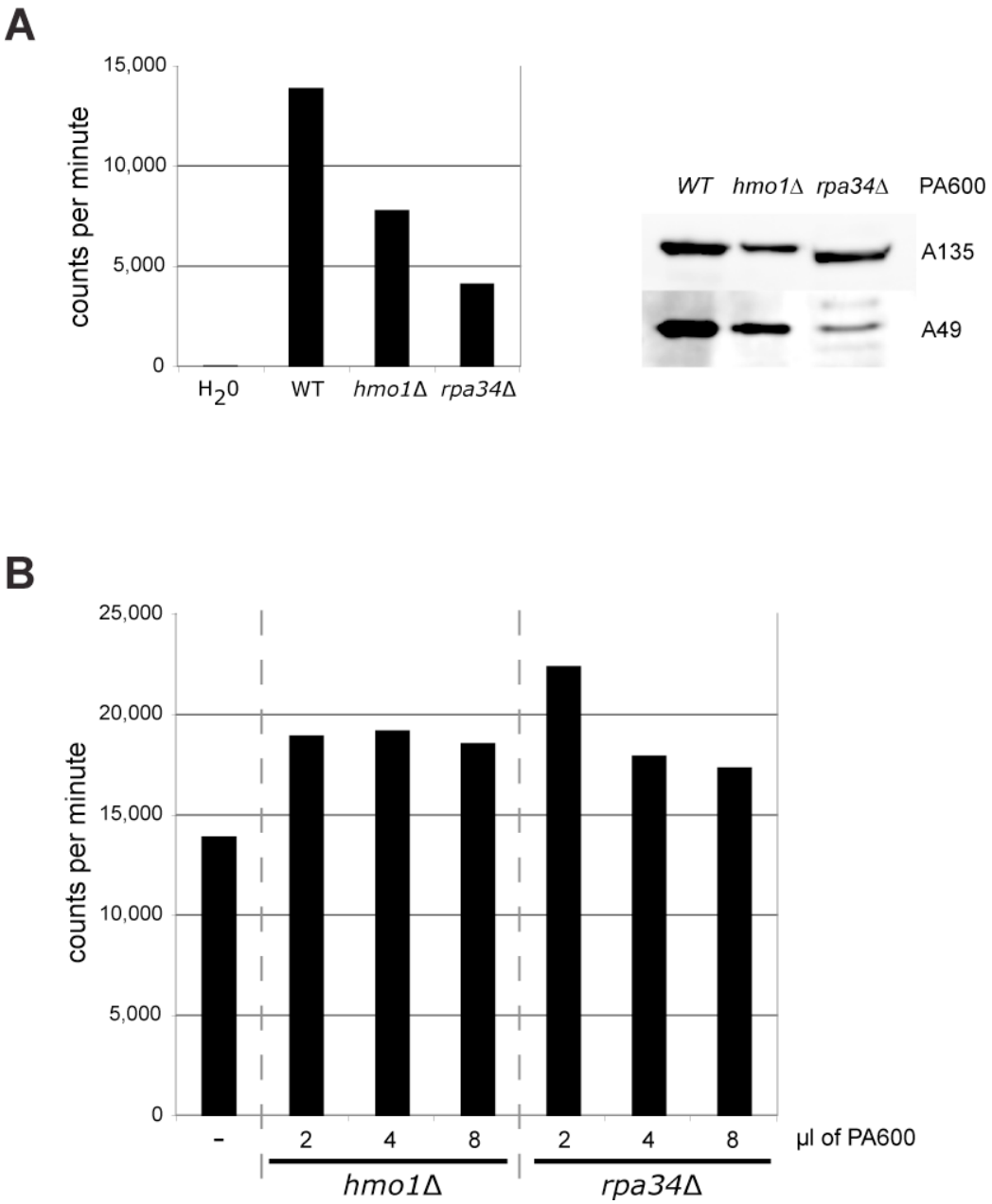

Figure 27. Transcription potential of Pol I fractions in promoter independent transcription assays.

Fractions were tested for their transcription potential by incubating them with 80 ng of DNase I digested calf thymus DNA in the presence of a ^{3}H-UTP containing NTP-mix. Transcription reactions were filtered through glass-fiber and incorporation of ^{3}H-UTP determined afterwards using a liquid scintillation analyzer.

A. Same amounts of PA600 as loaded on the western blot were tested for the transcription potential.

B. 1 µl of WT PA600 with varying amounts of mutant PA600 was analyzed to test for potential inhibiting contaminations in these fractions.

is then measured in a scintillation analyzer as previously described (Milkereit *et al.*, 1997; Schnapp *et al.*, 1990).

The results of these measurements are expressed as counts per minute (cpm) as shown in Figure 27. The same amounts of Pol I fraction loaded on the previous western blot have been assayed. Based on the amount of Pol I in each fraction as judged by western blot, the incorporation of UTP in the transcripts is comparable for Pol I purified from WT and *hmo1*Δ cells (two to three times less polymerase in the western, less transcript in the *in vitro* assay). The *rpa34*Δ fraction is impaired in its capacity to elongate as described before for Pol I* (Huet *et al.*, 1975).

From these assays, we can conclude that all three extracts contain active Pol I, but with differing transcription potential. Due to the lack of discrimination of the size of the transcript generated by each fraction, two scenarios are possible taking the results of these first experiments together: the extract is limited in initiation or promoter clearance or the extract lacks processivity.

An important control is to verify that the purified fractions do not contain inhibitory components. This has been tested by increasing addition of mutant extract to the WT extract. As can be seen in Figure 27B, addition of mutant extracts to the WT extract does not reduce its transcription competence. This suggests that the observed transcriptional defects of the *rpa34*Δ fraction, are indeed due to the mutant polymerase and not due to contaminants inhibiting the reaction, while the extract still possess WT potential.

We then wanted to see, if addition of ectopically purified Hmo1 could boost transcription as seen in the specific assay for WT extract, or compensate the slight (*hmo1*Δ fraction) or severe (*rpa34*Δ fraction) elongation defect. Using Hmo1 concentrations as tested in the initiation dependent assay, no clear increase of transcription potential could be observed for any of the three Pol I containing extracts (see Figure 28).

In conclusion, our three extracts do have transcriptional activity in promoter independent assays. However, *rpa34*Δ PA600 shows a clearly reduced elongation potential. The two mutant fractions do not contain contaminants hiding transcriptional activity of Pol I. We can only speculate about the observed such discrepancy between the non-specific transcription assay and promoter specific activity using the *hmo1*Δ and *rpa34*Δ fractions. These preliminary results point towards a specific requirement

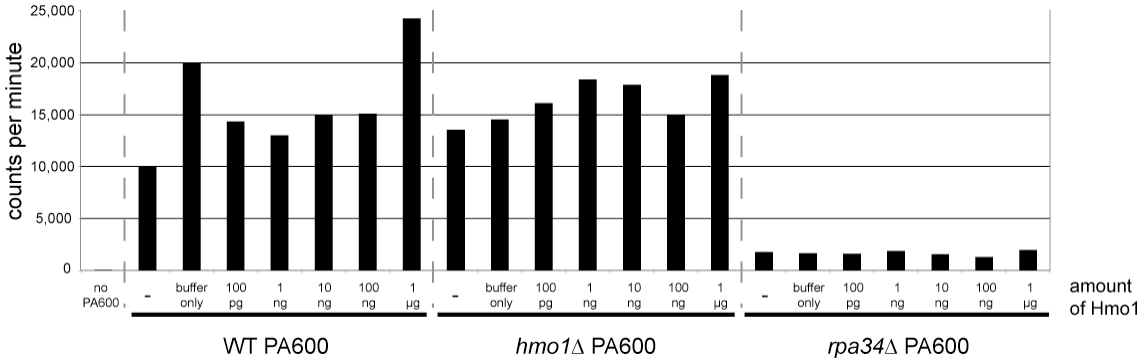

Figure 28. Hmo1 in PA600-driven promoter independent transcription.

Ectopically purified Hmo1 was titrated into promoter independent transcription assays at indicated amounts (final reaction volume 25 µl). WT PA600 (0.5 µl), *hmo1*Δ PA600 (2 µl) and *rpa34*Δ PA600 (2 µl) were assayed in parallel. In the reaction "buffer only" NaCl and imidazol have been added to reach the same final concentrations as in a reaction complemented with 1 µg of purified Hmo1 (10 mM NaCl, 2 mM imidazol final).

for both, Rpa34 and Hmo1 in initiation as well as in transcription elongation. Like for the promoter dependent assay, addition of recombinant Hmo1 has no effect on the decreased activity of the mutant extracts. However, in contrast to the promoter specific assay, Hmo1 does not seem to increase WT PA600 transcriptional activity, at least at protein concentration from 100 pg to 1 µg (see below).

