A study of chromatin mobility by live fluorescence microscopy in saccharomyces cerevisiae

NEUMANN, Frank

Abstract

In eukaryotes, the nuclear envelope separates chromosomes from the cytoplasm. Within the nucleus, chromosomes are non-randomly distributed and different chromosomal features localize to specific subnuclear compartments. This phenomenon is generally referred to as nuclear organization. Nuclear organization however is not static, but very dynamic. Here we discuss the tools and methods we developed to study the movements of chromatin in live yeast cells with high precision. We found that find that the context of the chromosomal fiber itself and chromosomal features such as telomeres and centromeres both constrain chromatin movement, whereas the chromatin state has no effect. Targeting of proteins to specific chromosomal loci was used to show that chromatin movements can be modulated. In particular targeting of the strong transcriptional activator VP16 or the catalytic subunit of a chromatin remodeling complex are sufficient to increase locus movements, suggesting that increased movement may be part of how these proteins influence nuclear functions like transcription or recombination.

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A STUDY OF CHROMATIN MOBILITY BY LIVE FLUORESCENCE MICROSCOPY IN SACCHAROMYCES CEREVISIAE

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par

Frank R. NEUMANN

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professeur (Deutsches Krebsforschungszentrum – Biophysics of Macromolecules Im
Neuenheimer Feld 280 – Heidelberg, Allemagne) et M. GARTENBERG, professeur (University
of Medicine and Dentistry of New Jersey – Department of Pharmacology – Piscataway,
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Nombre d'exemplaires à livrer par colis séparé à la Faculté : - 7 -
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## List of Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>ARS</td>
<td>autonomously replicating sequence</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CT</td>
<td>chromosome territory</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal DNA</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescent recovery after photobleaching</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyl transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HP1</td>
<td>heterochromatin protein 1</td>
</tr>
<tr>
<td>MSD</td>
<td>mean square displacement; (&lt;d^2&gt;)</td>
</tr>
<tr>
<td>NE</td>
<td>nuclear envelope</td>
</tr>
<tr>
<td>Pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>PSF</td>
<td>point spread function</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SIR</td>
<td>silent information regulator</td>
</tr>
<tr>
<td>SPB</td>
<td>spindle pole body</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TPE</td>
<td>telomeric position effect</td>
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<tr>
<td>wt</td>
<td>wild type</td>
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Chapitre 1: Introduction générale

L'information génétique des cellules eucaryote est distribuée sur des chromosomes linéaires qui sont groupés dans le noyau. Le noyau eucaryote est entouré par une double membrane caractéristique dans laquelle les complexes de pores nucléaires échangent des macromolécules entre le noyau et le cytoplasme activement et passivement. Un chromosome ne consiste pas seulement en une longue double hélice d’ADN très longue (qui chez l'homme mesure ~2 m, et chez la levure ~1.5 centimètre), mais a une structure évolution intrinsèque lui permettant d'être emballé dans le noyau d'une cellule (metazoas ~10 μm, levure ~2 μm en diamètre). L'ADN est enroulé autour des nucléosomes qui forment l'unité de base de la chromatine en formant la structure primaire de la chromatine qui ressemble à des "perles sur une corde". La fibre nucléosomique est condensée dans les structures secondaires et tertiaires peu caractérisées, pourtant l'ADN reste accessible pour beaucoup de facteurs dans ce contexte structural (Muchardt and Yaniv, 1999). En outre, des chromosomes et les domaines chromatiques sont placés dans le noyau d'une façon non-aléatoire. Je décrirai d'abord comment des nucléosomes et la fibre de chromatine sont modifiés pour accomplir leurs diverses fonctions nucléaires. Ensuite, je discuterai comment la chromatine s’organise dans le noyau d’une façon dynamique.

Un nucléosome se compose de 147 paires de bases (pb) d’ADN qui entourent dans 1,65 tours en superhélice un octamère qui se compose des quatre histones: H2A, H2B, H3 et H4. L’introduction des variantes d'histones (par exemple H2A.Z, H2A.X, macro H2A ou CENPA) qui s’intègrent dans des nucléosomes et la modification covalente
des queues d'histone offrent des possibilités illimitées à réguler et marquer la chromatine structurellement ou fonctionnellement (Luger, 2003). L’octamère d'histones qui forment le nucléosome se compose de deux dimères de H2A-A2B et de deux dimères stables qui forment le tétramère (H3-H4)2. Pendant longtemps, des nucléosomes ont été perçus en tant qu'objets immeubles sur l’ADN qui doivent être surmontés par les machineries de transcription ou de réplication. Cette vue a été changée drastiquement par la découverte des « facteurs de remodelage » qui possèdent la capacité de transférer l'octamère d’histones en cis le long de l'ADN en utilisant de l’ATP. Ces « facteurs de remodelage » sont des grands complexes de plusieurs sous-unités, conservés de la levure à l'homme (pour une revue voir Becker and Horz, 2002; Eberharter and Becker, 2004; Martens and Winston, 2003).

En générale, ces complexes jouent des rôles importants dans presque toutes les fonctions de la chromatine: la transcription, la réplication, la réparation des cassures d’ADN, la recombinaison, ou la cohésion des chromatides sœur. Puisque tous ces processus se déroulent en contexte de la chromatine, un système sophistiqué de contrôle au niveau de la chromatine s’est mis en place. Il n’est pas seulement conduit par « facteurs de remodelage » de la chromatine, mais également par beaucoup d'autres enzymes qui modifient des histones d’une façon covalente.

Comme le nucleosome simple, la formation d'une supra-structure secondaire ou tertiaire de la fibre chromatinienne se fonde également sur la qualité intrinsèque de la fibre et sur des interactions avec des protéines. Bien que les domaines de la "queue" N-terminale de l'histone (NTD) soient généralement considérés en tant que modules qui lient l’ADN, leurs rôles essentiels dans la compaction de la chromatine impliquent des interactions entre histones ou le recrutement des protéines inter-nucléosomiques responsables pour la formation des structures de chromatine compactée.
Une interaction entre histones bien décrite est celle de la NTD de H4 et avec H2A, qui est la base pour former la structure secondaire de la fibre de 30 nm (Dorigo et al., 2003; Dorigo et al., 2004). Les NTDs sont également impliqués dans la formation des structure tertiaires. L’interaction entre histones et le recrutement des facteurs diverses peut être réglés par des nombreuses modifications post-transcriptionnelles covalentes, comportant l'acétylation et la méthylation des lysines (K) et des arginines (R), la phosphorylation des sérines (S) et des thréonines (T), l'ubiquitinylation et le sumoylation des lysines aussi bien que le ribosylation. Les enzymes responsables de ces modifications et leurs spécificités pour des résidus sont fortement conservés (récapitulé dans Peterson and Laniel, 2004). Ces modifications forment la base pour le modèle des histones marqués (parfois désignées sous le nom du « code d'histone ») qui généralement, mais pas toujours, est en corrélation avec des fonctions spécifiques. Par exemple un « code » d'hétérochromatine conservé de la levure aux eucaryotes supérieures est le résidu de H4 K16 déacétylé, qui pour la levure est reconnue par des facteurs d’établissement et maintenance de la chromatine silencieuse (les protéines Sir, voir ci-dessous).

La chromatine silencieuse détermine des aspects importants de l'architecture nucléaire dans de la levure à l'homme (comme des compartiments périphériques). Des études cytologiques dans plusieurs organismes suggèrent que la relocalisation des gènes par rapport aux zones enrichies en hétérochromatine peuvent influencer le taux de transcription. Par exemple, l'interaction en trans des gènes régis par Ikaros avec l'hétérochromatine centromérique corrèle spécifiquement avec l'inactivation du gène relocalisé dans des cellules B de la souris (Brown et autres, 1999). On a également observé, que des allèles d'immunoglobuline se localisent près de la périphérie nucléaire dans des cellules souches embryonnaires, des cellules ancêtre.
haemopoietiques et dans des cellules pro-T, par contre dans le noyau des cellules pro-
B, le locus de la immunoglobuline est décalé dans des positions plus centrales, juste
avant le réarrangement V-D-J (Kosak et al., 2002). Ces observations, ainsi que
l’observation que les télomères de levure se groupent dans des régions silencieuses
près de l'enveloppe nucléaire, ont mené à la suggestion que la position dans le noyau
peut influencer la fonction du génome.

Dans les chapitres suivants, j’ai montré que l’organisation dynamique de la
chromatine (comme décrit dans l’introduction) a des différentes bases qui influencent
l’encrage, la mobilité ou la position d’un locus de chromatine spécifique. Dans le
chapitre 2, j’observe l’influence de la transcription et des « facteurs de remodelage »
sur la mobilité de la chromatine. Ensuite je focalise sur l’effet les protéines Sir
(comme exemple générale de chromatine silencieuse) sur la mobilité de la fibre
chromatinienne. Dans le chapitre 4 j’ai fait des investigations sur changements de la
position et mobilité de chromatine pendant l’interphase du cycle cellulaire. Je
discuterai spécifiquement des aspects de la phase S du cycle cellulaire dans laquelle
l’ADN est répliquée. Chapitre 5 finalement contient un vu générale sur la microscopie
de fluorescence dans la levure et décrit en détail les méthodes pour quantifier la
mobilité de la chromatine.


**Chapitre 2 : Les facteurs de remodelage de la chromatine augmentent sa mobilité**

Dans ce chapitre, j’étudie le rôle des « facteurs de remodelage » de la chromatine dans sa mobilité. J’utilise des méthodes *in vivo* pour regarder la position et la dynamique des chromosomes de la levure (cf. chapitre 5). L’analyse du mouvement de multiples sites dans le génome de la levure marqués au préalable en y insérant des séquences lac<sup>op</sup> bactériennes permettent de mieux comprendre les processus biologiques de la chromatine. Les cellules expriment une fusion Lac<sup>ς</sup>-GFP, ce qui permet la visualisation de foyers fluorescents aux sites marqués avec les lac<sup>op</sup>, et une fusion GFP-Nup49 signalant la forme circulaire de l’enveloppe nucléaire. J’ai observé les cellules cultivées en phase exponentielle à l’aide d’un microscope confocal, en prenant des séries d’images à intervalles réguliers le long de l’axe Z (stacks) toutes les 1,5 s. L’analyse du mouvement à haute précision était possible grâce à une collaboration avec Prof. M. Unser et D. Sage du laboratoire de traitement et analyse d’images de l’EPFL (Lausanne). En se basant sur un très grand nombre d’images et des séries temporelles, j’ai analysé le taux de diffusion, des grands déplacements rapides et le rayon de la contrainte des loci spécifiques. J’ai trouvé que la réorganisation des nucléosomes grâce au complexe Ino80, un « facteur de remodelage », était bel et bien une importante source de mouvement des chromosomes de la levure. Plus spécifiquement, l’activité ATPase de Ino80 était requise pour cette augmentation de mobilité. Par contre, l’inhibition de la transcription n’a pas changé la mobilité de la chromatine. En analogie avec des expériences en cellules mammifères, ceci indique que la transcription en soi n’influence pas directement la dynamique de la chromatine. Ce sont les premières indications mécanistiques que les facteurs de remodelage de la
La chromatine peut directement changer la mobilité de la fibre de la chromatine - un processus qui peut faciliter de diverses fonctions nucléaires, telles que la réplication, transcription, cohésion ou la réparation de cassures d’ADN. Changements dans la dynamique de l’ADN peuvent être un moyen de relocaliser la chromatine ou pour faciliter des contacts entre les régions subnucléaires distinctes.
Chapitre 3 : Mobilité et ancrage de la chromatine silencieuse

Le travail présenté dans le chapitre 3 a été effectué en collaboration avec le professeur Marc Gartenberg afin de montrer que l'ancrage des régions du chromosome à l'enveloppe nucléaire dépend de leur état de répression transcriptionnelle. Pour comprendre le comportement de la chromatine silencieuse en soi, nous arrivons à séparer la proximité du télomère et de la périphérie nucléaire du mécanisme de l'établissement de la chromatine silencieuse. Nous avons étudié le mouvement d’un fragment d’ADN libéré de son contexte chromosomique dans un état de répression ou dans un état de transcription active. Nous avons trouvés que L’ADN non-transcrit (l'hétérochromatine) reste dans la proximité de l’enveloppe nucléaire grâce à l’interaction de la protéine Sir4 qui fait partie de l'hétérochromatine de la levure. Sir4 interagit avec deux protéines: Esc1, une protéine d'ancrage, et yKu80, une protéine télomérique (Taddei et al., 2004). En éliminant ces deux sites de fixation, l'hétérochromatine est capable de traverser librement l’espace nucléaire.
Chapitre 4 : La dynamique de la chromatine dans l'interphase

Dans le chapitre 4, je présente mes études concernant l’effet des différentes phases du cycle cellulaire sur la mobilité de la chromatine. Je me concentre sur la phase de S du cycle cellulaire, pendant lequel une copie parfaite du génome, y compris ses marques épigénétiques, doit être reproduite, i.e. l’ADN est répliquée. Dans la levure, la réplication de l’ADN commence à des origines de réplication bien définis. Ceci nécessite l’assemblage d’un complexe pré-réplicatif fonctionnel au ACS (ARS consensus séquence). La réplication s’effectue d’une façon bien contrôlé en temps et lieu (revu par Bell and Dutta, 2002; revu par Mendez and Stillman, 2003). Plusieurs études basées sur la technique de pusses d’ADN génomiques, ont permis de définir l’utilisation et le temps de réplication de chaque origine de réplication dans la levure (Raghuraman et al., 2001; Wyrick et al., 2001; Yabuki et al., 2002). L’utilisation et la synchronisation du déclenchement d’une origine ne dépend pas de sa séquence primaire mais de son plus grand contexte chromosomique. Généralement, le déclenchement tardif d’origine souvent, mais pas exclusivement, corrèle avec des domaines de structure de la chromatine compacte (Friedman et al., 1996; Vujicic et al., 1999). Le temps de déclenchement et l’utilisation des origines est stablement transmis et implique des marques de chromatine et le positionnement dans le noyau, pourtant les mécanismes exacts qui retardent le déclenchement d’une origine sont encore mal compris. Puisque la fourche de réplication passe chaque nucléotide du génome, ses composants sont les cibles évidentes pour propager cet état. Mais le passage physique de la fourche de réplication n’est pas un préalable à l’établissement de la maintenance de chromatine silencieuse; pourtant le passage par la phase de S est exigé (Kirchmaier and Rine, 2001; Li et al., 2001). Il est probable que les régulateurs généraux de la
phase S agissent en concert avec des facteurs locaux pendant l'établissement de la chromatine silencieuse. La relation causale entre la réplication (et la synchronisation de réplication), la structure de chromatine bien que la mobilité générale de la chromatine et la localisation sous-nucléaire est un problème complexe. En tant que petites parties de ce puzzle, j'adresserai ici deux questions spécifiques au sujet de mobilité de chromatine et de position sous-nucléaire pendant la phase de G1 et de S du cycle cellulaire.

1. Est-ce que la mobilité réduite de la chromatine aux origines de réplication internes pendant la phase de S, une propriété générale du noyau de phase S où il est-elle est spécifiquement reliée à une structure près de la ? Nous reproduisons des modèles de localisation décrits près (Heun et al., 2001a) et trouvons en plus que la mobilité réduite des loci internes est une propriété générale de la phase S. Notre constatation que même un cercle extra-chromosomique répand avec une cinétique plus lente pendant la phase de S soutient l’hypothèse d’un changement globale.

2. Comment les niveaux globalement ou localement accrus d'acétylation d'histone influencent la position sous-nucléaire et la mobilité locale de la chromatine? J’ai découvert que des changements dans l'état d'acétylation des histones, qui servent également à réguler la réplication de l’ADN, influencent le positionnement d’un site chromosomique par rapport à la membrane nucléaire, mais probablement pas la mobilité de la fibre chromatinienne. Disruptions de l’histone déacétylase Rpd3 change la localisation de plusieurs origines de réplication, mais a des effets pléiotropes, comme elle est impliquée dans la répression de beaucoup de gènes. Le ciblage d’une histone acétyltransferase (HAT) introduit des changements minimaux et renforce l’idée donnée par la disruption de Rpd3. Le ciblage de le HAT Gcn5 cause la perte de la localisation périphérique des origines de réplication tardifs ainsi que le temps de
réplication de ce locus spécifique (Heun et al., 2001a; Vogelauer et al., 2002).
Chapitre 5 : Analyses par microscopie de fluorescence

Le chapitre 5 consiste d’une introduction courte au sujet de la microscopie de fluorescence dans la levure suivi par un papier que j’ai écrit pour un manuel de laboratoire (Cell Biology Laboratory Handbook, édité par J. Celis, 2005). Elle récapitule l’instrumentation et les analyses que nous avons utilisées dans l’analyse de positionnement et de dynamique des loci de chromatine dans le noyau de levure. Ce papier est suivi par des sections qui décrivent en grand détail les méthodes de acquisition, évaluation et de l’analyse développés pendant ma thèse. Elles contiennent des descriptions supplémentaires plus récentes ou plus détaillées de microscopie de phase de fluorescence sur des lieux chromosomiques étiquetés en levure. Celles-ci sont suivies d’une description plus détaillée d’analyse de dynamique de chromatine à l’aide de différents outils informatiques et établissant les paramètres significatifs. L’analyse de « mean square displacement » (MSD, déplacement quadratique moyen) a donné des informations robustes et détaillées sur des différents aspects du mouvement d’un locus chromatinienne, comme le coefficient de diffusion ou le rayon de contrainte. Finalement je discute le modèle du « random walk » dans un volume contraint pour décrire la mobilité de la chromatine. On trouve que ce modèle décrit précisément la mobilité d’un anneau de chromatine isolé, mais qu’il y a des contraintes supplémentaires qui limitent la « diffusion » de la fibre chromatinienne intégrale.
1. **GENERAL INTRODUCTION**

The genetic information of eukaryotic cells is distributed on linear chromosomes that are grouped together in the nucleus. The eukaryotic nucleus is surrounded by a characteristic double membrane packed with nuclear pore complexes which mediate active and passive exchange of macromolecules between the nucleus and the cytoplasm. A single chromosome consists not only of a very long double stranded DNA helix (which in humans measures ~2 m, in yeast ~1.5 cm), but has an intrinsic higher-order structure enabling it to be packed in the cell nucleus (metazoan~10 μm, yeast ~2 μm in diameter). DNA is wrapped around nucleosomes which form the basic unit of chromatin to form its primary structure resembling “beads on a string”. The nucleosomal fiber is further condensed into poorly characterized secondary and tertiary structures, yet the DNA can be accessed as needed within this structural context (Muchardt and Yaniv, 1999). Furthermore, chromosomes and specific sub-chromosomal domains are non-randomly positioned in the nucleus.

In this introduction, I will first describe how nucleosomes and the chromatin fiber are modified to fulfill their various nuclear functions. I will then focus on the general dynamic organization of chromatin within the nucleus.