1.2.2.2 Initiation independent assay using pure polymerases

We could observe a stimulatory effect of Hmo1 in promoter-dependent assays using Pol I containing protein fractions, but not for non-specific transcription.

The stimulatory effect of Hmo1 on Pol I activity in the specific assays can still be an indirect one, such as titration of an inhibitory factor or a co-activation with proteins from the same fraction, rather than a direct effect of Hmo1 on Pol I activity. Since the promoter independent transcription assays can be performed without the help of initiation factors, a highly purified polymerase can be used. The further addition of pure protein(s), such as Hmo1 in our case, allows an easier interpretation of the result, as compared to the much more complex polymerase containing fraction experiments.

The assays were carried out with RNA polymerase I (Gerber *et al.*, 2008; Kuhn *et al.*, 2007) and, to test for specificity, also with Pol II (kind gift of P. Cramer). Figure 29 summarizes the results obtained from three experiments, in which the transcription assays had been complemented with Hmo1. Hmo1 increases transcription efficiency specifically for Pol I. Contrary to the complementing experiments obtained in the initiation dependent assays using the PA600 fractions, the result in transcript increase is not linear (compare Figure 25), starting with very little amounts such as 10 ng of Hmo1 (4 ng / ml), but only shows an effect with bigger amounts like 1 µg (40 µg / ml), making a factor 10^4 difference. On the other hand, increasing Hmo1 protein concentration to 0.4 mg / ml (10 µg of protein) still increases transcription efficiency while the coupled increased salt and imidazol concentrations do not inhibit transcription in this assay. As a specificity control, RNA polymerase II does not show increased transcription potential when supplemented with Hmo1 in the assay (Figure 29, right side).

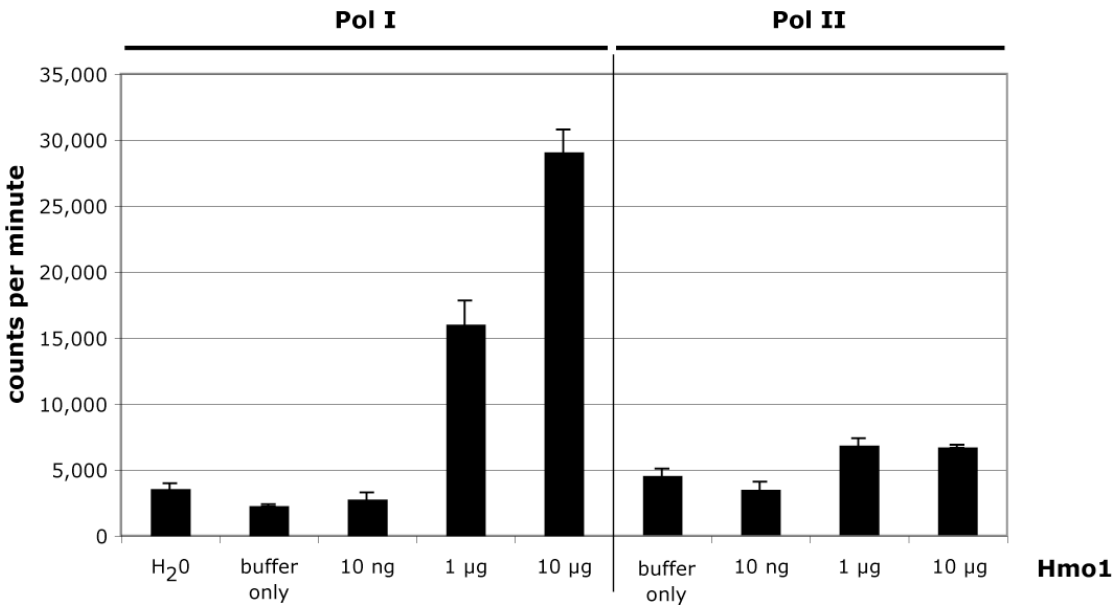

Figure 29. Hmo1 in a transcription system with pure Pol I and Pol II.

Hmo1 was titrated into promoter independent transcription assays at indicated amounts. Pol I (333 ng) and Pol II (500ng) purified to a high degree (see text for details), were assayed in 25 µl reactions in parallel. In the reaction "buffer only" NaCl and imidazol have been added to reach the same final concentrations as in a reaction complemented with 10 µg of purified Hmo1 (100 mM NaCl, 20 mM imidazol final). Standard deviations are based on 3 parallel experiments each.

In conclusion, we could detect two unexpected effects of Hmo1, both related to a stimulatory effect of recombinant Hmo1 in *in vitro* transcription assays. We detect a robust stimulatory effect of Hmo1 in initiation dependent assays using the wild type PA600 fraction. When using high amounts of protein, we could also detect a stimulation of Hmo1 in non-specific transcription assays using pure Pol I.

These findings are unexpected because a stimulatory effect for DNA binding proteins is not obvious. The described regulation of Pol I elongation by UBF is based on an inhibitory effect on transcription when UBF is bound to the DNA (Stefanovsky *et al.*, 2006a). Phosphorylation of UBF then allows Pol I to transcribe through UBF bound DNA (Stefanovsky *et al.*, 2006a).

The effects of Hmo1 on Pol I transcription remain to be confirmed using supporting assays making use of the G-less cassette plasmid described above, or immobilized tailed templates that allow to measure transcription efficiency from a single Pol I transcription round (Tschochner and Milkereit, 1997). However, we provide first evidence of a stimulatory effect of Hmo1 on both, Pol I initiation and elongation in *in vitro* transcription assays.

2. INFLUENCE OF HMO1 ON RIBOSOMAL PROTEIN GENE TRANSCRIPTION

2.1 Objective and Summary

Hmo1 had been genetically linked with the Pol I transcription machinery. At the same time, Hmo1 is an HMG-box protein with very little, if at all, sequence specificity (Bianchi and Agresti, 2005; Kim and Livingston, 2006). Global protein-protein interaction screens had found Hmo1 to interact with Fhl1, a yeast transcription factor specific to ribosomal protein gene transcription (RPG) (Ho *et al.*, 2002; Ito *et al.*, 2001). Furthermore Hmo1 had been shown to interact genetically and physically the peptidyl-prolyl cis-trans isomerase Fpr1 (Dolinski and Heitman, 1999), a protein required for rapamycin dependent TOR pathway inhibition (Heitman *et al.*, 1991). These indications led us to ask if Hmo1 fulfills functions in the cell different from its role in Pol I mediated rRNA transcription.

Since an *HMO1* deletion strain is viable, we decided to focus on a synthetic lethal screen. We assumed that an *hmo1*Δ mutant is viable because some redundant pathways allow the cells to maintain essential processes in the absence of Hmo1. Using a genetic screen we hoped to that the associated function of genetic interactors could give a hint of the essential pathway in which Hmo1 performs a non-essential function. Therefore, our aim was to isolate viable mutants that are lethal when combined with an *HMO1* deletion. During the course of this work, the lab of Alain Jacquier established a global genetic interaction screen based on a plasmid dependency assay. We could use this novel approach to investigate other putative functions of Hmo1 in the cell.

The global genetic interaction screen revealed interactions of *HMO1* with genes that could be grouped into classes. Besides Pol I associated genes, we found genes involved in stress response (especially conferring rapamycin sensitivity or resistance) and genes implicated in Pol II transcription and RPGs. To better understand the connection of Hmo1 to the Pol I transcription machinery beyond its genetic interactions, we mapped Hmo1 on the rDNA using chromatin immunoprecipitation (ChIP) analysis. We found that Hmo1 preferentially associates with the region transcribed by Pol I, suggesting a role in elongation rather than initiation (compare

result 1.2). Following the hint towards the TOR pathway, given by the SL interaction of *HMO1* with genes connected to rapamycin, we could demonstrate that a cell lacking Hmo1 is hypersensitive to rapamycin and that reducing the expression of an essential TOR complex 1 (TORC1) but not TORC2 constituent renders *HMO1* essential in this situation. The same depletion dependent necessity of HMO1 could also be established for the essential transcription factor Ifh1 that is specific to RPGs: depleting Ifh1 renders *HMO1* essential in this background. We next performed transcriptome analyses in an *hmo1*Δ mutant background or after short-term depletion of Hmo1. Global expression analysis showed a broad adaptation of the cell in an *hmo1*Δ background: more than 500 genes were found to be significantly up- or down-regulated including RPGs. We could then show that promoters of RPGs down-regulated in the absence of Hmo1 are highly bound by Hmo1, while promoters RPGs up-regulated in the absence of Hmo1 are not bound by Hmo1. The expression of much lesser number of genes is affected when Hmo1 is depleted for about a yeast cell doubling time (avoiding secondary and tertiary effects). However RPGs now show a mild but significant down-regulation.

These results demonstrated that Hmo1 binds to a subset of RPG promoters and can modulate the expression of RPG *in vivo*. However, the physiological consequence of the *HMO1* deletion was unclear. To test whether the nature of promoter bound RPG factors is different in the absence of Hmo1, we challenged the system by inhibiting the TORC1 pathway prior to our transcriptome analysis. This allowed us to test if RPGs are still able to rapidly shut-off their transcription upon TORC1 inhibition in the absence of Hmo1. Using this drug, we could demonstrate that the function of Hmo1 seems to allow rapid regulation of RPG expression under stress conditions. A published Pol I mutant strain, "CARA" (for *C*onstitutive *A*ssociation of *R*rn3 and Rp*A43*) also showed an alleviated response in terms of transcriptional down-regulation of RPGs upon rapamycin treatment (cf. introduction 1.4, (Laferte *et al.*, 2006)). To test if Hmo1 is required for the "CARA effect", we tested this mutant in an *hmo1*Δ background. We could show that the CARA mutant requires Hmo1 for its deregulation effect on RPG expression upon rapamycin treatment. Interestingly, Hmo1 is no longer bound to the rDNA nor to RPG promoters when cells are treated with rapamycin, suggesting a direct Hmo1 binding dependent regulatory mechanism.

2.2 Manuscript

November 2007

HMO1 IS REQUIRED FOR TOR-DEPENDENT REGULATION OF RIBOSOMAL PROTEIN GENE TRANSCRIPTION

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MOLECULAR AND CELLULAR BIOLOGY
Vol. 27, No. 22, pp 8015-8026

Link

(http://mcb.asm.org/cgi/content/abstract/27/22/8015)

2.3 Extended Discussion

rDNA association of Hmo1

We could show a specific association of Hmo1 with the rDNA region transcribed by Pol I. Interestingly it has now been demonstrated that the rDNA association of Hmo1 is dependent on Pol I. In a Pol I mutant (*rpa135*Δ), where rRNA is expressed from a plasmid under the control of a Pol II promoter, Hmo1 no longer associates with rDNA (Kasahara *et al.*, 2007). Additionally it has been shown that Hmo1 preferentially binds to active rDNA units, associated with Pol I (Merz *et al.*, 2008). The Hmo1 rDNA binding strength from our ChIP analysis is therefore likely to be underestimated compared to the RPG promoter values, by a factor of about two, since only about half of the rDNA units are actively transcribed in exponentially growing yeast.

Hmo1 has to date been mapped on the rDNA by several groups (Hall *et al.*, 2006; Kasahara *et al.*, 2007; Merz *et al.*, 2008). Although the results in some of these publications do not look that divergent compared to our results, their interpretation however is different. Most of the studies interpret their results as an association of Hmo1 "all over the rDNA unit". We describe a localization of Hmo1 binding that is specific to the region actually transcribed by Pol I. Our results do not exclude Hmo1 binding at the core promoter (positions -28 to +8 with respect to the transcription start site (Keys *et al.*, 1996). We can not discriminate between core element associated and only transcribed region associated, since our first amplicon showing strong, specific enrichment spans region +6 to +108 and due to the fact that DNA fragmentation during the ChIP procedure yields fragments of about 300 bp. However, a specific association of Hmo1 with the upstream control element (-146 to -100 (Keys *et al.*, 1996)), harboring the UAF, seems highly unlikely. Indeed, the amplicon covering this region (amplicon #5, manuscript figure 2) is detected about ten times less than the following amplicons (within the transcribed region). The reported Hmo1 binding to the 190 bp rDNA enhancer element 2 kb upstream of the Pol I transcription start site (Merz *et al.*, 2008) is supported by our data, although binding there, in our hands, is about 4-fold weaker than in the transcribed region (manuscript figure 2, amplicon #17). Therefore, we detect the strongest Hmo1 interacting sites within the

rDNA in the region transcribed by Pol I (including the core promoter reaching into the transcribed region).

Hmo1 interacts with TFIID

A recent publication reports a physical interaction of Hmo1 with TFIID (Kasahara *et al.*, 2008). The authors observed interactions between Hmo1 and Taf1 and TBP, two out of the 15 subunits forming TFIID. The authors discuss possible implications, like a function in transcriptional start site selection, of this discovery for Pol II transcribed genes. They do not discuss the fact that TBP also belongs to the Pol I promoter complex bridging the UAF and the CF. Taking this into account opens another interesting interpretation of this finding. We detect Hmo1 at the transcription start site, which also includes the core element of the promoter. Zomerdijk and coworkers proposed a role for UBF in promoter clearance (Panov *et al.*, 2006a). If Hmo1 localizes at the proximal part of the Pol I promoter where it still interacts with TBP, it could potentially fulfill this stimulating role of UBF in Pol I promoter escape in yeast. If this should be a function of Hmo1, it cannot be the only one as our preliminary transcription assays showed that Hmo1 stimulates transcription also under promoter independent conditions (see Figure 29). As for Hmo1 in yeast, UBF associates not only with the promoter, but also with the rest of the rDNA (O'Sullivan *et al.*, 2002). Accordingly, a function of UBF in regulating Pol I transcriptional elongation has been described (Stefanovsky *et al.*, 2006a).

Hmo1 DNA binding properties

We reported a dramatic loss of Hmo1 binding to its target sequences like the promoters of *RPS5*, *RPL16B*, or to the Pol I transcribed region, upon rapamycin treatment. However, other laboratories fail to reproduce our result of Hmo1 dissociation following rapamycin treatment (unpublished). A control that had been done at the time to exclude a TAP-tag specific problem like a conformational change upon rapamycin treatment, making it less detectable for the IgG-beads used during the purification, was a TAP-tagged version of Fhl1. We could still detect Fhl1 at RPG promoters in at least equal amounts compared to untreated ones, exponentially growing yeast. However the contrary observations of several independent laboratories

is alarming, and it would be very much required to repeat this same experiment, using a different affinity-tag for purification. An observation that is potentially favoring such a persisting association of Hmo1 with the rDNA upon rapamycin treatment is our observation of fluorescently labeled Hmo1 in live cells. After rapamycin treatment, the volume occupied by Hmo1 shrinks as does the total volume of the nucleolus (and presumably the rDNA that becomes more compact). In the case of Pol I, where it has been shown that the polymerase dissociates from the rDNA in the presence of rapamycin, the fluorescent signal expands to a diffusive localization, covering the whole nucleoplasm (Tsang *et al.*, 2003). Repeating the purification with another affinity tag would help to clarify this discrepancy.

Hmo1 post-translational modifications

The rapid release of Hmo1 from its target sites, although not totally clarified, needs to be regulated by some kind of molecular trigger. This could be mediated by post-translational modifications such as phosphorylation. UBF has been reported to be phosphorylated by CK2 (Voit *et al.*, 1992) and the MAP-kinase ERK (Stefanovsky *et al.*, 2001b). While ERK mediated phosphorylation of UBF has been connected to elongation efficiency (Stefanovsky *et al.*, 2006a), phosphorylation of UBF by CK2 has been described to stabilize both UBF binding on the rDNA and the interaction between UBF and SL1 (Lin *et al.*, 2006; Panova *et al.*, 2006). CK2 has now also been brought into play in the yeast system as being part of the mentioned CURI complex, potentially linking Pol I and RPG transcription (Rudra *et al.*, 2007). Analysis of post-translational modifications and especially the phosphorylation status of Hmo1 hence seems to be very interesting.

Hmo1 recruitment onto ribosomal protein gene promoters

The dependence of Hmo1 binding to RPG on Rap1 is a mystery. As for the initial observation that Fhl1 recruitment requires Hmo1, the assay showing the requirement of Rap1 for Hmo1 binding is based on a reporter construct (Hall *et al.*, 2006).