### 1.1 Chromatin structure and function

The chromatin fiber is a nucleoprotein complex which is made up of DNA, nucleosomes -which form the basic repeating unit of a eukaryotic chromosome- and additional linker proteins or nucleosome binding proteins. I first discuss the dynamic
behavior of nucleosome core particles. I will then review how the secondary and tertiary structure of chromatin is built and how it controls nuclear functions.

### 1.1.1. The nucleosome

A nucleosome consists of 147 base pairs (bp) of DNA wrapped in 1.65 superhelical turns around an octamer of the four core histones: H2A, H2B, H3 and H4. The introduction of core histone variants (e.g. H2A.Z, H2AX, macro H2A or CENPA) into nucleosomes and the covalent modification of histone tails have the potential to structurally and functionally alter and mark chromatin (Luger, 2003). The histone octamer component of the nucleosome is composed of two H2A–H2B dimers and a stable dimer of dimers (the H3–H4 tetramer). DNA, being a stiff molecule with a persistence length of ~150 bp (Hagerman, 1988) needs to be bent and distorted at high energy cost in order to be wrapped around the nucleosome. Direct histone-DNA interactions about every 10 bp stabilize this interaction (Luger et al., 1997).

For a long time, nucleosomes have been perceived as immovable objects in the path of the transcription and replication machineries. This view was changed dramatically by the finding that ATP-dependent chromatin remodeling factors are capable of translocating the histone octamer in cis along the DNA. These remodelers are large multi-subunit complexes, conserved from yeast to man (for review see Becker and Horz, 2002; Eberharter and Becker, 2004; Martens and Winston, 2003). ATP-dependent chromatin remodelers can be divided into a number of subfamilies, all related by the SWI2/SNF2 ATPase at their catalytic core. They can be further subdivided into classes of similar domains: e.g. ATPases of the Swi2 class contain a bromodomain, whereas the Ino80 and related enzymes are characterized by a split ATPase domain (Lusser and Kadonaga, 2003). In almost all eukaryotes, one or more
members of each subfamily have been identified. Budding yeast, with its comparatively small genome, contains eight different chromatin remodelers. The first complex identified in yeast was the \textit{SWI2/SNF2} chromatin remodeling complex (Winston and Carlson, 1992). Many more have been discovered since. Generally, ATP-dependent chromatin remodeling complexes play important roles in almost all functions of chromatin: transcription, replication, repair, recombination, sister chromatid cohesion, etc. Because all these processes take place within the context of chromatin, a highly sophisticated system of chromatin regulation which is driven not only by chromatin remodelers, but also by many other enzymes which covalently modify histones has evolved (discussed in 1.1.2).

Although nucleosomes were once thought as immobile structures of constant composition, we know that intact nucleosomes not only rarely remain in one place but also exchange dimers of core histones, all while keeping nucleosomal DNA wrapped. This is achieved by the constant action of remodeling factors, histone chaperones and thermodynamic motion. The transient removal of H2A–H2B dimers from assembled nucleosomes is an important pathway by which histone H2A variants are incorporated into chromatin in a replication-independent manner. H2A–H2B dimer removal also facilitates the passage of RNA polymerase through a chromatin template and enables transcription factor binding, and is closely linked to (or may even cause) nucleosome sliding. This can subsequently even lead to the complete loss of histones as described for the \textit{PHO5} promoter upon activation (Reinke and Horz, 2003). Here histones are first hypo-acetylated (unlike the majority of the promoter regions which become hyper-acetylated upon activation) before they were lost completely (Boeger et al., 2003; Reinke and Horz, 2003). Indeed two genome-wide studies suggest that
promoter clearance from nucleosomes is a general phenomenon in yeast (Bernstein et al., 2004; Lee et al., 2004).

H2A–H2B dimers are in constant exchange between different nucleosomes throughout the genome during interphase, whereas the (H3–H4)$_2$ tetramer is much less frequently exchanged. This process can be mediated by FACT (facilitates chromatin transcription), which is also required for chromatin assembly (Belotserkovskaya et al., 2003). In addition, also ATP-dependent chromatin remodeling factors (e.g. SWI2/SNF2) and histone chaperones (e.g. yNAP-1) are capable of promoting H2A-H2B removal and exchange (Bruno et al., 2003). Other protein complexes promote exchange of histone variants that are not coupled to replication. For instance in yeast, the Swr1 chromatin remodeling complex exchanges H2A-H2B dimers for H2A.Z-H2B (Mizuguchi et al., 2004). Similar mechanisms exist for other variant histones, such as H2A.X which requires the acetyl transferase and ATPase activity of the Tip60 complex for its exchange (Kusch et al., 2004). Finally, nucleosome mobility not only relies on large protein complexes, but nucleosomes appear to be highly dynamic on their own. Rapid (50 - 250 ms) unwrapping and rewrapping of the ends of nucleosomal DNA from the histone surface have been demonstrated in vitro (Li et al., 2005; Li and Widom, 2004). This time scale is sufficient to allow binding of transcription factors and recruitment of the transcription machinery.

1.1.2. The chromatin fiber

Like the single nucleosome, formation of a chromatin fiber superstructure also relies on intrinsic and protein-mediated assembly of secondary and tertiary chromatin structures. Although the highly basic histone’s N-terminal “tail” domains (NTD) are
generally viewed as DNA-binding modules, their essential roles in chromatin folding involve inter-nucleosomal histone-histone interactions or recruitment of the proteins mediating formation of higher-order structures (e.g. linker histones or other nucleosome-binding proteins (discussed below)). The H4 NTD directly contacts the H2A core, forming the secondary structure of the 30 nm fiber (Dorigo et al., 2003; Dorigo et al., 2004). NTDs also mediate tertiary structure formation. The histone-histone interaction and recruitment of histone binding factors can be precisely regulated by numerous covalent posttranslational modifications, comprising acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serines (S) and threonines (T), ubiquitinylation and sumoylation of lysines as well as ribosylation. The enzymes responsible for these modifications and their residue specificities (summarized in Peterson and Laniel, 2004) are highly conserved. These modifications form patterns of histone marks (sometimes referred to as histone code) which generally, but not always correlate with specific functions. For example a conserved feature of heterochromatin in yeast and higher eukaryotes is the requirement for an unacetylated H4 K16, which constitutes part of the site recognized by the yeast heterochromatin Silencing Information Regulator (SIR) protein complex (see below). Methylated H3 K9 also correlates with repressed genes only in most species (from fission yeast to man), while acetylation of histone tails (e.g. K9, K14, K18 K27 of H3 by Gcn5) is also a general mark for expressed genes.

The first histone modifying enzyme described was the histone acetyl transferase (HAT) Gcn5 which had been previously known for its role as co-activator of transcription (Brownell et al., 1996). Conversely, many co-repressors such as Rpd3 were found to have histone deacetylation (HDAC) activity. These findings were quickly followed by the discovery of other HATs, HDACs, histone methyl
transferases or histone kinases. The precise combination of locus-specific histone modifications is due to the combined effects of targeting histone modifying enzymes to specific loci, as well as to the inherent substrate specificity of the enzymes themselves. In the case of transcription, DNA sequence-specific activators directly interact with histone modifying enzymes. The yeast HAT complex SAGA (with its catalytic subunit Gcn5), for example, interacts with a variety of gene specific activators (reviewed in Timmers and Tora, 2005). Targeting histone modifications is not unique to transcriptional control but is involved in almost every function of chromatin; For instance the repair of DNA doublestrand breaks in all species correlates with the phosphorylation of either H2A or H2AX by ATM or ATR homologues. This in turn leads to the recruitment of the chromatin remodelers (e.g. Ino80) to the site of damage (Shen et al., 2000; van Attikum et al., 2004). By a mechanism discussed below, H3 K9 methylation is targeted to centromeric regions in mammals and fission yeast leading to the recruitment of heterochromatin protein 1 (HP1) and subsequent formation of centromeric heterochromatin.

Several nucleosome-interacting proteins, such as HP1, have been shown to mediate higher-order compaction. Examples of these are the linker histones (e.g. H1 and H5) or other nucleosome-binding proteins such as mammalian, human MeCP2, polycomb group protein complexes (PcG), or yeast Silent Information Regulator (SIR) proteins. Three different mechanisms are discussed below.

**HP1-mediated heterochromatin**

Heterochromatin surrounding mammalian and fission yeast centromeres is induced by HP1 binding to methylated H3 K9. This tri-methyl mark is seeded by small noncoding RNAs (siRNA). The centromeric regions are characterized by repetitive DNA sequences that are transcribed at low levels. The resulting double-stranded RNAs
provide substrates for processing by the RNA interference machinery which produces small, 21–23 nucleotide siRNAs. These small RNAs then associate with several chromatin components targeting the Suvar3-9/Clr4 histone methyl transferase (HMT) to centromeric repeats (Maison and Almouzni, 2004). Subsequent histone methylation leads to recruitment of HP1 which directs formation of the highly condensed, heterochromatin structure required for centromere function. Interestingly, HP1 is more effective in promoting condensation on nucleosomes containing H2A.Z than on normal H2A containing nucleosomes (Fan et al., 2004).

**PcG-mediated repression**

Silencing of telomeric and centromeric heterochromatin in *Drosophila* silencing depends on small RNAs and HP1. Stable repression of non-telomeric and non-centromeric genes, however, does not depend on HP1 but on polycomb group (PcG) proteins. They get recruited to polycomb responsive elements (PRE) within the DNA and are involved in the stable repression of many developmental genes, such as homeotic genes (Orlando, 2003; Pirrotta, 1998). PcG proteins were shown to directly bind nucleosomes in order to form a unique secondary structure *in vitro* (Francis et al., 2004). PcG proteins have also been shown to influence global and local chromatin architecture *in vivo* (Bantignies et al., 2003).

**Sir-mediated silencing**

In yeast, heterochromatin-like structures are formed by Sir proteins. They are required for stable repression of the homothallic mating type loci (*HMR* and *HML*) and variegated repression of subtelomeric regions (also known as TPE; see figure 1.1.A and B).
Figure 1.1. A) Stable silencing at the HM loci (near Tel3L and Tel3R) and B) variegated silencing at telomeres.

At the HM loci Sir proteins are recruited by factors bound to cis-acting sequences termed silencers. The silencer-bound factors comprise Sir1, subunits of the origin recognition complex (ORC) or the transcription factors Abf1 or Rap1. TPE on the other hand, is thought to be established by the recruitment of Sir4 and Sir3 by (TG1-3) telomere repeat-binding protein, Rap1 and by the DNA end-binding yKu heterodimer (Marcand et al., 1996; Mishra and Shore, 1999). The interaction of Sir2 with telomere-bound Sir4 subsequently leads to the deacetylation of histones H3 and H4 in the subtelomeric region. This step is the prerequisite for subsequent assembly and spreading of the Sir2-3-4 complexes from the telomere ends along the nucleosomal fiber. Ultimately, this results in the stable transcriptional repression of telomere-proximal genes (Hecht et al., 1995; Strahl-Bolsinger et al., 1997). In support of this model, it was shown that only Sir4 can bind telomeres independently of the other Sir proteins and that Sir2 recruitment requires Sir4. Furthermore, loading of Sir3 depends on the deacetylase activity of the NAD-dependent deacetylases Sir2 (Derbyshire et al., 1996; Hoppe et al., 2002; Luo et al., 2002). Consistent with the preferential binding of Sir3 and Sir4 to underacetylated H3 and H4 tails (Hecht et al., 1995), more recent studies suggest that the H4 K16 residue is an important physiological substrate of Sir2.
at telomeres, since an imbalance of acetylated and deacetylated H4 K16 in subtelomeric regions correlates with the loss or gain, respectively, of TPE (Kimura et al., 2002; Suka et al., 2002).

As is the case for all heterochromatic structures, the exact structure of SIR-dependent silent chromatin remains to be solved.

**General chromatin compaction**

Yeast chromatin is highly compacted, not only at silenced regions, but also in general. The average compaction of yeast chromatin has been estimated to be 40-80 fold compared to the B-form of DNA (Guacci et al., 1994, Bystricky et al, 2004). Nevertheless, chromatin must be accessible for DNA binding factors that regulate genome replication, transcription, recombination and repair of damaged DNA. Therefore dynamic modulation of compaction is a key regulatory principle at different organizational levels. The next section discusses how these changes in chromatin structure and chromatin organization on the nuclear level affect diverse nuclear functions.

**1.2 Dynamic organization of chromatin in the nucleus**

Some of the molecular mechanisms guiding changes in chromatin structure on the nucleosomal and chromatin fiber level have been described above. At the level of entire chromosomes and chromatin domains, it is well accepted that DNA is not randomly localized in the nucleus and that subnuclear localization can also affect local chromatin structure and function. Generally, we can distinguish between three different concepts of chromatin localization which are not mutually exclusive. The first reflects an order imposed by enzymatic functions inherent to the nucleus, often
the result of interactions between large multi-component complexes. The second is a result of sequence-specific interactions. Furthermore the global localization of entire chromosomes (e.g. into chromosome territories) can also influence sequence-specific or function-related subnuclear positioning. I will discuss the static view of subnuclear chromatin organization of the yeast and metazoan nucleus and will next examine dynamic aspects of this organization and give examples for the above concepts.

1.2.1 Subnuclear chromatin compartments in metazoan

**Chromosome territories**

In many metazoan nuclei, individual chromosomes occupy discrete “territories”, each defining a compartment within the nucleus (Bolzer et al., 2005 Fig 1.2; Boveri, 1909; Cremer and Cremer, 2001). However, the relative positioning of chromosomes does not reflect a species-specific map because no reproducible pattern of chromosomal territories (CTs) persists in all nuclei of a given multicellular organism. Moreover, Fluorescence in situ Hybridization (FISH) and real-time imaging have both documented extensions of chromatin from one CT in and out of a neighboring one (Mahy et al., 2002; Osborne et al., 2004; Volpi et al., 2000; Williams et al., 2002), an event correlated with active transcription in mammalian cells and allowing extensive chromosomal cross talk (see Kosak et al., 2002).
Figure 1.2. Chromosome territories in human G0 fibroblasts shown by 24-color 3D FISH. **A)** FISH is based on combinations of 8 chromophores (marked in the corresponding image). **B)** Individual chromosomes are shown in a false color midsection and **C)** 3D-views. Discrete volumes called Chromosomes territories (CTs) are clearly visible. Reproduced from (Bolzer et al., 2005).

Some principles of CT organization have been proposed. For instance, in several mammalian cell types a radial distribution is observed, such that gene-poor chromosomes are enriched at the nuclear periphery (Croft et al., 1999; Habermann et al., 2001), an arrangement that is not found in proliferating fibroblasts (Bolzer et al., 2005; Cremer et al., 2001) or quiescent cells (Bridger et al., 2000). Certain chromosomes showed a reproducible juxtaposition in quiescent human fibroblasts (Nagele et al., 1999) and mouse splenocytes (Parada et al., 2002), while other groups found random distributions (Cornforth et al., 2002; Habermann et al., 2001). Interestingly, certain chromosomal translocations that occur with high frequency in cancer cells have been correlated with a preferential juxtaposition of the relevant chromosomal domains in normal B lymphocytes (Roix et al., 2003). An important
remaining question about the CT theory concerns the mechanism of chromosome positioning. If specific proteins were involved in the positioning of chromosomes, mutations should be found that disrupt nuclear organization.

**Subnuclear localization of chromatin domains**

Chromatin has long been distinguishable as euchromatin and heterochromatin. While euchromatin decondenses after mitosis and contains the bulk of the transcribed genes, heterochromatin was initially observed by the cytological criterion to remain condensed in interphase (Heitz, 1928). With the development of new techniques, molecular rather than cytological features have been used to define heterochromatin. For example that heterochromatin often contains simple repeat sequences, such as that found at centromeres and telomeres, it generally replicates late in S phase of the cell cycle, and those found at the nuclear periphery and contains histone marks for repressive chromatin (*e.g.* methylation at H3 K9). Generally, heterochromatin tends to repress genes that are resident in or transposed near such simple repeat DNA, a phenomenon known as position-effect variegation (PEV) for genes integrated near pericentric heterochromatin in *Drosophila*. In most organisms, centromeric DNA repeats aggregate to form one or several clusters of pericentric heterochromatin called chromocenters. These also associate occasionally with other repetitive DNA sequences or position themselves around nucleoli (Haaf and Schmid, 1991). Chromocenters stain intensely with DNA intercalating agents, due to their sequence bias, and are enriched for heterochromatic markers such as HP1. Chromocenter organization depends on underacetylated histone tails, which are in turn necessary for HP1 binding (Taddei et al., 2001). Importantly, however, conditions that delocalize HP1 from heterochromatin do not induce a loss of chromocenter organization (Maison et al., 2002).
Although some chromosomal elements (such as subtelomeric regions or centromeres) are in a constitutive heterochromatic state, other genomic regions can switch from the more open transcriptionally competent euchromatic state to a compact and transcriptionally inactive heterochromatic structure. This transition from an active to a silent state for a given DNA locus is a typical character of epigenetic regulation and is of great importance for cell differentiation and development (see section 1.3.1 and Fisher and Merkenschlager, 2002).

In human cells as also in yeast (see below), some loci have been shown to have a constraint imposed on their mobility due to interactions with a nuclear structure (e.g. the nuclear lamina or nucleolus). Importantly, higher mobility is restored when the association with the nuclear structure is lost. Treatment with the transcriptional inhibitor DRB, which disrupts nucleolar structure, leads to an increase in the dynamics of a locus located close to rDNA repeats (Chubb et al., 2002).

Proteins involved in the positioning of chromosome domains other than CTs have been identified. This underscores the likelihood that positioning of chromatin domains can be of direct functional relevance. Studies in yeast reinforce this notion.

### 1.2.2 Subnuclear chromatin compartments in *S. cerevisiae*

*S. cerevisiae* (as also *S.pombe*) nuclei have a partially polarized chromosomal architecture during interphase. Their centromeres cluster near the spindle pole body, the yeast centrosome embedded in the nuclear envelope. Much like mammalian heterochromatin, yeast telomeres group at separate domains of silent chromatin at the nuclear periphery (Funabiki et al., 1993 Fig 1.3; Gotta et al., 1996). This grouping largely depends on the Sir-complex, while the anchoring relies on yKu (which binds
to chromosome ends) and Sir4 protein (which binds Esc1, a large acidic protein localizing to the nuclear envelope) (Hediger et al., 2002; Taddei et al., 2004). The strength of the anchoring pathways varies from one telomere to another but also during cell cycle. For example, the disruption of the yeast *yku70* gene leads to the release of telomere 6R from the NE and a significantly higher mobility of the locus. Importantly, not only telomeres but also the *HM* loci (which lie 11 kb and 23 kb from the opposing telomeres of chromosome 3) localize clusters of repressive chromatin at the nuclear periphery, probably due to interactions between Sir proteins, direct anchoring via Sir4 and indirect anchoring via the telomeres (Laroche et al., 2000; Palladino et al., 1993).

**Figure 1.3.** Subnuclear compartments of the yeast nucleus.

DNA was stained with DAPI; Immuno-fluorescence was directed against Rap1 to visualize telomeric foci and Nop1 to mark the nucleolus. Scale bar: 1 μm. Image provided by T. Laroche, M.Gotta and S.Gasser.

Another strictly localized part of the genome is the repeats containing the rDNA genes lying on yeast chromosome 12. These localize to the nucleolus, which in *S.cerevisiae* forms a crescent-like structure at nuclear periphery (Guacci et al., 1994, see figure 1.3). Furthermore, it is interesting to note that tRNA genes localize to the nucleolus when being transcribed (see below, Thompson et al., 2003).
1.3 Nuclear functions and dynamic chromatin organization

Specific chromatin compartments have been discussed above. In yeast, they comprise telomeric foci, the nucleolus or the cluster of centromeres. Here I show some functions linked to this chromatin organization and discuss how key nuclear functions dynamically affect chromatin organization.

1.3.1 Transcription

Some aspects of transcriptional regulation by histone modifications on the formation and subnuclear position of constitutive heterochromatin are described in the previous sections. Here I focus on ongoing transcription and transcriptional regulation by relocalization, especially during development.

Transcription factories

While transcription of rDNA and tRNA genes takes place in the nucleolus, RNA PolIII dependent transcription is dispersed throughout the nucleus. Little is known about its exact organization in yeast. In mammalian cells, however, transcription takes place at discrete foci called transcription factories. Their number varies from hundreds to thousands per cell depending on the cell type. Results from cultured cells indicate that multiple genes occupy one factory (Iborra et al., 1996; Jackson et al., 1998; Osborne et al., 2004). Osborne and coworkers have shown in mouse cells that even distal genes on the same chromosome can share the same factory with high probability depending on their active transcription. Taking into account the fact that even active genes are only transiently associated to an RNA polymerase (Janicki et al., 2004; Schmidt and Schibler, 1995), they further correlated the transcriptional “off”-state with
repositioning away from these factories (Osborne et al., 2004). It remains unclear, however, whether coordinately regulated distal genes have a higher incidence in sharing a transcription factory or whether these sites simply reflect the random juxtaposition of neighboring transcription units. Moreover, the mechanism which mediates physical interaction between RNA polymerases is not known, although internal lamins have been proposed to do this (Kumaran et al., 2002; Spann et al., 2002).

**Gene activation and repression during development**

As mentioned above, mammalian interphase chromosomes are organized into non-overlapping chromosome territories. Furthermore, heterochromatic regions of all chromosomes group at specific loci (e.g. at chromocenters for pericentric heterochromatin). FISH and real-time imaging on the other side have documented extensions of active chromatin from one CT into a neighboring one and more and more data indicate that this organization is variable with respect to gene expression (Mahy et al., 2002; Volpi et al., 2000; Williams et al., 2002). Moreover, a number of examples exist in which the position of a given transcribed or repressed chromatin locus changes relative to a heterochromatin domain or the nuclear periphery, in response to a developmental change in its transcriptional state. Some examples are discussed below.

First, the principle is illustrated during the maturation of thymocytes. When genes become silenced during B or T cell differentiation, they first lose the mark for active chromatin - i.e. the acetyl group is removed from lysine 9 from Histone H3 (H3 K9) and subsequently methylated lysine 4 (H3 K4) is lost. Changes begin at promoters and
spread along the chromatin fiber. Histone deacetylation appears to promote the repositioning of the locus to pericentric heterochromatin (Su et al., 2004).

A further example shows relocalization upon gene activation: using FISH combined with real-time RT-PCR and Chromatin Immunoprecipitation (ChrIP) assays in undifferentiated and differentiating ES cells, gene activation within the Hoxb cluster was studied. Activated genes show not only a preferential internal localization but also chromatin decondensation and an increase in histone modifications that mark active chromatin at early stages. This is followed by the apparent movement of the gene outside its chromosome territory at the time-point when transcript levels of the activated genes increase (Chambeyron and Bickmore, 2004).

Experiments in human cells, in which chromosomes were tagged through a large array of lac operators next to an inducible reporter gene, confirm the above observation. Marked loci were close to the nuclear envelope and compacted when inactive, but upon activation of transcription, chromatin decondensed and became localized more internally (Dietzel et al., 2004).

The unfolding of these large heterochromatic regions marked with arrays of lac operators could also be induced local targeting of a strong transcriptional activator. Recruitment of the activation domain of the *Herpes simplex* Viral Protein 16 (VP16) to a reporter gene integrated in a heterochromatic region of CHO cells led to decondensation of the region. On the nucleosomal level, targeting led to a sequential recruitment of different chromatin remodeling complexes and histone acetyl transferases (HATs) within the condensed chromatin (Memedula and Belmont, 2003). Interestingly, decondensation was not prevented when transcription was inhibited upon alpha-amanitin treatment - indicating that several steps lead to transcriptional activation, one of them being chromatin decondensation which acts upstream of
transcription (Tumbar et al., 1999). Indeed, targeting of a VP16 point mutant with minimal transactivation activity to a lac operator array in CHO cells still lead to decondensation of the entire domain implying that activation by VP16 requires the concerted action of decondensation and recruitment of activator proteins (Carpenter et al., 2005).

Generally, one can say that - especially during development - subnuclear localization follows the principle that highly transcribed genes are preferentially located in the nuclear center, whereas repressed genes are located in the regions of constitutive heterochromatin which are mainly peripheral. Separating repressed genes from active ones can be an advantage during differentiation and repositioning is thought to be an early step in transcriptional activation of previously repressed genes.

**Gene activation and locus relocalization in yeast**

In yeast the nuclear periphery has been associated both with active and repressed loci as described in the following section. Relocalization upon (or prior to) gene activation has become a popular concept for a subset of yeast genes. Best described are some galactose activated genes (e.g. *GAL1,7,10*) and the *INO1* gene which localize preferentially to the nuclear periphery when they are actively transcribed. (Brickner and Walter, 2004; Casolari et al., 2004). Interestingly, *GAL* genes and *INO1* require a functional Ino80 chromatin remodeling complex for their full activation (Ebbert et al., 1999). It remains to be seen if Ino80 is involved in the relocalization and if gene activation can also take place at the nuclear interior. Relocalization of genes has been shown not only for RNA PolII dependent genes but also for the PolIII dependent tRNA genes which were found in clusters and colocalized with the nucleolar 5S gene signal. This association is dependent on both the transcriptional status of the tRNA genes and
Chapter 1

Pol I-mediated transcription, which is necessary for nucleolar integrity (Thompson et al., 2003). The grouping of expressed tRNA genes at the nucleolus restricts the volume within which PolIII transcription factors function – which also implies, however, that the yeast nucleolus is accessible to all 16 chromosomes.

Two main questions concerning the relocalization remain to be answered. First, do active mechanisms or simple diffusion cause relocalization of chromatin loci? Second, is repositioning a cause or effect of transcriptional activation? Genetic analyses alone will answer the second question.

1.3.2 Replication

Replication of eukaryotic genomes is initiated at multiple sites dispersed along the chromosomes. Its spatial and temporal regulation as well as its link with epigenetic determination are well documented (McNairn and Gilbert, 2003). As a general rule, origins of replication near heterochromatic regions are late replicating whereas most eukaryotic regions replicate early in S phase. In yeast, where the temporal replication profile of the entire genome is described, subtelomeric origins and origins in the rDNA indeed replicate late. Nevertheless there are also internal late-firing origins near active genes (Raghuraman et al., 2001). Interestingly, the late-firing properties of a given origin are set up in early G1 phase of the cell cycle and correlate with preferential localization to the nuclear periphery (Heun et al., 2001a; Raghuraman et al., 1997). Replication occurs in discrete self-assembling foci, which are better documented in higher eukaryotes than in yeast. During progression of S-phase DNA their number and distribution changes (Cardoso et al., 1997 and references therein, Fig 1B; Dimitrova and Gilbert, 1999; Nakamura et al., 1986). These changes are of particular interest in respect to replication timing. In higher eukaryotes as well as in
*S. pombe*, replication foci are grouped at different subnuclear domains in early and late S phase (personal communication P. Meister; submitted). By counting foci and estimating the number of replication origins, it was proposed that between 5 and 10 bi-directional forks cluster in a replication focus in cultured HeLa cells (Jackson and Pombo, 1998), and on average, ~10 origins cluster at 20 - 40 foci in budding yeast (Lengronne et al., 2001; Pasero et al., 1997). Various observations led to the hypothesis that replication foci are sites at which polymerases remain stationary through which template DNA is pulled (Ma et al., 1998; Manders et al., 1996).

Genomic loci found in the same compartment during S phase are likely to be replicated at the same time and come in contact with the same chromatin factors right after replication. Generally, histone variants, chromatin modifiers or chromatin binding factors that influence factor accessibility are likely to act on newly replicated loci. These chromatin marks provide a means to re-establish a given transcriptional and/or spatial pattern of organization in daughter cells. Interestingly, disruption of several chromatin modifying enzymes changes the replication timing profile in yeast: E.g. disruption of the Rpd3 histone deacetylases advances timing of all internal origins, whereas in a *sir3* mutant subtelomeric origins are specifically firing earlier (Aparicio et al., 2004; Stevenson and Gottschling, 1999). Since very little is known about replication patterns in budding yeast, it remains to be seen how the spatial organization is affected by these mutations.

Subnuclear localization of replication factories, as such, may not be critical for immediate biological events, but could provide a mechanism for the accurate inheritance of transcription patterns by reinforcing epigenetic marks. If true, dividing and differentiating cells may have a more robust nuclear organization. The more a cell divides, the better its nuclear architecture and consequently patterns of replication
should be preserved and the more difficult it should be to go back to a more totipotent state. This is interesting for two reasons. First, the cloning of mammals was shown to be more efficient from quiescent than cycling cells, which suggests that epigenetic markers are more strongly present in dividing cells (Wilmut and Campbell, 1998). Second, during *C. elegans* embryogenesis, replication foci start to form only in the course of development, suggesting that differentiation (or proliferation) is responsible for their formation (personal communication P. Meister).

### 1.3.3 Insulators

Insulators provide a further example of a chromatin function that has been correlated with tethering or sequestering of sequences. The term insulator refers to two different activities that can be distinguished experimentally. The first is an enhancer-blocking activity, which shields a promoter from the action of a distant enhancer. The second is a silencing barrier activity, which protects genes from invasion by neighboring heterochromatin (reviewed in Burgess-Beusse et al., 2002). Both functions have been proposed to involve specific configurations of chromatin, such as a topologically defined loop tethered to nuclear pores (Blanton et al., 2003; Gerasimova et al., 2000; Ishii et al., 2002). In *Drosophila*, the element *Fab-7* contains an insulator next to a Polycomb Group (PcG) response element. This element has been shown to physically associate *in trans* with an integrated copy of the same element located on another chromosome (Bantignies et al., 2003). Interestingly, the absence of PcG protein not only compromises silencing but also association of *Fab-7* boundary elements. Similarly to *Fab-7*, two *gypsy* insulator elements inserted at distant sites have been reported to associate with each other (Blanton et al., 2003; Byrd and Corces, 2003) and random insertion of the *gypsy* has been shown to recruit the surrounding DNA to
the nuclear periphery (Gerasimova et al., 2000). However, the preferential peripheral association of *gypsy*-containing loci can be genetically separated from the insulator element and insulator can function in the nuclear interior (Xu et al., 2004). Thus *gypsy*-mediated anchoring is not a prerequisite for insulator function.

In vertebrates, a conserved sequence-specific factor (CTCF) is involved in the function of a many insulators. A transgene containing the CTCF insulator has been localized to the nucleolus and it is believed that CTCF insulator activity may reflect a higher-order level of genome organization (Yusufzai et al., 2004). This is reminiscent of activities isolated in a fusion protein screen in yeast, which can confer barrier activity by tethering to nuclear pores (Ishii et al., 2002).

It is important to note that to date –as described for the *gypsy* element- there is no genetic evidence that disruption of the spatial positioning affects native insulator function.

### 1.3.4 Quantification of chromatin mobility

In order to change position as described above, a given chromatin locus has to move within the nucleus. Indeed, one of the more surprising findings of recent years, documented through rapid time-lapse imaging of GFP-tagged chromosomal loci (Marshall et al., 1997), is how mobile chromatin can be when observed by high resolution fluorescence microscopy and single particle tracking (SPT). This technique revealed high local (Brownian-like) mobility but also long-range chromatin dynamics in interphase nuclei in species ranging from yeast to man. The most precise measurements of local chromatin mobility however are measured by fluorescence correlated spectroscopy (FCS) or two-photon standing wave fluorescence photobleaching (L.Gehlen and J.Langowski personal communication). The Bardeen
group showed in vivo that short-range chromatin mobility a) was very variable per se and b) reflected the DNA-histone interaction. Mobility increased when DNA-histone interactions were weakened in higher salt concentrations or decreased upon specific photo-crosslinking of histones (Davis and Bardeen, 2004). This is the first direct evidence that chromatin mobility and compaction are interdependent.

**Chromatin dynamics measured by single particle tracking**

Quantification of the movement of specific marked chromosomal loci shows that there is continual, but spatially constrained, movement. While the radii of constraint between fly, man and yeast are in the same range; their apparent diffusion coefficient varies between very comparable radii of constraint. The precise diffusion coefficient of different loci ranges from $1.25 \times 10^{-4}$ (5p14, man) to $1.8 \times 10^{-3} \mu m^2/s$ (LYS2, yeast) for non-repetitive open chromatin domains (Chubb et al., 2002; Heun et al., 2001b; Marshall et al., 1997). Using time-lapse imaging for chromosomal domains in budding yeast, we revealed the following, we could characterize movements on different scales: smaller, saltatory movements of less than 0.2 μm that occur constantly and larger, more rapid directed movements of more than 0.5 μm in a 10.5 s interval (Heun et al., 2001b). The smaller movements are observed for internal sequences as well as silent (mostly peripheral) regions. The larger movements are characteristic for internal non-silent loci and are less frequent at telomeric and silent regions. Moreover, changes in cellular energy levels (through depletion of glucose or addition of protonophores, which deplete membrane potentials) induce a decrease in or a complete abolition of large movements (Heun et al., 2001b). Chromatin movements thus seem to be influenced by energy-dependent processes.
The extent of spatial constraints imposed by the environment on a given locus, like the diffusion coefficient, also is calculated by plotting the mean squared displacement (MSD) over fixed intervals of time (Marshall et al., 1997; Saxton, 1997). For some loci, these MSD plots reach a plateau at time intervals of 60 - 100 s from which the radius of constraint of a given site can be derived and compared with the movement of other loci and different species. The movement of euchromatic loci in fly, man and yeast is confined to volumes of ~0.5-0.8 \( \mu \text{m} \) in radius, which constitutes a large fraction of a yeast nucleus (\( r=1 \ \mu \text{m} \)), but not of a mammalian one (\( r=10 \ \mu \text{m} \)) (Chubb et al., 2002; Heun et al., 2001b). These radii of constraint are in agreement with CTs in mammalian cells, but not in yeast.

Poorly characterized stochastic events may also influence the dynamics of some loci. Many of the observations described above would predict directional chromatin movement, but so far it has only been reported in a few cases. In interphase human centromeric regions are mainly static. Nevertheless, rare events of directed long range movements (at velocities of 7-10 mm/h) have been observed (Shelby et al., 1996). It has also been reported that, while most human telomeres move little, occasionally a telomere will make a rapid transition as if liberated momentarily from constraints (Molenaar et al., 2003). Spontaneous movement of yeast telomeres away from the NE has also been observed, possibly reflecting sporadic transcription or chromatin remodeling events (Hediger et al., 2002).

**Chromatin dynamics during cell cycle**

The degree of spatial constraint imposed on a locus depends on two further criteria: the cell-cycle stage and the nature of the locus observed. In *Drosophila* spermatocytes, there is both a random constrained movement and a long-range
movement in S and early G2 phase, which occurs over a much longer time scale. A
decrease in step size for the rapid movements has been recorded in late G2
spermatocytes just before they enter meiosis (Vazquez et al., 2002). This drop in
chromatin mobility correlates with a clear developmental change characterized by a
nuclear reorganization during which bulk chromatin moves from a central region to
three distinct perinuclear masses (Cenci et al., 1997). In budding yeast, different
marked origins of replication have shown a decrease in general mobility during
S phase (Heun et al., 2001b). The reduced mobility does not correlate with the
passage of a replication fork, and instead might either reflect a reduction in
ribonucleotide pools that accompanies S phase, or a trapping near replication foci.
As already mentioned, chromatin movement is constrained by nuclear substructure
and chromatin compaction. To a large degree, however, it is restricted in its mobility
by the contiguity of the chromatin fiber itself. Nevertheless the fact that this fiber
(even in its heterochromatic state) is accessible to most chromatin-bound factors
which is demonstrated by the rapid diffusion-based exchange rates detected for most
chromatin-bound factors by FRAP (reviewed in Misteli, 2001). The documented
mobility of chromatin may therefore facilitate searches for larger subnuclear
structures or for homologous DNA sequences for recombination or repair.
2. NUCLEOSOME REMODELING COMPLEXES CAN INCREASE CHROMATIN MOBILITY

2.1 Introduction

Major nuclear functions in interphase depend on the long-range mobility of chromosomes within the nucleus. In yeast, as also in metazoa, targeted integration of lac operators to specific chromosomal loci allows the observation of chromatin movements at any point in the cell cycle by live fluorescence microscopy (Robinett et al., 1996; Straight et al., 1998). Single particle tracking (SPT) of such marked chromatin loci has revealed that interphase chromosomes undergo rapid diffuse motion within the nucleus. Their mobility can be characterized by the kinetics of the diffusion process but also by the nuclear subvolume the chromatin occupies (Heun et al., 2001b; Marshall et al., 1997). Since an excised ring of chromatin shows no restriction in its mobility other than that imposed by the nuclear envelope (Gartenberg et al., 2004) this restriction in mobility reflects aspects of chromosome structure, which will be discussed below.

Chromatin mobility can also be measured by other fluorescence microscopy techniques. Using two-photon standing wave fluorescence photobleaching, the Bardeen group could show in vivo, that short range chromatin mobility was a) very variable per se and b) reflected the strength of the DNA-histone interaction (Davis and Bardeen, 2004). This is the first direct evidence that chromatin mobility and compaction are interdependent.