The question is why Hmo1 is for example not recruited by Rap1 to telomeric regions (Hall *et al.*, 2006). This could possibly be explained by the theory that different Rap1 consensus sequences modulate the structure of Rap1 after binding, making it

recognizable to different proteins (Piña *et al.*, 2003). But on the other hand, only nine out of the 138 RPG promoters are not bound by Rap1 (Lieb *et al.*, 2001). However, depending on the threshold applied, about 70 to 90 RPG promoters are majorly targeted by Hmo1. So the obvious question is, why is Hmo1 not recruited to the other 40 to 60 Rap1 decorated RPG promoters too?

Conversely, again depending on the applied threshold, two out of the nine promoters RPG promoters lacking Rap1 are nevertheless bound by Hmo1 (Hall *et al.*, 2006). How does Hmo1 get recruited to these RPG promoters?

In the absence of additional characterized DNA binding proteins that are shared by RPG promoters and the rDNA (and some additional Pol II gene promoters), we speculate that it might be mainly a characteristic chromatin structure common to promoters of highly transcribed genes, Rap1 binding sites and active rDNA repeats, namely the local depletion of nucleosomes (Bernstein *et al.*, 2004; Dammann *et al.*, 1993; Merz *et al.*, 2008) that is recognized by Hmo1.

Hmo1 function at RPG promoters

An *fhl1*Δ-mutant shows a strong slow growth phenotype. Over-expression of the essential gene *IFH1* compensates for *FHL1* absence (Cherel and Thuriaux, 1995). In an *fhl1*-deletion mutant, *IFH1* is no longer essential for survival (Cherel and Thuriaux, 1995), which can be interpreted with the missing "landing platform" for Ifh1 as well as for the co-repressor Crf1. A recent publication demonstrated that Hmo1 is strictly required for Fhl1 recruitment to RPG promoters (Hall *et al.*, 2006).

However, our study showed that *IFH1* is still essential in an *hmo1*Δ background making interpretation of these results not easy. In the manuscript, we speculated about an additional recruiting mechanism for Fhl1 besides Hmo1 mediated recruitment.

Supporting this idea, Kasahara and co-workers have now demonstrated that Fhl1 binds to many more RPG promoters than Hmo1 does (Kasahara *et al.*, 2007). They could show that on promoters associated with Hmo1, recruitment of Fhl1 is Hmo1-dependent, while it is not so for promoters not bound by Hmo1 (Kasahara *et al.*, 2007). They accordingly classed RPG promoters into either Hmo1-dependent Fhl1 recruitment or Hmo1-independent Fhl1 recruitment RPGs. Trying to explain the SL interaction of *hmo1*Δ and *IFH1*-depletion, we speculated about additional pathways that recruit Fhl1 onto RPG promoters.

This line becomes now very clear: The additional pathways seem to concern especially Hmo1-independent promoters. There, Fhl1 is still recruited in comparable quantities and these almost 40 RPGs still require Ifh1 for there expression.

3. MAPPING GENE LOCALIZATION IN LIVE YEAST WITH SUB-DIFFRACTION RESOLUTION

3.1 Objective and Summary

During my PhD, I performed a detailed study on Hmo1, a *bona fide* Pol I transcription factor, that has now been demonstrated to also be implicated in Pol II mediated RPG transcription. The association of Hmo1 with both, the nucleolus and RPG promoters led us to ask whether RPG promoters are physically tethered to the nucleolus. A similar model has been proposed and demonstrated for tRNA genes (Thompson *et al.*, 2003).

An explanation for nucleolar tRNA gene localization is the pool of Pol III and pre-tRNA processing factors concentrated within the nucleolus. The 138 genes coding for ribosomal proteins which are dispersed over the yeast genome could cluster close to the nucleolus, as shown for the tRNA coding genes. Our idea was to test whether the sub-set of Pol II transcribed RPGs that associates with Hmo1 localizes close to the nucleolus for co-regulation mediated via the HMB-box protein.

We chose to look at live cells, as this technique avoids artifacts introduced by harsh denaturing DNA-FISH conditions and leaves the option for later dynamic analyses. Looking at cells with a fluorescently tagged genomic locus, it becomes apparent why solid statistic approaches are required to describe the localization of a gene: as previously described (Heun *et al.*, 2001) a locus undergoes Brownian motion like fluctuations. Although these motions are “small scale” with regard to the small yeast nucleus, these jiggling motions explore an important proportion of the nuclear volume. In most gene localization studies the localization of the nucleolus so far has been neglected (exceptions are the tRNA-gene class labeling, or protein localizations suggesting indirectly a clustering of telomeres). Analyses of 3D data rather used radial distance measurements, thus reducing the 3D information to 1D results. In these types of analyses, a sample size of usually about 100 cells is evaluated to reach certain robustness in terms of statistical significance. Since we wanted to extend the analysis of gene localization adding the information of the position of a locus with respect to the nucleolus, which results in an effective 2D result, we need 100^2 = 10,000 cells to

obtain an equivalent robustness. This requires new methodological approaches for data processing, analysis and result representation: The existing analysis modules required extensive manual selection and analysis of each cell, making the handling of several thousand cells unattainable. 2D localization information (with respect to the nuclear envelope and to the nucleolus) of thousands of loci also required a new way to represent these results.

The following manuscript describes the developed tools, their validation and usage in addressing biological questions. Briefly, an automatic cropping module allowed us to extract ~200 cells per 3D image stack. Afterwards, localization data is extracted from each nucleus, such as the nuclear mass center, the mass center of the nucleolus, the locus itself, the nuclear envelope and an estimated nucleolar volume (using segmentation). The mass centers of the nucleus and the nucleolus allowed us to align all nuclei along the axis defined by these points (the SPB is lying on the same axis, see manuscript), leaving one degree of freedom, the rotation around this axis. This rotation symmetry allows projection of all gene positions within one single plane. Analyzing the gene density within this plane taking into account the volume (which is exponentially bigger, the further we move away from the axis), results in percentile or probability representation. Analyzing known yeast nuclear architecture approved the validity of the method and revealed a resolution of the "map" below the diffraction limit. We found that genomic loci are confined to small "gene territories" in a population of cells, demonstrating that gene localization within the yeast nucleus is non-random. The degree of confinement varies largely between different loci. We analyzed the position of two *GAL* loci (*GAL1* and *GAL2*), both of which have previously been reported to become relocalized to the nuclear periphery upon activation (Cabal *et al.*, 2006; Dieppois *et al.*, 2006). Speculations on whether these two loci become co-localized for co-expression, cannot completely be ruled out, but seem highly unlikely since the genemaps indicate a recruitment to opposite sites of the nucleus. Pol II transcribed genes involved in ribosome biogenesis show a diverge localization pattern that correlates with their localization on the chromosome and, for the small number of two studied genes, also with Hmo1 interaction profiles. Biophysical models and the biological relevance and outlook of the method are discussed.

3.2 Manuscript

December 2008

HIGH-RESOLUTION STATISTICAL MAPPING REVEALS GENE TERRITORIES IN LIVE YEAST

Axel B. Berger, Ghislain G. Cabal, Emmanuelle Fabre, Tarn Duong, Henri Buc, Ulf Nehrbass, J-C. Olivo-Marin, Olivier Gadal & Christophe Zimmer

NATURE METHODS
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Link

(http://www.nature.com/nmeth/journal/v5/n12/abs/nmeth.1266.html)

3.3 Extended Discussion

Genemaps and cell cycle.

Dynamic analysis of four yeast loci in G1 and S phase cells indicates that gene position can be different for some loci depending on the cell cycle stage (Heun *et al.*, 2001). This phenomenon seems plausible since, as for transcription, replication does not happen diffuse within the entire nuclear volume but concentrated in specialized replication foci (Gilbert, 2001). This needs to be taken into account when looking at the presented genemaps. This factor seems especially dangerous in cases where the described gene territory is either split (like in the case for *GAL1* under activating conditions, manuscript figure 3b) or very large (like for *RPS5*, manuscript figure 3k). In these cases, a different localization in S-phase cells can explain a second localization maximum or a more diffuse total gene localization volume while the volumes occupied in G1 and S phase are distinct and notably smaller. To eliminate this possibility for *GAL1* localization, we manually selected G1 and S phase cells and did not observe a difference in localization preference with respect to the nuclear periphery (data not shown). For *RPS5* we have not undertaken such efforts yet, leaving the possibility of smaller, cell cycle specific *RPS5* gene territories.