Although the yeast nucleus is small, it offers many advantages for describing chromatin dynamics with high precision. First, the round shape of the interphase nucleus facilitates accurate tracking, because the movement of the nucleus itself can
be reliably tracked independently of the tagged locus. Using the same techniques that are applied to fluorescent loci, the movement of the foci can be corrected for both nuclear and mechanical drift (Sage et al., 2003). A further advantage is the ease of genetic manipulations and the use of a relatively small array of lac repeats (~10 kbp). This sharply contrasts with the large arrays that are integrated in mammalian cells (which can range up to hundred Megabasepairs (Mbp); Li et al., 1998). Previous experiments have demonstrated that Brownian motion contributes in part to the chromatin movements. On the other hand various experiments show a tight connection between nuclear processes and chromatin mobility (see Chapter 1). In this chapter, we first address the question if chromatin mobility can be described based on a model of a random walk within a constrained volume or if more sophisticated models are needed to accurately describe chromatin mobility in yeast. We then go on to examine facilitated movement that correlates with the recruitment of a nucleosome remodeler to specific genes.

Mechanistically, changes in chromatin dynamics observed by fluorescence microscopy can have different causes: they range from active “motor-driven” processes such as mitotic chromosome segregation, to changes in the compaction or stiffness of the chromatin fiber, and can also arise from the presence of different “anchorage points” along the nuclear fiber. In *Saccharomyces cerevisiae* several sites of spatial “anchorage” have been defined. Notably centromeres and telomeres are preferentially located at the nuclear periphery. Telomere anchoring requires Silent Information Regulator (SIR) proteins, the yKu heterodimer and Esc1, a large acidic protein localized to the inner nuclear envelope. Attachment varies with the cell cycle stage (Andrulis et al., 2002; Gartenberg et al., 2004; Taddei et al., 2004). Centromere position on the other hand is determined by its interaction through microtubules with
the spindle pole body (Bystricky et al., 2005; Heun et al., 2001a; Jin et al., 2000). Indeed, centromere containing plasmid moves much less than an excised chromatin ring (Gartenberg et al., 2004; Marshall et al., 1997). The DNA in between centromeres and telomeres in many cases shows constrained mobility, yet no specific peripheral attachment (Gartenberg et al., 2004). Radii of constraint are in the range of 650 nm (Figure 2.2 and Chapter 5.2).

The source of chromatin movement is not known, but there is strong evidence that an active process is involved. Notably, addition of azide or energy depletion by addition of carbonyl cyanide chlorophenyl hydrazone (CCCP) an uncoupler of the mitochondrial F\textsubscript{1}F\textsubscript{0} ATPase and of plasma membrane potentials has been shown to drastically reduce chromatin mobility even of excised chromatin rings (Gartenberg et al., 2004; Heun et al., 2001b). Energy depletion in yeast cells had a similar effect: Free movement of tagged loci was reduced upon depletion of glucose, which also reduces ATP levels.

Similar results were observed for mammalian cells, where FRAP experiments showed a general compaction of nucleosomes that is thought to restrict chromatin mobility. In contrast to the reduced mRNP mobility, free GFP did not change mobility (Shav-Tal et al., 2004; FN unpublished observation). These differences between molecules of varying size can be explained by a “sieve model” in which small molecules are free to move, whereas larger ones such as mRNPs, or free chromatin rings get trapped.

In a previous study the effect of replication on chromatin mobility was investigated. Heun et al. (2001a) demonstrated that late firing origins of replication unlike early firing origins preferentially localized to the nuclear periphery in the G1 phase preceding replication. They further observed that chromatin mobility, in particular the large movements, were reduced in S phase for different internal (i.e. non-telomeric
and non-centromeric) replication origins (Heun et al., 2001b). This observation was also observed for internal loci distal from origins of replication (FN unpublished observation). We assume that reduced chromatin mobility reflects a general property of S-phase nuclei at least in part. This could be due to reduced NTP levels or the creation of replication foci that restrict movement of replicating DNA.

The effect of transcription on chromatin dynamics has not yet been directly investigated in yeast. Nevertheless, the notion that genes relocalize upon activation has been becoming a popular concept: Active tRNA genes were shown to relocalize from the nucleoplasm to the nucleolus (Thompson et al., 2003). For some activated RNA PolII dependent genes a preferential perinuclear localization was described: active GAL genes or the activated INO1 preferentially localize to the nuclear periphery (Brickner and Walter, 2004; Casolari et al., 2004). Interestingly, GAL1 and INO1 require a functional Ino80 chromatin remodeling complex for their full activation (Ebert et al., 1999). The underlying mechanism of relocalization of these specific set of transcriptionally active genes still remains to be investigated.

There are also several indications that chromosome organization in mammalian cells is variable with respect to gene expression. Active genes not only transiently localize to sites of ongoing transcription (Osborne et al., 2004) but they also stably change their subnuclear position towards the middle of the nucleus or vice versa (Chambeyron and Bickmore, 2004; Su et al., 2004). Further experiments performed in a mammalian cell culture system show that a heterochromatic locus marked with a large array of lac operators give a discrete fluorescence signal. Upon induction of transcription or upon expression of the VP16 transcriptional activator, this region decondenses and localizes more towards the nuclear interior. Interestingly, this decompaction was also observed in the absence of detectable transcription (Carpenter
et al., 2005; Tumbar and Belmont, 2001) suggesting that decompaction is a step preceding transcriptional elongation.

In yeast, transcriptional activation by acidic activation domains such as VP16 has been investigated intensively. Under physiological conditions, VP16 activation domain was shown to recruit two yeast HAT complexes, the SAGA (Spt/Ada/Gcn5 acetyltransferase) and NuA4 (nucleosome acetyltransferase of histone H4) complex with their catalytic subunits Gcn5 and Esa1, respectively (Biggar and Crabtree, 1999; Utley et al., 1998; Vignali et al., 2000). Recruitment seems to be mediated by Tra1, a common subunit of both complexes (Brown et al., 2001). In vivo crosslinking studies further confirmed direct interactions of VP16 with TATA-binding protein (TBP), TFIIB or SAGA (Hall and Struhl, 2002). VP16 not only interacts with HATs and basal transcription factors, but also with several components of ATP dependent chromatin remodeling complexes. In several in vivo and in vitro assays different acidic transcriptional activators (among them VP16) have been shown to interact with the Swi1, Snf5 and Swi2/Snf2 subunits of the yeast Swi2/Snf2 chromatin remodeling complex - stimulating transcription (Neely et al., 2002).

Swi2/Snf2 is the founding member of a large family of ATP dependent chromatin remodelers. Chromatin remodelers alter the positions and/or histone composition of nucleosomes and are involved in most if not all chromatin related functions, e.g. transcription, replication, recombination, repair or cohesion. The remodeling complexes are specifically recruited to their target loci, e.g. to promoters or double-strand DNA breaks. Many of these huge complexes have similar architecture and share common subunits (e.g. Actin related proteins), as reviewed in (Eberharter and Becker, 2004). For gene activation, the order and specificity of recruitment of HATs, chromatin remodelers, and general transcription factors, mediator and polymerase
complexes may vary among different promoters (Lemon and Tjian, 2000). Swi2/Snf2 itself is known to facilitate expression of several inducible genes, e.g. those expressed under stress but is not essential and generally not very abundant but other chromatin remodelers play a more critical role (Sudarsanam et al., 2000).

Another important and abundant chromatin remodeling complex is the Ino80 complex named for the large Swi/Snf like catalytically active ATPase subunit. The Ino80 complex is implicated both in repair and transcription. Importantly, in an in vitro assay, recombinant Ino80 facilitated chromatin remodeling by targeted VP16 in an ATP dependent manner (Shen et al., 2000). Generally, the Ino80 complex is required for the full induction of a subset of genes (e.g. GAL1 or INO1) and affects expression of ~1000 yeast genes leading to upwards or downwards >1.5x changes in both directions (induction and repression) which are distributed throughout the entire genome. Most of these genes are distinct from those affected by Swi2/Snf2 (Ebbert et al., 1999; Shen et al., 2000; van Attikum et al., 2004).

Here we want to investigate how transcription itself and chromatin modifiers influence local chromatin mobility observed by live fluorescence microscopy and single particle tracking (SPT). Local targeting of factors such as VP16 and Ino80 to the chromatin fiber will increase our understanding about how chromatin moves within the nucleus and by which functions it is regulated. Altered locus dynamics could be a cause or result of repositioning of activated genes but could also be implicated in other chromatin functions.
2.2 Results

2.2.1 Theoretical aspects of chromatin mobility

Using rapid 3D-timelapse microscopy on fluorescently tagged yeast chromosomes we describe the dynamic movement of specifically tagged chromosomal loci in interphase. Quantification of >60 minutes of movies (on a total of 8-16 independent cells) allows an accurate description of locus movement at a time resolution of 1.5 s. To correct for movement of the cell or nucleus, the absolute position in reference to the center of the nucleus is determined at each time point and its global movement is subtracted from that of the tracked locus.

Based on the coordinates of the time points (and the distances between them), we then performed a Mean Square Displacement (MSD) analysis which characterizes different modes of (obstructed) diffusion. For this, the mean distances between two positions of the fluorescent spot that cover a specific time interval (1.5 s – 150 s) are squared and plotted against the corresponding time interval for each movie. Due to the high number of distances averaged, this analysis turns out to be very robust. Averaging over individual movies gives a good qualitative image of the diffusive behavior of a certain locus. For quantitative analysis, the following two values which are based on the MSD analysis are taken into account: The radius of constraint ($R_C$) and the diffusion coefficient ($D$).

The $R_C$ is calculated from the MSD plot at longer time-points where it can form a plateau and reach a saturation value. For some loci this plateau is not reached within the time observed and its radius of constraint is calculated based on the maximal MSD value.

The diffusion coefficient ($D$) is calculated from the slope of the MSD graph (Berg, 1993). As a characteristic for obstructed diffusion, $D$ changes with time, as
demonstrated by the non-linearity of the MSD plots (Figure 2.1.D and 2.1.E). Nevertheless, the first time points show a quasi-linear relationship and the slope of this initial phase allows calculation of a diffusion coefficient (D) (Figures 2.1D-E, 2.2.A and D). Here, D is calculated based on the slope of a linear regression through the first 8 time points (1.5 s -12 s). Taking the slope of the first 8 data points which generally lie in a linear range of the MSD plot reduces tracking errors due to variable image quality, which are bigger for very short time intervals. The values for D are in the order of $10^{-3} \mu m^2/s$. From a study captured at 24 s time intervals, a diffusion coefficient of $3*10^{-4} \mu m^2/s$ has been described of the centromere proximal LEU2 locus (Marshall et al., 1997). This value is six times lower than the diffusion coefficients we find for the LYS2 locus if we examine $\Delta t=24$ s. This difference may reflect the anchoring of centromeres to the spindle pole body (SPB) by microtubules (Bystricky et al., 2005; Jin et al., 2000).

Locus mobility can be further characterized by the mean step size, which provides another measure of mean diffusion rates and the average number of movements which are larger than 500 nm in 10.5 seconds (normalized to a 5 minute interval). They may be a property of short-term directed motion since they are reduced in glucose depleted cells (Heun et al., 2001b).

Given that we accumulate 3D information over time, it was possible to analyze the movement either in 4D or as z-projections of the 3D time-lapse movies. A set of the experiments were analyzed in both ways, and we concluded that the latter produced a more robust and accurate description of locus mobility (Supplementary Figure 2.1.A and B), because 1) The optical resolution in z-axis is smaller than in the x and y dimensions, and 2) The high sampling rate of 6 images in 1.5 s with a spacing of 450 nm does not allow more sections in z without loss of acquisition speed. Because
the quality of information in the optical axis (z-axis) is low and because there is no oversampling spot mobility is most accurately followed in the xy-plane. The effect is visualized and quantified in supplementary figure 2.1.A-B, which shows that movements along the optical axis are overestimated in comparison to theoretical data (Supplementary Figure 2.1.C). Given these considerations, we have concluded that quantification of projected stacks (2D) over time is more accurate than quantitation in 3D.

**Figure 2.1.** Quantification of chromatin dynamics of the *LYS2* locus in the chromosomal context and as excised chromatin ring. **A.** Map of the marked *LYS2* locus on chromosome 2. Site-specific recombination leads to excision of the lac operators and parts of the *LYS2* gene and results in ring formation. **B.** representative 51
traces (3D projections) of two 7.5 minute movies. C. Corresponding kymograph representations of x- and y-axis along over time of a 7.5 min movie illustrate the tracking quality and differences in mobility. D and E. Averaged mean square displacement (MSD, blue lines) plots of the LYS2 locus (10 movies, 76 min) and the LYS2 chromatin ring (9 movies, 101 min) and the corresponding standard error of the mean (error bars). The red line shows the expected MSD plot based on the model of a random walk in a confined volume (step size and plateau level correspond to the experimental data). Details are described in the materials and methods section. F. Quantification of locus mobility: Diffusion coefficients (D) based on the slope of the first 8 data points and the corresponding standard error of the mean, the Radius of constraint (R<sub>C</sub>) calculated from the maximal MSD value, and the number of large rapid steps (>0.5 μm / 10.5 s) per 5 minutes.

Simulations of random walks in a confined volume further allowed to compare ideal trajectories in 3D and in 3D projections, showing that the 3D MSD plot is 1.5-fold higher than one derived from the projected stacks (Supplementary Figure 2.1.C and Materials and Methods). This applies to all data sets presented here and is taken into account for all values deduced from the MSD analysis.

The mobility of specific chromosomal loci in yeast depends on different factors. These are most fundamentally the physical properties of the chromosomal fiber and the nucleoplasm, which are represented by the persistence length of the fiber and the viscosity of the nucleoplasm (see chapter 5.4.4). Furthermore, specific functional chromosomal domains of yeast chromosomes, notably telomeres and centromeres, are anchored by protein-protein interactions at the nuclear periphery and therefore also restrict mobility of proximal loci (Bystricky et al., 2005; Gartenberg et al., 2004;
Hediger et al., 2002; Heun et al., 2001b; Taddei et al., 2004). Our initial aim was to better describe the characteristic diffusive movement of a non-tethered internal locus in interphase and to compare this with the diffusion of an excised ring of chromatin. To do this, the LYS2 locus located 342 kb from Tel2R was tagged by the insertion of lac op repeats and sites recognized by the recombinase R were inserted 13 kb apart (Gartenberg et al., 2004; Raghuraman et al., 1997; Straight et al., 1996). Upon induction of the recombinase, a ring of chromatin is efficiently excised and can be visualized by a lac repressor-GFP fusion which is constitutively expressed in the same strain. Within 10 minutes, the LYS2 locus diffuses within one third of the nuclear volume. This corresponds to a theoretical radius of constraint ($R_c$) of 0.66 μm (Figure 2.1.B, 2.1.D and 1.F).

We have previously calculated to what degree locus mobility is constrained by the context of the chromatin fiber (Gartenberg et al., 2004). Figure 2.1 shows a quantification of the constraint for the LYS2 locus: The subvolume in which the chromosomal locus moves is ~2.4x smaller than the one of the excised chromatin ring ($R_c$ ~0.66 μm and ~0.88 μm, respectively), i.e. the spatial constraint of a free chromatin ring almost exclusively relies on the confinement by the nuclear dimensions. Not only are the radii of constraint different in the two conditions but also the nature of movement. The excised ring has larger mean step size and an >3x increase in its diffusion coefficient ($D_{1.5,12s}=5.00*10^{-3}$ μm$^2$/s) over its chromosomal counterpart. In order to better understand these chromatin movements, we performed simulations based on the random walk model in a confined volume. Moreover, we were able to fit the function describing the MSD plot for given step sizes and plateau levels (see Materials and Methods). The excellent match of the experimental MSD
Contrary to an excised ring, the chromosomal locus movement does not conform to this simple model for random movement. At short time intervals, the slope of the curve is lower, i.e. actual diffusion is lower than the corresponding simulations of a random walk. This shows that the fiber itself imposes a strong constraint on a chromatin locus. Importantly, this reduction is seen for all chromosomal loci observed to date, many of which do not reach a MSD saturation value within the observed time period (see figure 2.2A and D). Irrespective of their presumable plateau level, the MSD plot does not fit a simple random walk model.

We speculate that the physical properties of chromosomal fiber and its anchoring points contribute to the non-random movements. Indeed, previous work has shown that telomere anchoring by Silent Information Regulators, Esc1 and yKu significantly restricts locus mobility (Hediger et al., 2002; Taddei et al., 2004) and that clustering of centromeres through microtubule linkage to the SPB restricts the mobility of centromere proximal tags (Bystricky et al., 2005; Heun et al., 2001b; Jin et al., 2000).
Figure 2.2. LexA-VP16 targeting increases chromatin dynamics of two internal loci.

A Scheme of the marked \textit{RPL9A} locus and the corresponding average MSD plots.

LexA- (11 movies, 82 min), LexA-VP16 (11 movies, 82 min) and LexA-Gal4
activation domain (GAD, 9 movies, 68 min). The LexA fusion proteins were recruited to 4 LexA binding sites at the target loci (red boxes). They are followed by 10 kb of Lac operators (green boxes) which are used to visualize the locus by expression of a Lac\textsuperscript{i} -GFP fusion protein. Error bars correspond to the standard deviation of the mean and are shown for the LexA- and LexA-VP16 targeting. For clarity, they are not included in the succeeding figures. Blow-ups of the first 8 time intervals (corresponding to 12 s) are shown in the bottom right corner. C. Two representative traces and kymograph representations of 7.5 minute movies showing the tracked marked locus along x- and y-axis along over the time of the movie. D-F. Analogous to A-C for the \textit{ATG2} locus (LexA-: 11 movies, 82 min; LexA-VP16: 8 movies, 54 min) G. Relative transcript levels were quantified by real-time PCR. The relative transcript levels are normalized to \textit{ACT1} and are based on the corresponding CT values. H and I. Relative transcript abundance of the \textit{RPL9A} and \textit{ATG2} gene upon targeting of LexA-, LexA-VP16 and LexA-GAD, respectively. All experiments were performed in triplicate and normalized to \textit{ACT1} expression.

2.2.2 Local recruitment of VP16 increases chromatin mobility

In order to monitor changes of chromatin as a result of recruitment of local factors we constructed strains in which we can target fusion proteins with LexA DNA binding domain to different chromosomal loci tagged with the lac\textsuperscript{i}/lac\textsuperscript{op} system (Straight et al., 1996; Taddei et al., 2004). We first sought to elucidate the effect of targeting of the strong transcriptional activator VP16 to the tagged locus. VP16 has been shown to interact with components of the basal transcription machinery as well as HATs and chromatin remodelers \textit{in vivo}. Its acidic domain was further shown to have chromatin
Chapter 2
decondensation activity on large arrays in CHO cells (Carpenter et al., 2005; Tumbar and Belmont, 2001; Tumbar et al., 1999). In our experiments we target LexA-fusion proteins constitutively expressed from a plasmid to four LexA operators adjacent of the lac operator-array. Using rapid time lapse fluorescence microscopy and precise tracking techniques (see Materials and Methods) we quantify locus mobility as described above in chapter 5.