A distinction of cell cycle stages is for sure a very interesting aspect that should be followed further. Parallel maps of the same locus in G1, S, and G2 phase cells would be ideal, however sorting of cells into these categories is not trivial.

One possibility to avoid imprecise, time-consuming manual selection of cells according to criteria like their morphology (bud emergence and size) or SPB duplication is to synchronize cells. Cells can for example be treated with alpha-factor or nocodazole leading to a cell cycle arrests in G1 and G2, respectively (Breeden, 1997; Guthrie and Wickner, 1988). We have tried to synchronize cells with alpha-factor, the treatment however led to a dramatically deformed nucleus that persisted for over 90 minutes after having changed the medium. Longer releases from the cell cycle block lead to an important fraction of de-synchronous cells (Breeden, 1997), making this in our hands an impropriate method for cell cycle dependent gene localization analysis. Other methods pose equally important doubts about their legitimacy: an example is nocodazole that depolymerizes microtubules leading to a late G2 arrest (Guthrie and Wickner, 1988). It is clear that chromosome arrangement in yeast is

determined to a large extent by the connection of all yeast centromeres with the SPB throughout the cell cycle. This makes it an obviously bad method of choice for synchronizing cells for gene localization analyses. Experiments using this drug also revealed a presumably secondary effect on nuclear morphology since the nucleolar volume decreases is size (P. Thérizols, E. Fabre, unpublished observation). The effect can be assumed for all cell cycle blocking agents: the cells no longer divide, leading to reduced ribosome production, leading to a reduced nucleolar volume. Although this latter effect might be corrected rapidly after drug removal (these experiments have not been performed yet), there are better possibilities emerging, namely fluorescent dynamic cell cycle markers (Sakaue-Sawano *et al.*, 2008). In this study, rapidly produced and degraded cell cycle specific proteins have been genetically fused to fluorescent proteins. The technique allows to follow cells and to tell from their overall color which cell cycle stage they are in. Cells could be automatically cropped and sorted corresponding to their expressed colored protein, allowing untreated, exponentially growing cells to be classed according to their cell cycle stage.

Ribosomal protein gene localization with respect to the nucleolus.

The two ribosomal protein genes chosen for our localization study have not been chosen randomly, but are the same two RPG that had been chosen as examples of RPG promoters that are bound (*RPS5*) or not bound by Hmo1 (*RPS20*) in genetic and biochemical analyses summarized in the first manuscript. Consistent with the ChIP data, *RPS20* is not in contact with the pool of Hmo1 situated in the nucleolus or its interface to the nucleoplasm. *RPS5* however, although diffuse, can contact the nucleolus much more frequently. To test if the localization was really dependent on the gene, the localizations of the *RPS20* and *RPS5* loci have also been tested while the gene itself had been deleted and a plasmid borne copy of the essential gene was provided (see Figure 30G). The already big gene territory of *RPS5* becomes even larger if the gene is deleted. Additionally the preferential localization versus the nucleolus is now lost, the gene seems to be distributed homogenously throughout the nucleoplasm while being excluded of the nucleolus. *RPS5* localization in an *hmo1*Δ background has been tested too, leading to a dramatic relocalization of the locus in our maps. However, the original localization phenotype could not be rescued

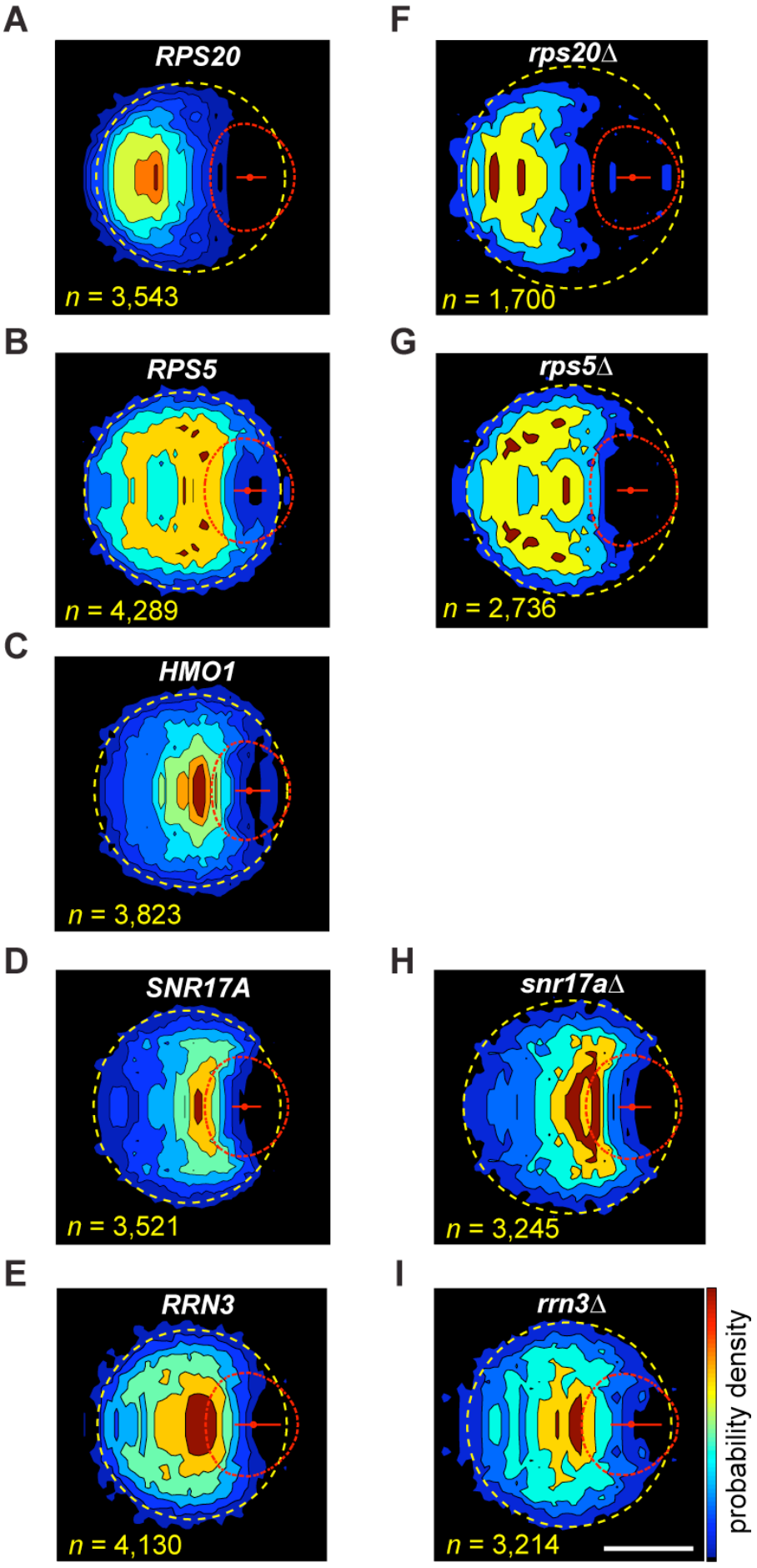
A
RPS20
n = 3,543
B
RPS5
n = 4,289
C
HMO1
n = 3,823
D
SNR17A
n = 3,521
E
RRN3
n = 4,130
F
rps20Δ
n = 1,700
G
rps5Δ
n = 2,736
H
snr17aΔ
n = 3,245
I
rrn3Δ
n = 3,214
probability density

Figure 30. Localization of genes involved in ribosome biogenesis.

A. to E. Probability maps of the localization of *RPS20* (**A**), *RPS5* (**B**), *HMO1* (**C**), *SNR17A* (coding for U3 snoRNA, **D**), *RRN3* (**E**).

F. to I. Probability maps of the same loci, but the corresponding transcribed and flanking (usually +/- 500 bp) sequences were deleted. *rps20* (**F**), *rps5* (**G**), *snr17A* (**H**), *rrn3* (**I**).

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providing back *HMO1* on centromeric plasmid under its native promoter (see also discussion below). The result is therefore only preliminary and needs further experiments to be validated.