Although the mean step size does not change significantly in the presence or absence of VP16, a clear difference can be observed for the radius of constraint and for the number of large rapid steps. Targeting of VP16 leads to a general significant increase in mobility (Figure 2.2A-F). Local recruitment to two different internal loci showed not only that the diffusion coefficient is \(\sim1.2\)-fold higher than in the control situation but also that the volume occupied by the locus is 1.5-fold larger, which is deduced from \(R_C\) (Figure 2.2.B and E).

We monitor two loci, \(RPL9A\) and \(ATG2\), which are located internally on chromosomes, i.e. not in the proximity of a telomere nor a centromere (see map in figure 2.2.A and D). They differ however in the basal transcription levels (Figure 2.2.G) and in one case the VP16-fusion protein is targeted to a site 1kb upstream of the promoter (\(RPL9A\)), while in the second (\(ATG2\)) the activator is bound 900 bp downstream of the \(ATG2\) gene. This is 5.6 kb downstream from its transcription initiation site. To show that the observed increase in mobility does not reflect an artefactual VP16 “squelching effect” in trans, we monitored the effect of LexA-VP16 on a locus that has no integrated LexA operators. VP16 expression did not lead to any significant change in mobility demonstrating that the local recruitment of VP16 is necessary to increase chromatin mobility (Figure 2.3.D and I).
Figure 2.3. Locus mobility and transcription. **A and E.** Schemes of the lac^op^ marked YNL217W/ARS1413 and ATG2 loci. LexA fusions were targeted to LexA binding sites (red boxes) followed by 10 kb of Lac operators (green boxes) which are used to visualize the locus by expression of a Lac^I^-GFP fusion protein. **B-D and F.** Average MSD plots. (YNL217W/ARS1413: rpb4Δ 37°C n=6, wt 37°C n=4, rpb4Δ 25°C n=4, 50 mm 6-AU: n=8, 0.1% DMSO n=3; ATG2: LexA n=11, LexA-Gcn5 n=10). Blow ups of the first 12 s intervals are shown in the bottom right corner. Transcription was
inhibited by the use of 50 μM 6-Azauracil (6-AU, Sigma). Temperature was regulated using a temperature controlled box. G-I. Quantification of D (based on the slope of the first 8 data points), R_C (calculated from the maximal MSD value) and the number of large rapid steps (>0.5 μm / 10.5 s) per 5 minutes.

It is worth while noting that the RPL9A gene is induced proportionally to a much lesser extent than ATG2 – probably due to the very high level of basal activation in the rapidly growing cells. The basal transcript levels of ATG2 and RPL9A (normalized to ACT1) differ by almost 100 fold in wildtype cells (Figure 2.2.G). Nonetheless, the effect of VP16 is roughly the same. To see if the increase in mobility reflects the nature of a transcriptional activator per se, we targeted the Gal4 activation domain (GAD) to the ATG2 gene. Surprisingly, locus mobility did not change significantly upon recruitment of a LexA-fusion protein to GAD. Consistent with the similar mobility of the two differentially transcribed loci, we conclude that transcriptional activation per se is not the cause of locus mobility. Indeed both VP16 and Gal4-AD induce expression of a β-galactosidase reporter to similar extent (data not shown). This shows that local activation of transcription does not automatically lead to increased chromatin mobility. The differential increase in mobility by different transcriptional activators may be due to different modes of action. In contrast with GAD, VP16 stabilizes a transcription reinitiation complex containing transcription initiation factors and mediator (Stafford and Morse, 2001; Yudkovsky et al., 2000). Eventually, the difference is also due to recruitment of different histone acetyl transferases or chromatin remodelers involved in the transcriptional activation.
2.2.3 Inhibition of transcription and temperature do not suffice to change chromatin mobility

In CHO cells the decondensation of chromatin upon VP16 targeting was observed even when transcription was inhibited (Tumbar et al., 1999). It is thus interesting to know whether or not in yeast, transcriptional elongation contributes to general chromatin mobility – or if chromatin becomes less mobile when transcription is inhibited – a question which could not be addressed by targeting VP16 to a highly transcribed locus as described above. To monitor locus mobility during the inhibition of elongation, we made use of a complete rpb4 deletion, which is temperature sensitive for RNA PolII transcription at the non permissive temperature (Woychik and Young, 1989)). Two marked loci were monitored (LYS2 and YNL217W in the late replicating cluster of chromosome 14). We also used the IMP dehydrogenase inhibitor 6-azauracil (6-AU) which depletes pools of GTP and prevents elongation by RNA PolII (Exinger and Lacroute, 1992; Uptain et al., 1997). No drop in mobility was seen in presence of 6-AU or at the restrictive temperature. We even detect a slightly larger radius of constraint for both strains observed (Figure 2.3.C and H).

2.2.4 Chromatin remodelers can alter mobility by themselves - histone acetyl transferases cannot

We find that elongation of transcription is not the direct cause of increased chromatin mobility through recruitment of VP16 and previous experiments also ruled out DNA replication as a source of mobility. We therefore wanted to test other activities related to that transcriptional activator. VP16 has been described to recruit histone acetyl
transferase complexes, different chromatin remodeling activities and components of the basal transcription machinery (Biggar and Crabtree, 1999; Hall and Struhl, 2002; Memedula and Belmont, 2003; Vignali et al., 2000). To distinguish between these two different activities—local change in acetylation levels of histone tails and chromatin remodeling, candidate proteins of each group were tested in the mobility assay. Several in vitro and in vivo studies show that activation and remodeling upon VP16 targeting is accompanied by sequential recruitment of HATs (SAGA and NuA4) and nucleosome remodelers (Brown et al., 2001; Hall and Struhl, 2002; Memedula and Belmont, 2003; Neely et al., 2002). Interestingly, Gcn5 which is a co-activator of GAD and VP16 has been shown to be required for remodeling by VP16 only (Stafford and Morse, 2001). We therefore tested if Gcn5 alone could promote increased chromatin dynamics upon recruitment to the ATG2 locus; however, we observed no significant changes in MSD plots (Figure 2.4.A). Quantitative real time PCR confirms an increased transcript level implying that the HAT locally activates transcription (Figure 2.4.G-H)). Supporting the above observation, the targeting of histone deacetylase Rpd3 (which prevents recruitment of SAGA and Swi/Snf) also did not change mobility significantly (Supplementary Figure 2.2.A; Deckert and Struhl, 2002).
Figure 2.4. Chromatin modifiers and locus mobility at RPL9A locus (map). A. Effect of snf2 disruption on chromatin mobility; Average MSD plots. The inlayed boxes shows a blow-up of the first 8 time points (snf2Δ LexA- n=6, snf2Δ LexA-VP16 n=9).

B. Ino80 targeting and chromatin mobility. Average MSD plots (LexA-: n=11; LexA-Ino80: n=9). C. Table with D (based on the slope of the first 8 data points), R_C (calculated from the maximal MSD value) and the number of large rapid steps (>0.5 μm / 10.5 s) per 5 minutes corresponding to graphs A and B.

Memedula and Belmont (2003) have shown that VP16 not only recruits HAT but also SWI/SNF in order to reposition nucleosomes. There is also biochemical evidence that VP16 can reorganize chromatin independently of Swi/Snf or ATP suggesting that is has other effects. To test the dependence on Swi/Snf we performed the mobility assay on snf2 disrupted cells. Indeed, snf2 disruption in the absence of VP16 led to a decrease in diffusion coefficient, but the radii of constraint remained unchanged.
Local recruitment of VP16 in the snf2 strains on the other hand did induce a similar increase in mobility as observed for the wt control. Consistent with a Swi/Snf-independent chromatin remodeling activity demonstrated for VP16, we note that Swi/Snf is also not required for the increased mobility upon VP16 targeting. The ambiguity of disruption assays is that one cannot exclude a role for redundant activators. We therefore next tested whether direct recruitment of a chromatin remodeling complex is sufficient to increased chromatin mobility. This was performed with the catalytic subunit of the Ino80 complex, called Ino80. The Ino80 complex is known to affect expression of ~1000 yeast genes (>1.5x change in expression) throughout the entire genome (Shen et al., 2000; van Attikum et al., 2004). Furthermore, in an in vitro assay recombinant Ino80 facilitates chromatin remodeling by targeted VP16 in an ATP dependent manner (Shen et al., 2000). LexA-Ino80 was recruited to the RPL9A and ATG2 locus and the mobility was scored as described above. Here we observe a significant increase of the radius of constraint as well as an increase in large rapid steps to almost the level of observed for VP16 recruitment (Figure 2.4.E and F). To determine if increased mobility is a reflection of the activity of the Ino80 complex, we targeted the Arp8 subunit of the complex and analyzed its effect on locus mobility. The actin related protein Arp8 is an unique component that is known to recruit Ino80 complex and remodel nucleosomes (Shen et al., 2003). Indeed, the change in mobility upon targeting Arp8 was nearly identical to that observed upon Ino80 recruitment (Figure 2.5.A and B).
**Figure 2.5.** The Ino80 complex increases chromatin mobility, depending on its enzymatic activity. A. and B. LexA- and LexA-Ino80 are repeated from 2.4 B. (LexA-: n=11; LexA-Ino80: n=9; LexA-Ino80^K737A: n= 10; LexA-Arp8: n=6) C. Map of RPL9A locus. D. Table with D (based on the slope of the first 8 data points), R_C (calculated from the maximal MSD value) and the number of large rapid steps (>0.5 μm / 10.5 s) per 5 minutes corresponding to graphs A and B. E. Relative transcript levels of the RPL9A upon targeting of LexA-, LexA-VP16 and LexA-Ino80, LexA-Ino80^K737A, and LexA-Arp8 respectively. All experiments were performed in triplicate and normalized to ACT1 expression.

To ascertain that the ATPase activity of the Ino80 complex is required for the higher mobility of the RPL9A locus, we targeted a form of Ino80 carrying a point mutation in the ATP binding pocket of Ino80 (lysine 737 is substituted by alanine). This point
mutant is still able to bind the other subunits of the Ino80 complex but is not able to remodel chromatin (Shen et al., 2003). Recruitment of this mutant Ino80 (Ino80K737A) does not show an elevated radius of constraint and large rapid steps which was observed for Ino80 and Arp8 (Figure 2.5.A and B). This suggests that ATP dependent chromatin remodeling can lead to increased chromatin mobility. This correlates with the general requirement for energy for chromatin mobility (Gartenberg et al., 2004; Heun et al., 2001b).

2.3 Discussion

We have described the mobility of internal loci on yeast interphase chromosomes by live fluorescence microscopy with high precision. Based on single particle tracking and on simulations of random walks in a confined volume, we were able to define the constraints exerted by the chromatin fiber (Figure 2.1). Our analysis is based on mean square displacement analysis and the following critical parameters: diffusion coefficient (D), large rapid directional movements and the radius of constraint which defines a subvolume a given locus explores during a given time interval. We find that local recruitment of the transcriptional activator VP16 and of the Ino80 chromatin remodeling complex significantly increases all three parameters of locus mobility (Figure 2.2, 2.4.E and 2.5). Importantly, the Ino80 induced mobility increase is largely dependent on its ATPase activity (Figure 2.5A and B). Elongation by RNA PolII however has no influence on locus mobility, suggesting an involvement of processes prior to transcription elongation (Figure 2.3).
2.3.1 VP16 and Ino80 alter locus mobility

The order of events in transcriptional activation by VP16 has been studied extensively. It consists of the sequential recruitment of Swi2/Snf2 and HATs and basal transcription factors to target promoters. We therefore disrupted Swi2/Snf2 in order to see if a) VP16 targeting would still lead to increased chromatin mobility and b) if its disruption would lead to a change in general chromatin mobility. The increase in mobility upon VP16 targeting was also observed in the \textit{snf2} mutant strain, a finding which correlates with biochemical data showing that VP16 can reorganize nucleosomes independently of Swi2/Snf2, several other chromatin remodelers and even ATP (Robinson and Schultz, 2005). Interestingly, unlike all other general properties of movement, the diffusion coefficient over short time intervals was 25% smaller in the mutant strain than in wildtype, an effect which is also seen when VP16 is targeted (Figure 2.4.C-D). Therefore we can speculate that Snf2-activity directly or indirectly contributes to short range chromatin mobility in the nucleus, but not to VP16-coupled activation. On the other hand Swi2/Snf2 is clearly not required for the increase in mobility upon VP16 targeting.

The Ino80 chromatin remodeling complex was shown to facilitate in vitro remodeling induced by VP16 and further changes transcription levels of many genes (Shen et al., 2000; van Attikum et al., 2004). Therefore two unique subunits, Ino80 and Arp8, were tested in the locus mobility assay (Figure 2.5.A-D). Indeed, both of them showed increased diffusion coefficients, radii of constraint and large rapid displacements. This raised the question if indeed the enzymatic function of the Ino80 complex was involved. Targeting of Ino80$^{K737A}$, a mutation eliminating ATPase function, indeed abolished the mobility shift. This is the first indication that chromatin remodeling can change locus mobility observed by fluorescence microscopy and sheds light on new
aspects of their functions in transcription or DNA repair. Previous experiments which showed energy dependence of chromatin movement substantiate this observation. Depletion of energy by the use of the protonophore CCCP drastically reduces movements of chromosomal loci and chromatin rings (Gartenberg et al., 2004; Heun et al., 2001b). Obviously, this harsh treatment affects all energy dependent processes of the cell which also contribute to the effect of reduced mobility. It is important to state that the nucleoplasmic viscosity was not generally increased because mobility of free GFP measured by FRAP remained unaffected by the CCCP treatment (FN unpublished results). A similar effect has been described in mammalian cells, where chromatin compacts upon energy depletion (Shav-Tal et al., 2004). It is suggested that upon energy depletion chromatin condenses forming a sieve in which small molecules can still freely diffuse, whereas larger molecules get trapped.

### 2.3.2 Transcription elongation does not change chromatin mobility

We present three lines of evidence showing that active transcription does not change local chromatin mobility as it can be observed by fluorescence microscopy. First, general inhibition of transcription - by treatment of the cells with 6-AU or by a temperature sensitive PolIII subunit - did not change chromatin mobility significantly (Figure 2.3). Secondly, we locally targeted two transcriptional activators and monitored transcriptional activation and mobility. While VP16, the Gal4 activation domain and the HAT Gcn5 lead to elevated transcript levels, only VP16 was able to shift locus mobility (Figure 2.2.A-B and 2.4.A-B). And third, we see an increased mobility upon local targeting of two components of the Ino80 chromatin remodeling complex, although we do not detect any activation of transcription. These findings correlate with observations in mammalian cells, where VP16 could induce
decondensation and relocalization of a heterochromatic region in absence of transcription. Upon inhibition or massive reduction of transcription using alpha-amanitin or by targeting truncated VP16 with minor transcription activation activity decondensation was still observed (Carpenter et al., 2005; Tumbar et al., 1999). It is probable that decondensation, as well as increased chromatin mobility, are early steps involved in transcriptional activation which here could be separated from active transcription. Interestingly, we have observed that the Gal4 activation domain (GAD) did not change locus mobility. Several differences in the mode of activation could account for this difference: unlike GAD, VP16 is stabilizing a reinitiation complex (containing general transcription factors and mediator) at promoters (Yudkovsky et al., 2000). A stabilized reinitiation complex facilitates high transcription rates and could be required to observe an increase in chromatin mobility. Furthermore, the fact that VP16 can reposition nucleosomes independently of ATP, several chromatin remodelers and SAGA could further account for distinct modes of action of both activators (Robinson and Schultz, 2005).

2.3.3 Does increased locus mobility correlate with large scale chromatin decompaction and repositioning?

The finding that local chromatin mobility can be altered through targeting a few molecules can provide important insights to the mechanism how changes in large scale chromatin structure could be achieved in higher eukaryotes either during cell cycle progression or gene activation (Csink and Henikoff, 1998; Li et al., 1998; Tumbar et al., 1999). As discussed above, it is possible that the decondensation and delocalization of large heterochromatic chromosome regions in higher eukaryotes is caused by the same mechanisms which also lead to increased local chromatin
movement in yeast. Whether or not targeted chromatin remodelers, will lead to decompaction and repositioning of heterochromatin in higher eukaryotes, still has to be seen. Contrary to these experiments, we do not observe any significant difference in the size of the LacI-GFP signal, even though also in yeast, active chromosomes seem to be ~40-fold compacted, in average to a structure similar to the 30 nm fiber (Bystricky et al., 2004). This could be due to the huge size differences of the lac operator arrays. We can therefore not exclude that also in yeast increased mobility could lead to further opening of this fiber. This could again facilitate the contact to subnuclear structures required for transcription, i.e. it could facilitate looping out of activated genes from a chromosome territory (Chambeyron and Bickmore, 2004; Osborne et al., 2004). Interestingly, two genes which require Ino80 for their full activation (INO1 and GAL1) are localized to the nuclear periphery when activated (Brickner and Walter, 2004; Casolari et al., 2004). In order to carefully study directional movements e.g. along or in the direction of the nuclear envelope, analysis has to be performed in 3D and not on 3D-projections. For that a better resolution along the optical axis (which is achieved through an increase of the number of z-sections) is essential (Supplementary Figure 2.1.B). Therefore, further studies will have to investigate if Ino80 or VP16 targeting lead to any directional movement towards or along the nuclear periphery.

### 2.3.4 Mobility of chromosomes in the interphase nucleus

As shown by Davis and collaborators, locally and on short time scales, chromatin mobility can be directly related to the strength of the DNA-nucleosome interaction (Davis and Bardeen, 2004). On longer timescales, there are additional constraints imposed on the chromatin fiber. We could show that a movement of a free chromatin ring can be described by the simple model of a random walk in a constrained volume.
Contrary to the free ring, mobility of a chromosomal locus does not fit this model (Figure 2.1). The diffusion coefficients are below the expected values which indicates additional degrees of constraint. Well described examples of additional constraints are illustrated by the comparison of the centromere proximal \textit{LEU2} locus or subtelomeric loci with internal loci as described here (Hediger et al., 2002; Marshall et al., 1997). There the anchoring to the spindle pole body or to the nuclear envelope constrains movements even more. Mobility changes of a locus during cell cycles has first been described by Heun et al. for internal replication origins which showed reduced mobility in S-phase (Heun et al., 2001b) – an observation which has been also described for internal loci distal to origins of replication (FN unpublished observation). We assume that reduced chromatin mobility is at least partially a general property of the nucleus during S-phase.

Interestingly diffusion of chromatin below the time resolution used here seems to be even faster. The facts that the MSD plots do not increase linearly and that a linear regression intersects with the x-axis presume these faster movements (see small boxes in figure 2.2.A). Indeed these faster movements have been quantified by FCS measurements and reinforce the above hypothesis (L.Gehlen and J.Langowski, unpublished results). Generally, the variability of published diffusion coefficients can be due to different local chromatin structure, compaction or anchoring but also due to different time scales or techniques.