To test if Hmo1 had "pulling forces" on the RPS5 locus, we labeled a centromeric plasmid that contained or contained not the RPS5 gene (ORF +/- 500 kb flanking sequences) with tetO repeats. The obtained genemaps for the two plasmids are almost identical (see Figure 31). A plasmid recruiting phenotype towards the nucleolus had been described by Engelke's group: when they inserted a tRNA coding gene (*SUP53*) onto a centromeric plasmid the plasmid became localized to the nucleolus (Thompson *et al.*, 2003). We tested their plasmids (containing either *SUP53* or an mutated, inactive *SUP53* variant) using the tetO/TetR-GFP DNA labeling method and not FISH. Using this approach, we cannot reproduce a nucleolar localization of the *SUP53* containing plasmid (data not shown). We cannot exclude that the tetO-insertions disturb the system, but in living cells, we could not reproduce these results.

In fact all the so far observed centromeric plasmids no matter what gene composition they are containing are all localized in a very similar volume, juxtaposed to the SPB. This raises the question if the strong forces exerted on the plasmid by the connection of its centromere with the SPB can be overcome by potential gene recruiting mechanisms. Genetic screens, based on plasmid based gene localization observations, as recently published (Vodala *et al.*, 2008), should therefore be judged with caution, since the observed localization will most likely be based on a competition between the pulling forces of the SPB and any other potential structure.

An interesting challenge is to estimate the major forces that are actually responsible for endogenous *in vivo* gene positioning. The centromere is an important determinant. It can be assumed that several kb flanking the yeast centromere can hardly be

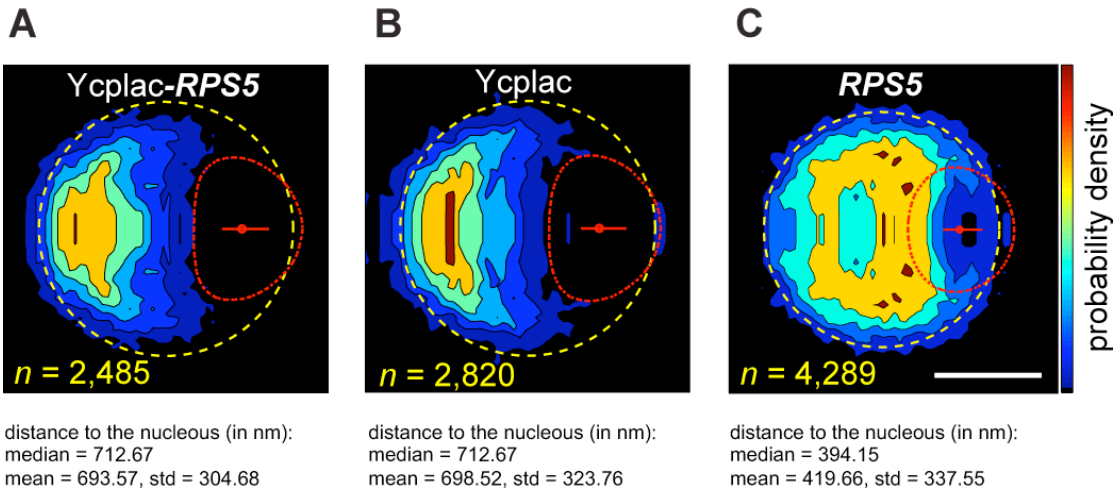

Figure 31. Localization of a plasmid borne *RPS5*.

A. Strain Nucloc2 containing plasmid Ycplac33-RPS5 (Ferreira-Cerca *et al.*, 2005) has been transformed with *Eco*RI linearized plasmid pTetO-NAT-ura3Δ, yielding plasmid Ycplac33-RPS5-TetO-NAT. Cells were grown in YPAD containing clonNAT, and were diluted in YPAD for 3 hours prior to microscopy. The localization probability map of the extracted gene positions is shown.

B. As in **A.**), but Nucloc2 contained the empty vector Ycplac33 that has been modified via homologous recombination of linearized plasmid pTetO-NAT-ura3Δ *in vivo*.

C. For comparison the localization probability map of the endogenous *RPS5* locus has been added. Scale bar is 1 µm.

localized adjacent to the nucleolus for example. The rDNA on the right arm of chromosome XII seems to be another such determinant. *GAL2*, although situated roughly in the middle between the rDNA and *CEN12* on this chromosome (about 150 kb to each side), localizes very close to the nucleolus, indicating that the chromatin structure between *GAL2* and the rDNA seems to be considerably different compared to the chromatin between *GAL2* and *CEN12*. In this line, one hypothesis was that the linear localization of RPG on the chromatin fiber could be important for its characteristic of being bound by Hmo1 or not. Hmo1, as shown in Figure 19, is a protein solely detected on or around the rDNA by fluorescence microscopy. If the protein is predominantly sequestered within the nucleolus, this could imply that RPG close to the SPB could not localize towards this pool. There are five RPG that are closer than 40 kb from their corresponding centromere in yeast: *RPL14B* (1 kb), *RPL14A* (10 kb), *RPL4B* (20 kb), *RPL27A* (20 kb) and *RPS20* (30 kb). Consistent with the predicted influence of centromere-close localization, as for a centromeric plasmid, *RPS20* indeed localizes in close proximity to the SPB, reminiscent with the territory occupied by a centromere (compare manuscript figures 2c and Figure 30). Analyzing the ChIP-on-chip data of genome wide Hmo1 binding, it is interesting to note that all of these five RPGs have been assessed as not being associated with Hmo1 (Hall *et al.*, 2006). In the same study, from the remaining 130 analyzed RPG, 76 (58 %) were thresholded as not being associated with Hmo1. For distances larger than 40 kb away from the centromere, there is no linear relationship between Hmo1 association and genomic position.

The statistical significant of these thoughts is not established (P = 0.078), but they form the basis for a hypothesis that should be followed up further.

Localization of other Pol II transcribed genes involved in ribosome biogenesis.

Hmo1 is involved in several steps of ribosome biogenesis: the transcription of the precursor containing the large rRNAs (Gadal *et al.*, 2002), potentially the co-transcriptional processing of the rRNAs (Hall *et al.*, 2006) and transcription of RPGs (Berger *et al.*, 2007). The promoter of the gene *HMO1* has been additionally shown to be bound by Hmo1 itself, suggesting a role in auto-regulation (Hall *et al.*, 2006). We therefore tested *HMO1* for its localization pattern within the yeast nucleus. *HMO1*

resides with high probability centrally, just in front of the nucleolus (see manuscript figure 3i). As for other loci, we wanted to measure the impact of the gene on the localization of the chromatin segment it resides on. But, as already observed for *RPS5* tested in an *hmo1*Δ background, we tend not to believe the drastic relocalization phenotype also observed in this case unreserved. The problem is that it seems as if the lack of Hmo1 in the cells drastically decreases the signal intensity of the locus labeling. This could be either due to a loss of the originally 112 tetO copies, or the tetO repeats are less accessible due to the absence of the HMG-box protein, which leads to decreased TetR-GFP targeting and a loss of fluorescent intensity. The way the algorithm is designed, to determine the locus, a "spottiness score" is calculated first: the curvature of the intensity distribution is multiplied with the local intensity of pixels. The pixel with the highest score is used to initially determine the rough gene position (see manuscript supplementary figure A). If the intensity of the locus labeling drops, the "spottiness score" difference between the locus and the NPC clusters decreases, making false positive detection of a bight NPC cluster instead of the gene much more likely, which ultimately results in a false, much more peripheral mapping of the locus. The questionable, preliminary results of gene localization in an *hmo1*Δ background are therefore not shown nor further discussed.

SNR17A codes for snoRNA U3, involved in the early maturation steps of the 35S rRNA precursor. The candidate was chosen since the gene has been demonstrated to localize to Cajal bodies in human cells (Gao *et al.*, 1997). As mentioned in the introduction, the function of metazoan Cajal bodies, such as snoRNA maturation, seems in yeast to be executed within the nucleolus. The peri-nucleolar localization of *SNR17A* (see manuscript figure 3j and Figure 30A) therefore fits the observation in human cells, indicating a potential evolutionary conservation.

Similarly, we tested *RRN3* localization in yeast. As can be seen in Figure 30B, *RRN3* localization probability peaks just in front, or even at the interface of the nucleolus. The yeast model system now offers the possibility to analyze localization determinants due to its easy accessibility for genetic modifications.

Chromatin localization in dependence of one single gene. Implications.

To assess the influence of the single locus on this positioning, we again deleted *SNR17A* and *RRN3*. The general localization distribution does not change importantly for any of the two if the genes are removed from the genome (essential *RRN3* was provided borne on a plasmid) (see Figure 30F and –G).

This demonstrates, in several examples (*GAL2*, *SNR17A*, *RRN3*, *RPS20*), the marginal influence of a gene to determine the local positioning of the chromatin fiber. It is hard to imagine that each of the about 6,000 genes densely packed into the yeast genome has a distinct localization preference that it needs to reach for optimal transcriptional regulation: while *GAL1* needs peri-nuclear localization for optimal expression, a adjacent gene coding for tRNA (*tT(AGU)B*) needs to be localized to the nucleolus, while again another gene requires potentially central localization at the same time to regulate its transcription status.

If there is an optimal site for a gene to regulate its transcriptional status, for repression and / or activation, a linear functional arrangement of genes within the compact yeast genome would facilitate this optimal localization. Recombination events are very frequent in yeast and genes could have functionally arranged into co-expressed domains. Such an organization has originally been postulated by George Church's laboratory after having correlated genome wide expression analyses with genomic gene positions (Cohen *et al.*, 2000). A recent study confirm this initial analysis (Janga *et al.*, 2008). In our few examples, *GAL2* resides, as previously discussed, on the same chromosome arm as the rDNA. Although situated linearly at a large distance from the rDNA, the *GAL2* domain in front of the nucleolus (see manuscript figures 3e to 3h). *SNR17A* coding for U3 snoRNA is only separated by less than 2 kb from *RPL33B*. Deletion of *SNR17A* does not significantly change the locus' position with respect to the nucleolus (Figure 30A and –F). And last, *RRN3* is localized between two tRNA coding genes, *tK(CUU)K* at 5 kb and *tA(AGC)K1* at 11 kb respectively; again, deletion of the *RRN3* and its cis-regulatory elements does not affect the localization of this chromatin stretch (Figure 30B and –G).

Another possibility is that genes only need brief contacts with a sub-nuclear volume enriched in metabolism-specific factors, like for transcription initiation events. Occasional "kissing" events are not captured using our static mapping methodology.

The observed random movement with only rare long range (apparently directed) movements (Heun *et al.*, 2001) allows a local chromatin stretch to explore a big volume of the nucleus. In agreement with such an interpretation is the observation that gene transcription from bacteria to humans occurs in stochastic transcriptional bursts (Raj *et al.*, 2006; Raser and O'Shea, 2005). In yeast, even if all clonal cells within a population are shifted from *GAL* gene repressing glucose containing medium to activating galactose medium, *GAL* gene transcription happens in each individual cell randomly in short transcriptional bursts or pulses (Blake *et al.*, 2003). For the specific case of the *GAL1* locus, a transient short activation at the periphery can be excluded as single particle tracking revealed that the locus stays confined at the nuclear periphery when actively expressed (Cabal *et al.*, 2006). This confinement last for several minutes which is also the reason that we can detect this relocalization in our genemaps.

Potential "recruiting" factors.

The *GAL1* locus has been demonstrated to be virtually anchored by the histone acetyltransferase complex SAGA to the NPC (Cabal *et al.*, 2006). While this on first sight suggests that the gene gets recruited there first to then be expressed, the peri-nuclear localizations also get lost in the *mex67*Δ mRNA export mutant (Dieppois *et al.*, 2006), and mutants of co-transcriptionally recruited exosome component (Vodala *et al.*, 2008) The latter phenotypes rather suggest a "reverse recruiting" mechanism where the gene is anchored via its nascent mRNA that gets co-transcriptionally processed and exported. Such a mechanism can also be imagined for U3 snoRNA coding gene *SNR17A*, if the nascent pre-snoRNA is co-transcriptionally processed within the yeast nucleolus. Or, with *RPL33B* being expressed at not even 2 kb from *SNR17A*, a combination of the two positioning elements: while *RPL33B* becomes activated at the local Hmo1 pool, *SNR17A* is pulled via its nascent transcript towards the nucleolus.

It is likely that the positioning determinants apart from the biophysical guidelines of the chromatin fiber are specific to individual genes or DNA segments. Therefore, each localization phenomenon would need to be analyzed separately, at best isolated. Such possibilities are discussed a bit more in detail in the "perspectives" section.

CONCLUSION AND PERSPECTIVES

My thesis is divided in three parts. In the first one, I characterized the budding yeast protein Hmo1 using especially biochemical approaches. My preliminary results from *in vitro* transcription assays suggest that Hmo1 plays a role in RNA polymerase I initiation and elongation, however these results have to be confirmed. The stimulatory effect of recombinant Hmo1 in promoter independent assays which could be observed for homogenous Pol I, but not for Pol II indicate that Pol I is a specific target for Hmo1 action . My results furthermore indicate that the recombinant HMG-box protein is not capable to support transcription in vitro of a DNA template associated with nucleosomes. Furthermore, using ChIP assays I could show that Hmo1 interacts physically with the region transcribed by Pol I. Hmo1 has been proposed to be the homolog of the metazoan HMG-box protein UBF that also associates with the rDNA (Gadal *et al.*, 2002). At the rDNA, UBF has been described to be involved in pre-initiation complex assembly (Moss *et al.*, 2007), promoter clearance (Panov *et al.*, 2006a) and control of Pol I elongation (Stefanovsky *et al.*, 2006a). It is hence possible that Hmo1 also acts at multiple levels of Pol I transcription.

In the second part of my thesis, I characterized the function of Hmo1 using genetic, molecular and cellular biology approaches. To test the potential functional conservation between Hmo1 and UBF, I expressed human UBF in yeast. UBF is well described in metazoans. Hence a functional similarity would allow to draw conclusions on the function of Hmo1. Although very poor in sequence conservation, fluorescently tagged hUBF localizes to the yeast nucleolus. Genetic complementation experiments demonstrated that UBF cannot fully compensate the slow growth phenotype of an *hmo1*Δ strain. However, in lethal *hmo1*Δ - Pol I double mutants hUBF1 was able to restore growth. Our results demonstrate the partial functional conservation between the two proteins in relation to Pol I. A global genetic screen pointed to three different gene classes interacting with *HMO1*: a) genes involved in Pol I transcription (already described before (Gadal *et al.*, 2002)), b) genes involved in Pol II transcription and genes coding for ribosomal proteins and c) genes associated with stress response pathways. I could confirm that *HMO1* becomes essential when TOR complex 1 function is comprised as well as when the essential RPG activator Ifh1 is depleted. I then performed global expression analyses with a strain deleted or depleted of Hmo1. Microarray experiments demonstrated a very broad response to *hmo1* deletion, resulting in a significant transcriptional up- or down-regulation of

about 1/10th of all genes. Depletion of Hmo1 for about one cell cycle affected much lesser number of genes and showed a mild, but significant down-regulation of RPGs. Expression analysis of *hmo1*Δ cells after TORC1 inhibition by rapamycin revealed that yeast show severe defects in RPG down-regulation under these conditions. The effect was similar to a described, constitutively active Pol I mutant that was equally unable to down-regulate RPG expression after rapamycin treatment suggesting a Pol I – Pol II cross-talk (Laferte *et al.*, 2006). Deletion of *HMO1* in this mutant strain background largely alleviated the observed effect, indicating that Hmo1 plays an important role in mediating this polymerase cross-talk. The results also demonstrated the bi-modal behavior of RPG in the absence of Hmo1: while most RPG showed the described de-regulation, others appeared to be unaffected. We next wanted to analyze if the gene position of Pol II transcribed genes with respect to the *bona fide* nucleolar protein Hmo1 contributes to expression regulation, a mechanism previously demonstrated for Pol III transcribed tRNA coding genes (Thompson *et al.*, 2003).