These general properties of chromatin mobility seem to be overlaid by local changes in dynamics as described above. If increased local chromatin mobility is indeed facilitating a specific nuclear function remains speculative. Future experiments will address the question if the mobility increase underlying the Ino80 complex favors transcription activation of specific genes or repair by the non homologous end joining
machinery and if changing chromatin dynamics is a generally mechanism of chromatin remodelers at work.

### 2.4 Materials and methods

#### 2.4.1 Growth conditions and media

Yeasts were grown in minimal and rich media (SD, YPD) as described in (Guthrie 1991). The use of alternative carbon sources (Raffinose, Galactose (Fluka) is indicated in the text. 6-Azauracil (6-AU; Company) was dissolved in water and used at a 50 μM final concentration.

#### 2.4.2 Microscopy

Movies were acquired as described in (Hediger Methods in Enzymology). Cells were either mounted on a depression slide (Milian SA, Cat. No. CAV-1) upon 1.4% agarose (Eurobio Cat. No. 018645) containing minimal medium with 4% glucose (Fluka) or in a liquid cell observation chamber in the corresponding growth medium (Ludin chamber, Life Imaging Services, Reinach (BL), Switzerland) by fixation on a 18mm coverslip treated with 10 μl of Concanavalin A dissolved to 1mg/ml in H₂O (ConA, Sigma, Cat. No. C-0412). Confocal microscopy can be performed on a Zeiss LSM510 Axiovert 200M, equipped with a Zeiss Plan-Apochromat 100x/ NA=1.4 oil immersion objective. The stage was equipped with a hyperfine motor HRZ 200. Temperature was stabilized to 25°C, 30°C or 37°C using a temperature regulated box surrounding the microscope (The Box, Life Imaging Services, Reinach (BL), Switzerland). The settings for the Zeiss LSM510 were as follows: laser: Argon/2 458, 488 or 514 nm; tube current 4,7 Amp. Output 25%; filters: Channel 1, Lp 505;
channel settings: Pinhole 1 – 1.2 Airy unit (corresponding to optical slice of 700 to
900 nm); detector gain: 930 to 999; amplifier gain: 1-1.5; amplifier offset: 0.2V–0.1
V; laser transmission AOTF = 0.1 - 2 %; scan settings: speed 10 (0.88 μs/pixel), 8 bits
one scan direction, 4 average/Mean/Line; Region of Interest (ROI): < 40x36 pixel;
zoom 1.8 (pixel size: 100 x 100 nm); imaging intervals: 1.5 s; z-settings: 6 optical
slices at 450 nm spacing. In order to use minimal laser transmission the pinholes were
regularly aligned. Typically one cell was monitored for 7 minutes, after which cells
were still able to grow with normal kinetics for more than one generation. Statistical
significant results were obtained from > 50 min of movies per condition. Experiments
were generally performed on at least two different days.

2.4.3 Movie tracking and analysis

3D-time lapse movies were processed using a maximal projection in z-dimension in
the LSM software (Zeiss) and exported as TIFF image. In these projected time series,
the GFP-signal of the tagged locus was tracked using the Spottracker plug-in (Sage et
al., 2003) for the public domain software ImageJ (Rasband NIH). The specific
settings in the Spottracker plug-in were: Cone Aperture: 5; Normalize Factor: 80;
Center Constraint: 20-25; Movement Constraint: 20; active subpixel resolution. The
coordinates of the tagged locus and the center of the nucleus were further processed
using a notebook programmed in Mathematica 4.1 (Wolfram Research) to calculate
the distribution of step sizes, the number of large steps (defined as displacements
larger than 500 nm in 10.5 s per 5 minutes) and the MSD plots with the corresponding
errors (standard error of the mean). For legibility, error bars are only shown in
figure 2.1 and 2.2. The average length of the movies taken (450 time points in
7.5 min) determines the interval in which MSD plots meaningful to maximal time
intervals of 150 s. The calculation of the diffusion coefficient (D) and the radius of
constraint \((R_C)\) are based on the MSD plots. For the time-dependent diffusion coefficient, a time scale between 1.5s and 12s was defined since it is the best compromise to be still in the linear range of the curve and to be in the robust range of the curve. It was calculated by making a linear regression through the first 8 time points. The error is shown as the standard deviation of the mean from \(D\) of all individual movies of a given condition. The radius of constraint is a theoretical value derived from the plateau of the MSD plot. For our geometry, i.e. projected 3D time lapse, the plateau of the MSD \(= 4/5 \ R_c^2\) (personal communication, Jonas Dorn, Scripps La Jolla, CA, USA). Even though some MSD plots do not reach a plateau within the shown time interval, in this paper we define \(R_c\) as the radius of constraint corresponding to the highest value measured in an MSD graph.

### 2.4.4 Random walk simulations and calculations

Simulations of random walks in a constrained volume were programmed in Perl (www.perl.org). To reduce border effects, a step size of 10 nm was used. A number of 120 10 nm steps corresponds to the measured average step size of 100 nm per second. The radius of constraint was set according to the measured maximal MSD value.

GNUplot (http://www.gnuplot.info) was used to fit the experimental curves, which were found to fit the function \(<d^2>(\Delta t) = a*1-e^{-\Delta t/\tau}\), where \(a\) is the plateau level of the MSD plot \(<d^2>(\infty)\). In order to find a correlation between MSD plots derived from data in 3D and from 3D-projections, the connection for free random walks was calculated. Both curves differ by a factor of 1.5, a value which could be shown experimentally to apply also for constrained random walks.
2.4.5 Quantitative real-time PCR

mRNA was extracted using RNasey kit (Qiagen) from a 5 ml culture grown in 50 ml Falcon tubes to an OD$_{600}$ of 0.4 – 0.6. RNA concentrations were determined using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies).

Reverse transcription was performed on 200 ng of mRNA using the i-script reverse transcriptase (Biorad). cDNA was quantified by real-time PCR using the Perkin-Elmer ABI Prism 7700 Sequence Detector System as described (Cobb et al., 2003). All experiments were performed in triplicate and normalized to the $ACT1$ message (Schawalder et al., 2004). Individual experiments were normalized to the LexA-expressing control strain. Sequences of the primers/probes that amplify $RPL9A$ and $ATG2$ regions in the $S. cerevisiae$ genome are available upon request.

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**Supplementary Figure 2.1.** Comparison of data evaluated in 3D and on 3D projections on the LYS2 chromatin ring. **A.** 6 movies of the LYS2 ring (45 min) have been tracked in 3D (red) and in 2D obtained by a projection in z-dimension (3D projection, blue). The corresponding MSD curves and standard errors of the mean are plotted. **B.** Kymograph presentations of a representative a 7.5 minute movie tracked in 3D. **C.** The ideal MSD plots were generated from coordinates (in 3D or 3D projections) obtained by simulations of random walks in a confined volume and were based on the measured step size and radius of constraint (step size: 200 nm, RC=880 nm). The difference between 3D and projected 3D MSD values equals 1.5.
Supplementary Figure 2.2. Targeting of histone deacetylases Rpd3 does not change chromatin dynamics. A and B. MSD plots (LexA-: 8 movies, 54 min; LexA-Rpd3: 11 movies, 82 min) and corresponding characteristic values are shown in C.
3. SILENT CHROMATIN AND LOCUS MOBILITY

The following chapter is based on a very intensive collaboration with Marc Gartenberg (UMDNJ, Piscataway, NJ, USA). He initiated the work, was responsible for all strain construction and carried out initial experiments. All live microscopy was performed by me, while immunofluorescence images were acquired by Thierry Laroche. Marek Blaszczzyk helped with the statistical analysis of locus mobility. All methods are described in more detail in Chapter 5.

The paper is an important contribution to the understanding of chromatin dynamics in *S. cerevisiae*. In agreement with (Taddei et al., 2004), we show that anchoring of silent chromatin depends on the anchoring via Esc1 and yKu70. Our data further support the model that silencing is established and maintained by nucleation through binding of a few Sir proteins. When the local concentration of silencing factors is high enough, the recruitment of more Sir complexes and their spreading along the chromatin fiber is favored. In the wildtype situation, this positive feedback loop takes place in the telomeric foci where pools of Sir proteins lead to variegated silencing of subtelomeric regions (TPE). In a mutant where Sirs are delocalized from telomeric foci, TPE is lost. In this situation even weak internal silencers can attract sufficient levels of Sir proteins in a way that also at this locus silencing can be maintained or established (Taddei et al., 2005).

In an Esc1/yKu70 double mutant TPE is lost and Sir proteins are dispersed throughout the nucleus. If in these conditions, a ring of silent chromatin is excised, we observe very high mobility although its silent status is maintained.
Sir-Mediated Repression Can Occur Independently of Chromosomal and Subnuclear Contexts

Marc R. Gartenberg, Frank R. Neumann, Thierry Laroche, Marek Blaszczyk, and Susan M. Gasser

Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

The Cancer Institute of New Jersey, New Brunswick, New Jersey 08901

Department of Molecular Biology and Frontiers in Genetics NCCR Program, University of Geneva, 30 Quai Ernest Ansermet, CH-1211 Geneva, Switzerland

Institute of Applied Mathematics, University of Lausanne, CH-1015 Lausanne, Switzerland

Summary

Epigenetic mechanisms silence the HM mating-type loci in budding yeast. These loci are tightly linked to telomeres, which are also repressed and held together in clusters at the nuclear periphery, much like mammalian heterochromatin. Yeast telomeres anchoring can occur in the absence of silent chromatin through the DNA end binding factor Ku. Here we examine whether silent chromatin binds the nuclear periphery independently of telomeres and whether silencing persists in the absence of anchorages. HMR was excised from the chromosome by inducible site-specific recombination and tracked by real-time fluorescence microscopy. Silent rings associate with the nuclear envelope, while nonsilent rings move freely throughout the nucleus. Silent chromatin anchorage requires the action of either Ku or Escl. In the absence of both proteins, rings move throughout the nucleoplasm yet remain silent. Thus, transcriptional repression can be sustained without perinuclear anchoring.

Introduction

The three-dimensional architecture of the nucleus provides an additional layer of epigenetic control that is superimposed on the regulation conferred by transcription factor binding sites and local chromatin structure. In an increasing number of examples, transcriptional repression can be correlated with a gene's juxtaposition in trans to constitutive heterochromatin (Fischer and Merkel wła, 2002). The molecular mechanisms underlying these spatial arrangements are not yet understood. Real-time microscopy has, nevertheless, provided a robust view of nuclear architecture not only by cataloging the relative positioning of chromosomes, but by documenting the movement of individual domains and the constraints imposed upon their mobility (Marshall, 2002). Chromatin movement has generally been found to exhibit characteristics of an obstructed random walk, meaning that a chromosomal locus moves constant, randomly directed movements within a defined radius of constraint (r; Marshall et al., 1997; Vazquez et al., 2001). The source of this constraint is unknown, but it could arise either from intrinsic viscosity of the nucleoplasm, from association with other chromatin fibers, or possibly from interactions with less mobile proteins.

In all cases reported to date, chromatin mobility was confined to zones of radII far smaller than the nuclear radius. Moreover, the movement of heterochromatin in mammalian cells, and of telomeres and centromeres in yeast, is significantly more restricted than the movement of transcriptionally competent chromatin (Chubb et al., 2002; Heun et al., 2001; Marshall et al., 1997). Yeast telomeric and centromeric sequences appear to be tethered to specific elements within the nuclear envelope that may contribute to their limited mobility (Heun et al., 2001). Analogously, mammalian heterochromatin is often found around nucleoli or near the nuclear lamina or can be clustered in a structure called the chromocenter in Drosophila larvae and other cells. Still, molecular mechanisms for the anchorage of mammalian heterochromatin remain obscure, and it cannot be ruled out that heterochromatin positioning simply reflects its exclusion from transcriptionally active nuclear zones.

In budding yeast, silent information regulatory proteins Sir1-4 are required for transcriptional repression of the silent mating-type loci, HML and HMR, which reside near opposite ends of chromosome 3 (Rusche et al., 2003). A related Sir-dependent form of repression occurs in telomere-proximal sequences of most chromosomes. At the HM loci, Sir proteins are recruited by factors bound to cis-acting sequences termed silencers. At telomeres, Sir recruitment is provided by Rap1 and the DNA repair/telomere binding protein Yku80 (Mancard et al., 1996; Laroche et al., 1998; Mishra and Shore, 1999; Roy et al., 2004). Following recruitment, subsequent spreading of a Sir2,3,4 complex leads to a repressed domain that is more stable at HM than at telomeres.

Telomeres in many lower eukaryotes are grouped together during interphase near the nuclear periphery (Gotta et al., 1995; Scherf et al., 2001). In yeast, Ku plays a central role in this process (Laroche et al., 1998). In cells lacking either heterodimeric subunit of Ku, some telomeres lose this association with the nuclear periphery and become highly mobile while others remain tethered by a Sir4-dependent mechanism (Hediger et al., 2002). Thus, redundant pathways anchor telomeres in yeast. Moreover, targeting either the Yku80 subunit or a subdomain of Sir4 to DNA is sufficient to drag a randomly positioned, internal chromosomal locus to the nuclear periphery (Tscheli et al., 2004). Whereas the nature of the Ku docking sites remains obscure, an associ-
ation between a domain of Sir4 and the perinuclear protein Esc1 (enhancer of silent chromatin 1) is sufficient to ensure such colocalization (Andrulis et al., 2002; Taddei et al., 2004).

RISSH studies have shown that HML resides at or near the nuclear periphery where it colocalizes frequently with telomeric clusters (Laroche et al., 2000). Corresponding studies on the relationship between chromosomal position and silencing have shown that the repression of HML depends in part on its proximity to a telomere in cis (Thompson et al., 1994; Mallet et al., 1996). Synthetic targeting studies by Sternglanz and coworkers also showed that Sir-dependent transcriptional repression could be restored to a crippled silencer by tethering the locus artificially to the nuclear envelope (Andrulis et al., 1996). In recent experiments, it has been suggested that the effects of perinuclear anchorage on transcriptional repression, but the simplest is that silencing of the HM loci benefits directly from the elevated local concentration of Sir proteins found at telomeric clusters (Mallet et al., 1996). In conflict with this notion, however, Sir protein overexpression did not obviate the need for the membrane anchor in this targeted silencing assay (Andrulis et al., 1998), and Sir3 foci were shown to persist in mfd1 mfd2 double mutants, although targeted repression was compromised (Feuerbacher et al., 2002; Hediger et al., 2002b).

Here we explore the mechanisms that control the nuclear localization of silent chromatin using HMR as a representative silenced domain. To understand the behavior of silent chromatin on its own, we uncouple the mating-type locus from its neighboring telomere by inducible recombination. We find that rings of silent chromatin possess an intrinsic ability to associate with the nuclear periphery via Ku and Esc1. Quantitative live imaging shows that nonsilent chromatin rings diffuse rapidly and randomly throughout the nucleoplasm. We conclude that both protein-mediated anchorage and linkage to an intact chromosomal fiber restrict motility of chromatin in an additive fashion. Surprisingly, we find that disruption of both the Ku and Esc1 perinuclear anchors produces highly mobile chromatin rings that remain silent. We conclude that transcriptional silencing can persist without perinuclear attachment.

Results

Extrachromosomal Rings of Silent Chromatin

Localize to the Nuclear Periphery

Heterochromatin in many species appears to cluster underneath the nuclear envelope, particularly in differentiated cells. Given the potential importance of telomere positioning for chromosome stability and regulated gene expression in yeast (Ai et al., 2002; Haime et al., 2004), we were prompted to investigate whether a discrete domain of silent chromatin can anchor itself to the nuclear periphery.

HMR is situated sufficiently close to the right end of Chr 3 (~5 kb) that anchoring of Tel 5R would readily mask or override any intracellular positioning information intrinsic to the locus at the resolution of light microscopy (~30 kb; Bystricky et al., 2004). To overcome this limitation, we used inducible site-specific recombination to uncouple the locus from its normal chromosomal context (Cheng et al., 1998). The region containing HMR, including its silencers, was flanked by a pair of target sites for recombination on the R site-specific recombinase. An array of 256 lac operators (lacO) was inserted between the right silencer, HMR-I, and the proximal recombination site (Figure 1A). Excision, which was 95% complete 2 hr after the induction of recombination, produced a transcriptionally silent 16.8 kb HMR-derived ring that bound a coexpressed lac repressor-GFP fusion (Cheng et al., 1998 and data not shown; see Figure 6). The nuclear envelope, visualized by tagging a nuclear pore protein Nup49 with GFP, served as an internal frame of reference for monitoring HMR position (Figure 1B). By tracking the locus in real time with live fluorescence microscopy, the impact of both silent chromatin and chromosomal context on HMR localization could be assessed.

Three-dimensional (3D) focal stacks were collected on living cells from four strains that differed by the presence or absence of a galactose-inducible R recombinase and a functional SIR3 gene (Table 1). All strains were cultured in galactose under identical conditions. HMR positions were quantified by measuring the shortest distance between the center of the HMR focus and the nuclear envelope in the focal plane that contained the strongest lac-GFP signal. To account for variations in nuclear size, each distance measurement was normalized to the nuclear diameter (measured by midplane signal) measured in the plane of focus (Hediger et al., 2002a).

The cell cycle stage was determined for each cell, yet G1 and S phase nuclei yielded similar results and were therefore pooled. G2 phase cells were excluded due to a limited sample size. HMR foci were scored for their positions relative to three concentric zones of equal surface (Figure 1B), with zone I defining the outermost ring. Any deviation from randomness (33% per zone) was tested for statistical significance.

The spatial distribution of the chromosomal locus (unexcised) was determined in the strain lacking recombinase. In 91% of the cells, HMR was found within zone I, reflecting an exceptionally high enrichment of the silenced chromosomal domain at the nuclear periphery (Figure 1C). In a silencing-defective sir3 deletion strain, the perinuclear fraction of HMR remained at 91%, indicating that a silencing-independent mechanism is able to anchor the right end of Chr 3. This result is consistent with the finding that numerous telomeres (Tel 5R, Tel 8L, Tel 14L, and truncated Tel 5R) remain perinuclear in sir-deficient strains, confirming that there are silencing-independent mechanisms for anchoring chromatin in yeast (Hediger et al., 2002a; Thurm et al., 2001). When HMR was uncoupled from the chromosome, the silent excised ring was found within zone I in 86% of the cells (Figure 1C), while excision in a sir3 deletion strain produced a ring that distributed throughout the nucleoplasm (45% in zone I and 36% in the innermost zone III; Figure 1C). This demonstrates that perinuclear enrichment of HMR is an intrinsic property of the locus, achieved without direct coupling to the neighboring telomere. Moreover, immobilization of HMR at the nuclear periphery requires Sir3-containing chromatin. HMR anchorage is therefore distinct from telomere anchorage, which persists in the absence of silencing due to Yku (Gotta et al., 1996; Hediger et al., 2002a; Thurm et al., 2001).
Figure 1. Influence of Silencing and Chromosomal Linkage on HMR Localization in Living Yeast Cells

(A) Experimental Design. Site-specific recombination at engineered target sites (RS sites) uncouples the lac-replicated HMR locus from the chromosome. The strains carry lac-GFP, Nup99-GFP, and a galactose-inducible R recombinase.