This question led to the third part of my PhD work, in which I participated in establishing a novel statistical imaging approach adapted to the morphology of the yeast nucleus considering e.g. the nucleolar volume. This approach was necessary due to the small size of yeast nuclei and the stochastic motion of genomic loci (Heun *et al.*, 2001). The 3D localization of a locus is therefore never absolute but rather needs to be described in terms of statistical localization probabilities. Our imaging approach makes use of the nucleolus by aligning the nuclear and nucleolar mass centers. This allows to orient nuclei and to superpose thousands of gene positions, resulting in the probability distribution of a locus in a population of cells. The generated final "gene map" revealed a high degree of spatial confinement of a locus in live cells. The size of this "gene territory" varies largely depending of the tagged locus. Gene territories can also be remodeled if the tagged gene is transcriptionally activated. I then started to analyze Pol II transcribed genes required for ribosome biogenesis. Looking at the same genes as in the earlier detailed expression analysis, I could observe that *RPS5* (which interacts with Hmo1) localizes largely in front of the nucleolus, while *RPS20* (which does not interact with Hmo1) occupies a more central territory close to the spindle pole body. Deletion of *RPS5* from the chromatin fiber led to a redistribution of the locus throughout the nuclear volume. However, placing *RPS5* on a centromeric plasmid did not change its localization pattern away from the SPB, arguing that in this

case forces exerted through the microtubule are stronger than potential attracting forces in the nucleolus. Unfortunately, preliminary results of loci observed in an *hmo1*Δ background are difficult to interpret since the signal intensity of the locus in this mutant background is drastically decreased leading to detection problems. It furthermore seems as if other loci, like the gene coding for U3 snoRNA or *RRN3* also localize non randomly and in close proximity to the nucleolus. Further investigations are needed to elucidate the functional significance of this localization.

During my thesis I characterized the protein Hmo1 using a variety of approaches. Additional experiments are needed to further merge the different aspects of the results. However, the presented results and the preliminary data allows us to speculate that gene positioning has an impact on RPG – Hmo1 interactions and / or *vice versa*. This interaction is indicative of the participation of Hmo1 in RPG – Pol I cross-regulation by acting on RPG promoters and Pol I initiation and elongation events. This model also illustrates the redundancy of the important regulation of RPG and Pol I expression. Multiple pathways participate in this regulation. Manipulation of one of them can be balanced by the others. This idea is also supported by the transcriptome analyses: genes coding for regulatory factors (such as Rrn3 for Pol I) are significantly up-regulated in the absence of Hmo1. Moreover, about half of the RPGs is up-regulated in an *hmo1*Δ background presumably since over-expression of redundant factors lead to increases in gene expression.

However these are only speculations, more experiments are needed to determine the exact role of Hmo1 in the Pol I transcription cycle and to explain and generalize the observed gene positioning phenotypes.
More specifically, future *in vitro* experiments should make use of templates with which it is possible to distinguish between initiation and elongation. This could be achieved using a yeast rDNA promoter containing immobilized template. The promoter follows a short stretch lacking deoxycytidine that allows arrest of the ternary complex in the absence of CTP in the reaction buffer. After washing the complex, the addition of reaction buffer containing all four NTPs and the protein(s) of interest then chases the transcript (Tschochner and Milkereit, 1997). The produced transcripts originate from one single initiation event per polymerase, allowing to separate

initiation from elongation and processivity. An alternative approach is to make use of the G-less cassette plasmid. Similarly, this template makes use of a stretch of deoxyguanosine free DNA following the transcription start site to block elongating polymerase after the initial 20 nucleotides. A possibility is to mark this short transcript by incorporation of a different nucleoside labeled with an alternative phosphate isotope, such as ^{33}P-ATP. The ternary complex blocked at position +20 can then be chased by addition of GTP (containing also ^{32}P-labeled GTP) and an access of unlabeled ATP. Analyzing the ^{32}P to ^{33}P ratio allows to determine the elongation rate per initiation event.

Mass-spectrometry of Hmo1 purified from untreated or rapamycin treated cells could be used to study potential post-translational modifications of Hmo1, which could be responsible for the changed DNA binding properties. These two Hmo1 fractions could be also tested in transcription assays. Pre-incubation of the reaction buffer with kinases, phosphatases, acetylases, deacetylases etc. could allow to test for common protein modifications on a candidate approach.

Similar, pre-incubation reaction buffers could be used to test for a potential. Furthermore the influence of chromatin remodeling complexes that are largely available in the laboratory of Herbert Tschochner could be tested in context with Hmo1-activity on nucleosomal templates.

To further analyze the distribution of genes within nuclear space and the factors that actually determine this localization requires a variety of approaches. First of all, more RPGs need to be tested for their *in vivo* localization pattern to test the hypothesis RPGs that are associated with Hmo1 are close to the nucleolus, while RPGs not bound by Hmo1 are not. This would then result in a more detailed description of the *in vivo* localization.

Multiple, complementary experiments can be envisioned to approach the determinants of gene positioning. As in the presented examples, neighboring genes seem to have an important influence on the localization of a given locus. Furthermore, computational analysis revealed that co-regulated genes seem to preferentially cluster in the yeast genome (Cohen *et al.*, 2000; Janga *et al.*, 2008). It can therefore be imagined that this close linear co-localization results also in a 3D clustering. Removing genes adjacent to the locus of interest could help to show, how far and how strong these nearby

positioning influences are. Conversely, the gene of interest with appropriate flanking sequence can be moved to other parts of the genome. Different target integration sites should be chosen to insert the gene, such as a gene-poor chromosome segment localized centrally on a chromosome arm, or a position close to sub-telomeric regions or one close to the centromere. Parallel expression analyses would additionally allow to test for functional consequences of this translocation. Furthermore, the locus of interest could be flanked with lox-sites, allowing excision of the segment. The episome could then be followed and its preferential localization studied. A recent study followed a similar idea, though the authors only excised the centromere of a centromeric plasmid. They observed a subsequent relocalization and association of the remaining episomes with NPCs (Shcheprova *et al.*, 2008). However, the plasmid was still not cleared of genes, carrying at least the gene coding for the selection marker. Cre recombinase could be induced by tetracycline regulatable promoter. This system is already available in the laboratory. Likewise the fluorescent labeling of genes with lacO/LacI-GFP is established, a necessity for tetracycline-induction in the same cell. One such experiment would be to study the position of the *GAL1* locus. The gene cluster shows the most obvious relocalization phenomenon (see manuscript 2, figure 3b), that would be interesting to follow with the locus excised from its chromosomal context. This study could for example reveal if it is really the activated, peripheral localization of the locus that needs to be maintained. On the contrary, it is possible that the central localization away from the nuclear periphery under repressive conditions needs to be actively achieved.

Another potential of our *in vivo* gene labeling method is to study the interesting aspect of the dynamic behavior of a locus. Staying with the example of *GAL1*, we could address the question if the observed bimodal distribution pattern is due to two discrete cell populations, one close to the periphery and one rather central, or if there is a rapid relocalization of genes between the two locations. A module for such dynamic analyses has already been integrated in the software, however the representation of hundreds of trajectories still needs further improvements.

Treatment of cells that results in global effects is a general problem for the study of potentially relevant localization determinants. Rapid gene relocalization has been described to be energy dependent as shown by ATP depletion (Heun *et al.*, 2001). However, depleting the cell of ATP has enormous effects that make such observations

difficult to interpret. Indeed, global chromatin restructuring has been observed after ATP depletion that could explain many previously observed changes in nuclear particle mobility (Shav-Tal *et al.*, 2004). Similarly, one needs to be very cautious for future analysis, when addressing the implications of microtubule mediated anchoring of the centromeres, treating cells with microtubule depolymerizing drugs (discussed in 3.3). The idea of treating cells with rapamycin to study the dependence of gene localization on transcription is equally problematic. Transcriptional arrest leads to blockage or strong decrease of ribosome biogenesis resulting in a fast reduction of the nucleolar volume. Accordingly, one can observe an increased distance of the gene of interest from the nucleolus. The main difficulty in such structure/function analyses is that the different parameters are all linked. Our methodology is the only one that allows to measure gene position together with the size of the nucleus and the size and position of the nucleolus. We could split the population by sorting cells according to their morphological alteration, and uncouple this change from other nuclear characteristics.

An advancement of the methodology would be to be able to detect the RNA produced from a tagged gene additionally to all other extracted parameters. A detection module for gene-to-gene distance measurements using two different fluorophores is already integrated in the software platform (data not shown), but would need to be adapted for RNA localization signals. RNA could be labeled *in vivo* by using e.g. MS2-binding sites targeted by a fluorescently labeled MS2 coat protein (Bertrand *et al.*, 1998a). This will allow correlating each of the nuclear parameters with RNA production. From such analyses we could statistically determine which criterions determine RNA production. Thus drug treatment analyses would be even more informative.

In conclusion, the aim of my work was to correlate biochemical and genetic analyses of an HMG-box protein with gene positioning in nuclear space. Though I could not reach a final conclusion, I observed an interesting connection between gene positioning and gene association with this HMG-box protein. Furthermore, all the tools to follow-up on these first observations are now available. Bridging nuclear architecture with gene expression clearly is an exciting topic for future studies and HMG-box proteins remain promising candidates for a functional interconnection.

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