(B) An equatorial section of GFP fluorescence. Distances between the lacGFP-tagged HMR spot and Nup99-GFP at the nuclear envelope (5) are normalized to the nuclear diameter (g) and binned according to three zones of equal surface area. In a 2 μm diameter nucleus, zone I corresponds to the outermost ring of ~100 nm width.

(C) Distribution of HMR in the three zones is plotted as a percent of the total number of cells counted (p) for each strain. Red bar at 90% represents an idealized random distribution, p values are calculated by y2 analysis comparing actual values to a hypothetical random distribution.

Strains used: MRG2251 SIR+/chromo, MRG2251 sir3/chromo, MRG2249 SIR+/ring, MRG2250 sir3/ring (Table 1).

Silencing Imposes Constraints on Intracellular Chromatin Motion

In still images, chromosomal sequences that appear to be enriched at the nuclear periphery are not necessarily immobile. Most telomeres, for example, undergo dramatic movements along the nuclear envelope without leaving the perinuclear space defined by zone I (Hediger et al., 2002a). To determine the degree of constraint on the motion of silent chromatin rings, we used laser scanning confocal microscopy to capture rapid time-lapse images of nuclei in G1 cells. Three-dimensional stacks (6 × 0.45 μm focal sections) were obtained at 1.5 s intervals (3D + time = 4D movies) and then projected onto a single plane for analysis. Here, the Nup99-

### Table 1. Strains List

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<th>Name</th>
<th>Designation</th>
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<th>Genotype</th>
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<tr>
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<td>β2::ring</td>
<td>GA2627 [LEU2::GAL1::RS]Δlep2-2,112</td>
<td>–</td>
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</tbody>
</table>
GFP fusion was replaced with a Tet repressor-GFP fusion that produces a uniform, low-level nucleoplasmic fluorescence. The calculated center of the tet-GFP signal intensity (i.e., the nuclear center of mass) provides an internal reference point from which the coordinates of lac-GFP-tagged foci can be measured accurately with a novel semiautomated spot tracking software package (SpotTracker, http://bigwww.epfl.ch/spotsoft/spottracker). This frame of reference removes noise due to the translational movement of cells. Moreover, FRAP studies (fluorescence recovery after photobleaching) indicate that nuclear rotation is not a significant factor over the timeframe of our observations (K. Bystricky et al., submitted).

For each of the primary strains, multiple 5–7.5 min 4D movies totaling ≥65 min (≥2600 frames) were obtained. Simple visual inspection of the movies showed that the nonsilent HMR ring is highly mobile relative to the locus in the other three strains (see Supplemental Movies S1–S7 at http://www.cell.com/cgi/content/full/119/7/955/DC1/). Representative images from a time lapse series of the ring in Sir + and sir3-deficient cells are shown in Figure 2, where a red trace is used to indicate the locus’ trajectory over 108 s. Even within this short time interval, the nonsilent ring makes frequent large steps, sampling a larger fraction of the nuclear volume than the silent ring. This is illustrated with a graphical treatment of the time lapse data in which the sites sampled by the tagged HMR locus over a representative 5 min period are projected onto a single plane (Figure 3A). For each movie, we calculated the minimum percentage of the total surface that encloses 95% of the sites (see Figure 3A, red oval; Experimental Procedures). The average value over all movies for each strain is indicated as a surface coefficient. The nonsilent ring exhibits the highest mobility, sampling on average 68% of the projected nuclear surface, while the silent chromosomal locus is the most constrained, sampling only 17%. The silent ring and the nonsilent chromosomal locus yield nearly equal intermediate values, confirming other mathematical treatments of this data (see MSD below). Thus, HMR dynamics are constrained in an additive manner by silent chromatin and by HMR’s presence in a contiguous chromosomal fiber.

The data sets were further characterized in terms of diffusion coefficients, step sizes, and average velocities (total track length divided by elapsed time). Data summarized in Supplemental Figure S1 on the Cell website show that the nonsilent ring not only exhibits less spatial constraint, but moves with larger step size and higher velocity, with maximal values of ~32.97 μm/min. This exceeds the values reported for other chromosomal sites in either human, fly, or yeast cells (Chubb et al., 2002; Heun et al., 2001; Marshall et al., 1997).

Assuming that chromatin movements in interphase nuclei resemble a random walk, the most robust method to determine the spatial constraints imposed on this movement is an analysis of mean square displacement (MSD; Marshall et al., 1997; Vazquez et al., 2001). The MSD plot describes a linear relationship between the square of the distance traveled by a particle (<ΔΦ2>) and increasing time intervals. Deviation from linearity, manifest as a plateau, provides a measure of spatial constraint (i.e., obstructions to the free movement of the locus) on otherwise unconstrained diffusion (see Figure 3B, for example). The height of the plateau reflects the volume in which the locus’ movement is restricted, which is directly dependent on the radius of constraint (r; Supplemental Data on the Cell website).

Furthermore, the slope of the MSD curve is proportional to the diffusion coefficient, which decreases with increasing r due to spatial constraints on the particle dynamics.

MSD analyses for HMR displacement were averaged over all time lapse movies of each strain and reach plateaus at larger time intervals (Figure 3B). The nonsilent ring moves with the least constraint; its MSD value reaches a plateau at 0.5 μm², which represents an r of ~0.8 μm. This value approaches the average nuclear radius of haploid yeast cells (0.9 μm). Thus, comprehensive quantitative analysis confirms that the nonsilent ring moves freely throughout the entire nuclear volume. Similar values were obtained for the ring in a strain lacking sir4. For the silent ring and the nonsilent chromo-
somal locus, on the other hand, \( r = 0.60 \) \( \mu \)m and 0.65 \( \mu \)m, respectively, while that of the silent chromosomal HMR locus is \( \approx 0.48 \) \( \mu \)m. This value is lower than that of an active chromosomal locus found in the middle of Chr 2 (LYS2, \( r = 0.65 \) \( \mu \)m, see below) but is comparable with the constraint on telomere-proximal tags, which range from 0.43 to 0.45 \( \mu \)m (values for Tel 3L, 6R, and 6L; K. Bystričky et al., submitted).

The hierarchy of spatial constraints for HMR locus mobility is also reflected in the frequency with which the tagged locus makes large directional movements, defined arbitrarily as movements spanning at least 0.5 \( \mu \)m within 10.5 s (Heun et al., 2001). These large steps are quantified over the entire time lapse data set and show that loss of either silencing or chromosomal linkage increases the average frequency of large steps from five to seven per 5 min. In contrast, the nonsilent ring averages 15 to 19 large steps over the same interval (see sir3 and sir4 ring data, Fig. 3C). The higher mobility of the ring in the sir4 null relative to the sir3 null may reflect the residual binding of Sir4 to HMR silencers in the absence of Sir3 (reviewed in Rusché et al., 2003). This higher mobility is also reflected in the different slopes of the MSD plots for sir4 and sir3 rings (Fig. 3B).

Chromatin Ring Movement
Is a Facilitated Process
The driving force behind interphase chromatin motion is unclear. Such movement has been described previously as “Brownian-like” due to an absence of long-range directionality (Marshall et al., 1997), yet previous analysis suggests that large chromatin steps are energy
dependent (Hou et al., 2001). In budding yeast, the depletion of intracellular ATP levels can be achieved by either natural glucose deprivation at high cell densities or incubation with carbonyl cyanide m-chlorophenylhydrazone (CCCP), a protonophore that collapses both mitochondrial and plasma membrane potentials. CCCP exposure, like depletion of glucose, eliminates large movements, although steps ≤0.2 μm persist and possibly reflect residual Brownian motion (Hou et al., 2001).

To ask whether the uncoupling of HMR from the chromosome relaxes the dependence of chromatin movement on the energy status of the cell, we performed time lapse imaging and quantitative analyses on the non-silent HMR ring in the presence of CCCP, or its solvent DMSO. Surprisingly, the increase in the frequency of large movements that characterizes the non-silent ring is abolished when ATP is depleted by CCCP (Figure 3C). Significant reductions occur in both the slope (diffusion coefficient) and the height of the plateau (r² drops from 0.8 to 0.45 μm). We conclude that the rapid dynamics of the extrachromosomal ring depend on the energy status of the cell, which may reflect a general change in chromatin distribution within the nucleus (Shav-Tal et al., 2004).

The rapid, unconstrained movement of the chromatin ring in the silencing-deficient strains is unlike any in vivo mobility reported for chromatin to date and is particularly remarkable considering the mass of the particle (at least 38 MDa). To rule out that these dynamics reflect some general effect of sir3/sir4 deletion on nuclear structure, we generated a non-silent ring in a sir² strain by excising a portion of the transcriptionally active LYS2 gene from its native position on Chr 2 (Figure 4A). Visual inspection of movies indicates that unlinking LYS2 from the chromosome dramatically increases its mobility (see traces in Figure 4B). We note that the LYS2 ring moves throughout the nucleoplasm in an energy-dependent manner, with a radius of constraint approaching the nuclear radius, very similar to that of the HMR ring in the sir² background (see MSD analysis, Figure 4C). In contrast, the chromosomal LYS2 locus moves within r² = 0.65 μm, which agrees closely with the r² value of the non-silent chromosomal HMR locus. Movement of the LYS2 ring is not likely to reflect transcriptional elongation, since the excised fragment lacks the LYS2 promoter. This result indicates that the rapid, unconstrained mobility of the non-silent HMR ring is not an artifact of sir deficiency, but instead reflects the true mobility of chromatin fragments unhindered by linkage to the chromosome. We conclude that both silent chromatin and linkage to the chromosome limit the ability of chromatin to diffuse freely throughout the nucleus, to nearly equal degrees.

Silent Chromatin Rings Colocalize with Telomere Clusters

The finding that HMR is highly enriched at the nuclear envelope raised the possibility that the silent loci can associate with telomeric foci in trans. To address this question, we measured the extent of colocalization between telomeric foci visualized by Sir4 immunofluorescence and the lac-GFP signal of HMR (Gotta et al., 1996; Laroche et al., 2000). Figures 5A and 5B show that the excised locus coincides with a telomeric cluster in 39% of the nuclei, and the two are immediately adjacent in an additional 33%. Comparable values were obtained in the absence of excision, when HMR and its neighboring telomere were physically linked in cis or when Sir4 colocalization was scored for a control strain carrying a GFP-tagged telomere (Tel14L). In contrast, the colocalization of perinuclear clusters of Sir4 with nucleoporins showed only 12% overlap (Taddei et al., 2004), and the overlap of a nontelomeric lac-GFP tagged ARS element was around 14% (Figure 5B). We conclude that the silent HMR ring associates specifically with telomeric clusters that sequester silent information regulatory factors at the nuclear periphery. Since 86% of the silent excised rings are perinuclear but only 67% colocalize with Sir4 foci, the difference may either reflect telomere-independent binding sites for Sir4 rings or rings that localize to Sir4 pools that fall below our threshold of detection.

Ku and Esc1 Immobilize Silent Chromatin through Independent Pathways

Colocalization of silent HMR rings with telomere clusters suggested that their anchoring might involve common perinuclear docking sites (Figures 5A and 5B). The anchorage of yeast telomeres relies on two parallel pathways, one requiring the DNA end binding Ku complex and the other requiring the perinuclear anchor, Esc1 (Hediger et al., 2002a; Andriulis et al., 2002; Taddei et al., 2004). Ku and Esc1 also influence the partitioning behavior of circular plasmids that contain telomeric sequences or a targeted Sir4 domain known as PAD4 (Andriulis et al., 2002, A. Ansari, S. Nagai, S.M.G., and M.R.G., unpublished data). To test whether these proteins also facilitate HMR anchoring, time lapse confocal microscopy was performed with strains lacking either Esc1 or yku70. Diffusion constants, radii of constraint, and the frequency of large steps increase only slightly in both strains, indicating that chromatin anchoring persists in the absence of either Esc1 or Ku (Figures 5C–5E).

When these analyses were extended to the esc1 yku70 double mutant, we found that the excised HMR ring was now completely released from perinuclear anchorage (Figures 5C–5E). Both the frequency of large steps and the radius of constraint on the HMR ring in the esc1 yku70 double mutant were identical to those of the sir- null allele, and the radius of constraint approached the radius of the nucleus (Figure 5C). Thus, genes that provide two parallel anchoring pathways for the Sir4 domain in an artificial targeting assay (Taddei et al., 2004) also constrain the mobility of native silent chromatin on excised rings. Not only does this indicate that Ku and Esc1 provide efficient docking sites for Sir4 when it is incorporated into silent chromatin, but it shows that there are no other chromatin anchors at HMR, a conclusion that could not be drawn from domain targeting assays (Taddei et al., 2004).

Silencing Persists in Chromatin Rings Released from the Nuclear Periphery

Inevitable questions are raised by this analysis: does the esc1 yku70 double mutant compromise HMR silencing when the ring is released from the nuclear periphery? Can silencing persist in the absence of the usual association with telomeric pools of silencing factors? To address this question, we monitored the ability of the esc1
yku70 double mutant to maintain repression of the aT gene at the excised HMR locus by performing Northern blot analysis on strains from which the active MAT locus was deleted. As expected, the chromosomal aT gene was derepressed in the sir3 strain and repressed in the single yku70 and escf1 mutants (Figure 6A). Importantly, however, the gene was also fully silenced in the escf1 yku70 double mutant, not only at its endogenous locus but also when borne on the excised ring. Silencing under these conditions was shown to be Sir4 dependent and to persist at least eight hours after induction of the recombinase (data not shown). From these observations, we conclude that perinuclear tethering is not required to maintain HMR silencing, if the two essential anchors for silent chromatin are eliminated.

Ku helps recruit Sir proteins to telomeres, thereby restricting the ability of these factors to repress genes more centrally located in the nucleus (Misha and Shore, 1999; Roy et al., 2004). In the absence of Ku, telomeric silencing is reduced, but HMR silencing and silencing at internal chromosomal sites improves slightly (Maillet et al., 2001). It was of interest, therefore, to examine how the escf1 deletion affects Sir factor distribution, particularly in a yku70-deficient strain.

As shown above, immunofluorescence localizes the majority of the Sir4 signal to four to eight perinuclear foci, which correspond to clusters of telomeric DNA (Gotta et al., 1996). In both the escf1 and yku70 single mutants, we see that these clusters are preserved, although there is a significantly higher background of diffuse Sir4 staining in cells lacking Ku (Figure 6B; Larroche et al., 1998). In the escf1 yku70 double mutant, however, Sir4 is completely released from telomeres and is found uniformly distributed throughout the nucleoplasm. The results are consistent with the notion that mobilized rings remain silent because the dispersal of telomeric Sir proteins compensates for loss of perinuclear anchorage. Unlike a situation in which Sir proteins are overexpressed, we note no growth defects in the escf1 yku70 double mutant, ruling out indirect effects on nuclear architecture. We thus complement the previous finding that transcriptionally active telomeres can reside at the nuclear periphery (Gotta et al., 1998; Tham et al., 2001) by showing that silent chromatin can persist in the absence of perinuclear association (Figures 6A and 6C).

Discussion

Silent Chromatin as a Determinant of Nuclear Architecture

In organisms from yeast to man, cytological studies suggest that the relocalization of genes to zones enriched
Figure 5. Factors Controlling Association of Silent Chromatin with the Nuclear Periphery
(A) Representative image of silent HMR rings (green) and telomere clusters (red) by multichannel confocal laser microscopy, as described in Laroche et al. (2000). Affinity-purified rabbit anti-Sir4 detects telomere clusters, and direct GFP fluorescence identifies the tagged HMR locus.

Background nucleoplasmic staining (blue) was from coexpressed tet-GFP.
(B) Images as in (A) were analyzed for overlap or juxtaposition of Sir4 and lac-GFP-tagged loci in the indicated number of cells (n). Signals were classified as colocalizing if centers of intensity were <0.3 μm, or classified as adjacent if signals touch but do not overlap significantly. Identical analyses were performed on GFP-tagged HMR in strains MRG2201 Sir1-counter, MRG2197 Sir7-counter, as well as GFP-tagged ARS607 and TEL14L (Best et al., 2002b).
(C) Mean squared displacement analysis of HMR was performed on strains MRG2197 Sir1-counter, MRG2201 Sir1-counter, MRG2214 sir1-counter, MRG2205 sir1-counter, and MRG2227 sir1-counter. The slope of this plot, $\langle \Delta x^2 \rangle / \Delta t$, is proportional to the diffusion coefficient $D$, which is plotted as a function of $\Delta t$ in (D).
(E) Tally of large movements, as in Figure 3C, determined for the time-lapse series used in Figure 3C.
Archonage of Silent Chromatin

in heterochromatin can influence transcription rates. For instance, the interaction of Ikarus-regulated genes with centromeric heterochromatin in trans correlates with the cell type-specific inactivation of the relocated gene in cycling mouse B cells (Brown et al., 1999). Relocalization to centric heterochromatin was also observed in Drosophila larval cells for a mutant allele of brown (bw), which succumbs to a variate expression state upon association with centromere repeats (Csink and Henikoff, 1996; Demburg et al., 1998). As a further example, immunoglobulin alleles found near the nuclear periphery in embryonic stem cells, haemopoietic progenitors and pro-T cells, shifted to more central positions in the nucleus of pro-B cells just prior to VDJ rearrangement (Kosak et al., 2002). Finally, in the case of imprinted genes, asynchronous replication patterns could be correlated with distinct nuclear positioning prior to the establishment of repressed or open transcriptional states (Gribnau et al., 2003). These observations, together with the finding that silent yeast telomeres cluster near the nuclear envelope, have led to the suggestion that sub-nuclear position and in many cases positioning near repeat sequences can influence genome function.

Despite these correlations, there has been no molecular mechanism identified to date for heterochromatin positioning, and it has remained unclear whether the transcriptional status of chromatin was a cause or a result of its position. In the case of Drosophila bw, an HP1 mutation influences heterochromatin structure and weakens the bw-centromere interaction (Csink and Henikoff, 1996). However, HP1 is a highly mobile protein that localizes to many sites throughout the nucleus (Cheutin et al., 2003; Fettstein et al., 2003), and no structural ligand for HP1 has been found that restricts its interphase distribution. Even in yeast, where recent studies have shed light on mechanisms that tether yeast telomeres (Hediger et al., 2002; Taddei et al., 2004), it was not known if silent chromatin would occupy an inherent subnuclear position and if so by what means.

Using inducible recombination to uncouple HMR from Chr 3, we found that the repressed HMR locus anchors to the nuclear periphery through a pathway that requires silent chromatin in cis. Our work is the first unambiguous demonstration that integral components of silent chromatin structure, as opposed to associated enzymes such as active polymerases or splicing factors, can determine a chromatin domain’s subnuclear position. This finding suggests that the 3D positioning of chromosomes within the nucleus may be largely self-determined, organized by the binding of general repressors that are integral components of the chromatin itself. Spontaneous heterochromatin anchoring could account for the formation of the chromocenter, of nucleoli, and other associations of repetitive DNA in trans.

Ku and Esc1 as Silent Chromatin Anchors
We have identified Ku and the oncoprotein E1c as anchors for silent chromatin in yeast, although neither protein plays an essential role in the repression of HMR. Elimination of both Ku and Esc1 allows the release of the HMR ring from the nuclear periphery without loss of silencing, showing for the first time that Sir-mediated repression can occur independently of a chromosomal and subnuclear context. This complements the formal separation of anchoring and silencing in the yeast nucleus: not only can telomere components anchor

Figure 6. HMR: Silencing and Sir4 Protein Distribution in Cells Lacking Esc1 and Ku (A) Northern blot analysis of the HMR 1α transcript. Cultures were grown according to standard ring induction procedures, and samples were harvested immediately before galactose addition (labeled chromosomal) or 4 hr thereafter (labeled ring). Lanes 1 and 6—MRG2262 SIR1, lanes 2 and 7—MRG2263 a1, lanes 3 and 8—MRG2264 esc1, lanes 4 and 9—MRG2265 yku70, lanes 5 and 10—MRG2266 esc1 yku70. Blot was hybridized simultaneously with probes to α1 and ACT1 as an internal control. (B) Immunolocalization of Sir4 (red) and nuclear pore (green). Affinity purified anti-Sir4 rabbit serum and Ma1414 (mouse anti-pore) were used on the strains used in Figure 5C after growth and fixation with formaldehyde in YPD, as described (Lacheco et al., 2000). (C) Schematic representation of conditions allowing localization-independent silencing in yeast. In wild-type yeast cells, telomere bound yku anchors chromosome ends creating unequal distributions of Sir proteins due to multiple telomeric Repl1 binding sites. Silent chromatin is recruited to these subcompartments by interaction of Sir4 with yku and Esc1. Without these proteins, Sir proteins are released and HMR remains silent despite a lack of anchorage.
without silencing (Mediger et al., 2002a; Taddei et al., 2004; Tham et al., 2001), but also silencers can repress without anchoring. Our experiments are incompatible with previous claims that nuclear envelope components are required for chromatin-mediated repression (Galy et al., 2000). Finally, we can argue that the positioning of silent chromatin at the nuclear envelope is not a default result of exclusion from internal zones that favor active chromatin.

The action of Esc1 and Ku in anchoring silent chromatin is likely to be direct: both proteins interact with Sir4 (Andrulis et al., 2002; Roy et al., 2004), and both proteins promote silencing when targeted to DNA (Andrulis et al., 2002; Martin et al., 1999). Using mutant forms of these proteins it could be shown that both proteins can tether non-silent DNA to the nuclear periphery in a relocalization assay (Taddei et al., 2004). Sir4 is an essential silent chromatin component and as such provides a molecular link between silenced domains and anchorage. Although telomeric positioning also depends on Ku and Esc1, telomere and HMR anchoring differ in at least one critical respect: telomere anchoring does not require silent chromatin because Ku also associates with the extreme ends of chromosomes in the absence of Sir proteins (Graval et al., 1998; Martin et al., 1999).

The release of the silent HMR ring in esc1 yku70 double mutants correlates with a redistribution of Sir4 from telomeric foci where it is normally sequestered (Figure 6B). These data reinforce earlier evidence showing that telomeres and HM loci compete for Sir proteins and that the release of Sirs from telomeres can increase repression at internal sites (Buck and Shore, 1998; Mallet et al., 1996; Marcond et al., 1996). We propose that the repression of the mobile HMR ring is attributed to the uniform nucleoplasmic distribution of Sir proteins seen in this double mutant (Figure 6B). Further, we suggest that the only critical role for perinuclear anchorage in silencing is to provide a high local concentration of Sir proteins. We cannot, however, rule out the formal possibility that establishment of silencing requires transient perinuclear localization.

The unconstrained mobilities of silent HMR rings in the esc1 yku70 strain, as well as those of nonsilent rings, are striking and unexpected demonstrations that there is no significant impediment to chromatin movement in the yeast nucleoplasm. The rings appear to be subject to Brownian motion since no pattern or directionality to the movement could be detected and since the diffusion coefficients of the rings were found to be similar to the theoretical limit predicted by the Stokes-Einstein equation (2.6 × 10^{-12} μm^2/s, assuming a minimal mass of 39 HDa and a nuclear viscosity 5-fold greater than water (Wachsmuth et al., 2000). If the motion is indeed Brownian, then the role of ATP may be indirect in rendering the nucleoplasm and/or ring competent for free diffusion. Microtubule poisons have ruled out filament-based motors as the essential dynamic force (Houn et al., 2001), and inhibition of transcriptional elongation also has no effect (A. Taddei, F.R.N., and S.M.G., unpublished data). Irrespective of the source of chromatin mobility, it is noteworthy that chromatin can rapidly sample the entire nucleoplasmic space, as this may facilitate interactions between distal chromatin sites and homology-based search events.

Our improved conditions for chromatin tracking allow an accurate evaluation of single particle movement in the nucleoplasm. By comparing the mobility of the LYS2 locus before and after excision, we reveal the constraint imposed on chromatin mobility by its colinearity/linkage with the chromosome (Figure 7). By comparing the differences in mobility of anchored versus nonanchored silent HMR rings, we expose the constraints imposed by protein-protein interactions, in this case, by Sir binding to yKu and/or Esc1 (Figure 7). These restraining forces probably contribute to the definition of chromosome territories in interphase nuclei (Marshall, 2002; Chubb et al., 2002; Fisher and Mirkenschlager, 2002).

Subnuclear Silencing Compartments as Facilitators of Genetic Control

If yeast cells can repress mating-type loci without telomeric pools of Sir proteins, then why are such loci formed in the first place? We propose that telomere anchoring and its incumbent sequestration of Sir factors serves as an antisilencing mechanism for the rest of the active genome. The creation of subcompartments enriched for Sir proteins may allow the cell to ensure that most other loci remain active, while nonetheless exploiting the power of a general, promoter-independent, transcriptional repression mechanism. This rationale has also been proposed for the methylation of lysine 79 in histone H3, a modification that is found throughout the active genome, and which appears to be incompatible with silencing (van Leeuwen et al., 2002). Similarly, the histone variant Htz1 is thought to help insulate the rest of the genome from inappropriate silencing events (Manoehini et al., 2002). By creating subcompartments that favor repression through sequestration of Sirs, the cellular concentration of Sir proteins can be maintained at relatively low levels. This is undoubtedly important, as overexpression of Sir2 or Sir3 was shown to be lethal for budding yeast (Holmes et al., 1997). Although strains lacking both Yku70 and Esc1 are fully viable, microarray analysis shows a significant deregulation of a large number of internal chromosomal genes (A. Taddei and S.M.G.; unpublished data).

Grouping telomeres and distal silent chromatin domains in nuclear subcompartments could facilitate genetic control in a number of ways. Bringing silent loci together raises the local concentration of silencing factors that are otherwise limiting in the nucleus (Buck and Shore, 1995; Mallet et al., 1996). Factors dissociating from one locus might bind more frequently to a nearby locus poised for silencing rather than to other distant and active regions of the genome. This is likely to become critical in the propagation of a repressed structure as chromosomes duplicate. Furthermore, the juxtaposition of silent loci at the periphery could also promote formation of higher order structures creating trans interactions between unlinked silent domains.

The clustering of telomeres may also impact native subtelomeric gene expression. Recent studies have shed light on the physiological importance of regulating subtelomeric genes in a range of unicellular pathogens, including Candida, Trypanosoma, and Plasmodia. In these organisms gene families involved in cell adhesion and virulence are subject to variegated expression due
Figure 7. Chromosomal Linkage and Protein Interactions Constrain Chromatin Mobility  
Reconstruction of the paths of GFP-tagged foci from representative 5 min 3D time lapse movies. The tracking and nuclear shell were visualized using Imaris SURPASS and Time modules (-release 4.9. Bitplane). The top row images demonstrate the constraint imposed upon the LYS2 locus by the chromosomal fiber (compare chromo with ring), while the lower images demonstrate the constraint imposed upon the excised HMR locus by the interaction of Sir4 with Esc1 and Yku70.

to their subtelomeric position (De Las Penas et al., 2003; Scherf et al., 2001). In budding yeast, a similar form of reorganization was recently proposed for the subtelomeric FLO genes, which encode cell wall glycoproteins involved in flocculation. Expression of these genes is subject to a position-dependent variation that is heritable yet reversible, and which responds to challenges from the environment (Halter et al., 2004). This is likely to be related to the regulation of another set of subtelomeric genes, the PAU genes, which respond to external stresses through a rapamycin-regulated phosphorylation of Sir3 (Al et al., 2002). While mechanisms remain to be clarified, it appears that perinuclear pools of general transcription regulators, like Srs, may provide an epigenetic tool that not only silences mating-type information in a stable manner, but ensures genetic flexibility by variated expression of other subtelomeric gene families in the face of environmental stress.

Experimental Procedures

Plasmid and Strain Construction
A pAF52 (Slater et al., 1999) derivative that contained TRP1, a -14 kb array of 250 lac operators, and an HMR proximal fragment (Chr 2 coordinates 294689-295241) was integrated by homologous recombination between HMR-J and a telomere proximal RS site in a Δsir1Δhis3 derivative of W303-1A (YAL1) bearing recombinease sites at Chr 3 coordinates 292453 and 295252. Full-length Sir3 expressed from its own promoter was reintroduced to this strain at its normal chromosomal locus using pMM164. The resulting locus, SIR3::URA3::Δsir1Δhis3, was converted to Sir3 in strain MRG2259 by selecting for spontaneous elimination of URA3 and HIS3. The LYS2 excision cassette in YMC17 (Cheng et al., 1998) was modified by homologous integration of a pAF52 lac derivative to be described elsewhere (F.R.N., M.R.G., and S.M.G., unpublished data). Lac-GFP and tet-GFP were introduced by integration of pGIV40 (Bystriyuk et al., 2004) at the chromosomeal ade2-1 locus. Nup49 was tagged internally with GFP as described (Heun et al., 2001). The untagged copy was eliminated only from MRG2259. A GAL I-R recombinase expression construct was integrated as a tandem pair at ade2 by integrating pHNT (Saghirahman et al., 1997), his3::lacs, was converted to his3 by transformation with a PCR product containing the gene. MATA, ECH1, YKU70, and SIR4 were replaced with antibiotic resistance markers using a single-step PCR approach (Goldstein and McGuirk, 1999) to create null alleles of esc1::YKU70, and sir4.

Growth of Cultures and Single Z Stack Microscopy
Freshly streaked cells were grown in SC-lox media containing 2% dextrose for approximately 8 hr before diluting 1/20 into SC-his + 2% raffinose. When cultures reached approximately 0.25 OD at well-mixed overnight growth, galactose was added to 2%. After 2 hr, 1 ml of cell culture was harvested by centrifugation and either placed on microscope slides bearing 1.4% agarose plugs containing 4% galactose (pH 5.0) or 4% dextrose (pH 7.0) as needed or were mounted in a Lubin chamber flushed with appropriate media (Hediger et al., 2004). Data collection was limited to 2 hr after mounting.

All cultures were prepared similarly since carbon source and cell density are known to influence chromatin dynamics (Heun et al.,...
For ATF depletion experiments, cells were treated with CCCP dissolved in DMSO 1 h before harvesting and resuspended in agarose plugs containing drug and solvent at 40 uM and 0.1%, respectively. GFP-tagged locus positions were determined as described (Tades- dei et al., 2004; Hidrager et al., 2006a). Statistical significance of distributions was compared with random distributions by z-analy- sis. Student’s t-tests were used to determine the similarity of zones 1 values.

Chapter 3

Acknowledgments

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References


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Chapter 3

A  Box plot comparison of step size (nm)

B  Box Plot of Mean Velocity

C  Diffusion coefficient ($<\Delta d^2>/\Delta t$) vs $\Delta t$

Gartenberg et al., Suppl. Figure 1
4. CHROMATIN DYNAMICS IN INTERPHASE

4.1 Introduction

This Chapter discusses several observations which relate to cell-cycle specific changes in mobility and subnuclear position of chromatin loci. I focus on the S phase of the cell cycle, during which a perfect copy of the genome, including its epigenetic marks, must be generated. All these processes undoubtedly influence chromatin dynamics. In yeast, DNA replication starts at well defined origins of replication. This entails the assembly of a functional pre-replicative complex at the ARS (autonomously replicating sequence) consensus sequence and takes place in a temporally staged manner (reviewed in Bell and Dutta, 2002; reviewed in Mendez and Stillman, 2003). The genome-wide mapping of origins of replication by the use of several microarray based techniques has been a powerful tool for understanding the regulation of origin choice and timing (Raghuraman et al., 2001; Wyrick et al., 2001; Yabuki et al., 2002). The use and timing of origin firing does not depend on the exact sequence of the origin but on its larger chromosomal context. Consistently, late origin firing often, but not exclusively occurs within domains of altered chromatin structure(s) and silenced regions (Friedman et al., 1996; Vujcic et al., 1999). Timing patterns are stably inherited and will undoubtedly involve both chromatin marks and subnuclear positioning, yet the exact mechanisms that delay initiation are still poorly understood.
4.1.1 Chromatin structure and replication timing

Among the histone modifying enzymes that alter timing patterns are the yeast histone deacetylase Rpd3 or SIR proteins. Deletion of Rpd3, the major histone deacetylases in yeast (or its interacting partner Sin3) causes early activation of late origins at internal chromosomal loci, but did not alter the initiation timing of early origins or of late-firing, telomere-proximal origins. This led to a faster progression through S phase. Interestingly RPD3 deletion suppresses the intra-S replication checkpoint, so that late origins were not prevented from firing in a clb5Δ or upon HU treatment (Aparicio et al., 2004). The hypothesis that histone acetylation (at least partially) determines timing of origin firing is strengthened by the fact that locally increased acetylation of histones in the vicinity of the late origin ARS1412 (through targeting of the histone acetyltransferase Gcn5) caused ARS1412 alone to fire earlier (Vogelauer et al., 2002). Subtelomeric origins in the telomeric Y’ or X elements, the timing of which remained unchanged by mutations in RPD3, were affected by deletion of SIR3 (Stevenson and Gottschling, 1999). This inhibitory effect of silent chromatin on origin timing could also be observed by the local recruitment of Sir4 to the early origin ARS 305 (Zappulla et al., 2002).

4.1.2 Subnuclear organization and dynamics of replication origins

Isolated origins of replication fire early by default. The property of late firing is set up in early G1 phase of the cell cycle and correlates with a preferential localization of the late origin to the nuclear periphery (Heun et al., 2001a; Raghuraman et al., 1997). Interestingly, the preferred perinuclear position was not necessarily preserved in the subsequent S phase. Taking advantage of the R-recombinase system described in
Chapter 3 and 4, it could be shown that an origin which was excised from its chromosomal context in mitosis became early replicating and shifted to a random distribution in the nucleus. If however the late origin was excised in late G1 phase \((i.e.\) at which point it is preferentially perinuclear), it maintained its late firing properties in the subsequent S phase. Since the information of replication timing does not lie in the DNA sequence and the default state of an origin is early firing, it was speculated that a given origin would be “marked” \((e.g.\) by histone modifications) in a window in early G1 phase for late replication.

Heun et al. have investigated the effect of replication on mobility of replication origins. Interestingly, they found that internal loci are generally less mobile during S phase, especially with respect to large displacements \((\text{Heun et al., 2001b})\). This decrease in mobility was lost upon inhibition of replication \((\text{by addition of hydroxyuracil or aphidicolin})\) suggesting that this treatment leads either to the release of a tether \((\text{such as release of the fiber from a replication factory})\) or that there is a general change in the physiology of the nucleus. The mechanism and the physiological significance of this observation remain to be understood.

### 4.1.3 Subnuclear organization, structure and mobility of chromatin during in S phase – cause and effects

Subnuclear position and of different chromatin states have been discussed in Chapter 1. In S-phase DNA has not simply to be copied but also its transcriptional state has to be remembered. Since the replication fork passes every single nucleotide of the genome, fork components are obvious targets for propagating this state. Indeed there are many replication proteins involved in the establishment of silent chromatin. However, there is increasing evidence that the distinct mechanism that couples a
chromatin state to S-phase progression is independent of a passing fork. On one hand a direct connection was suggested, for mutations of several proteins present at the replication fork (PCNA, RF-C, DNA pol ε) can restore silencing of a crippled silencer, and for a mutant allele of the replicating clamp PCNA is silencing defective (Ehrenhofer-Murray et al., 1999; Zhang et al., 2000). On the other hand, two studies demonstrated that a cassette excised from the chromosome could be silenced without undergoing active replication. Even though physical passage of the replication fork is not a prerequisite for the establishment of silencing, passage through S phase was still required (Kirchmaier and Rine, 2001; Li et al., 2001). It seems likely that general S-phase regulators act in concert with local factors during the establishment of silent chromatin.

The causal relation between replication (and replication timing), chromatin structure (and local targeting of remodeling activities) as well as general chromatin mobility and subnuclear localization is a complex problem. As small parts of this puzzle, I will address here two specific questions about chromatin mobility and subnuclear position during G1 and S phase of the cell cycle.

1. Is the reduced chromatin mobility at internal replication origins during S phase, a general property of the S-phase nucleus or is it specifically related to a replication dependent structure?

2. How do globally or locally increased histone acetylation levels influence subnuclear position and local mobility of chromatin?
Chapter 4

4.2. Results and discussion

4.2.1 Cell cycle differences in chromatin dynamics

Analyses based on radial movements of yeast chromosomal loci (see Chapter 5) suggested that GFP-tagged loci in yeast have reduced mobility during S phase of the cell cycle (Heun et al., 2001b). This reduction in S-phase mobility was observed for marked internal origins of replication (ARS1413 and ARS907) but not for a tagged centromere or Tel 6R, both of which are anchored by mechanisms described in Chapters 1 and 3.

Movements have been quantified using radial MSD analysis which quantifies the radial component of the locus movements, i.e. their movement relative to the nuclear envelope.

In a subsequent analysis, however, absolute displacements were used because they give a more precise description of actual movements (see Chapter 5). Absolute displacements are used in this analysis. For Telomere 8L, which generally shows a lower mobility than most internal loci, did not change significantly between G1 and S phase. For the more mobile telomere 14L however, less mobility could also be detected in S phase (personal communication G. Van Houwe and S. Gasser). In order to address the question if changes in mobility require a nearby origin, we tagged the LYS2 locus which lies 15 kb away from the next origin and termination zone. As the origins of replication described above, its mobility is also compromised in S phase (Figure 4.1.B and C). Quantification by MSD plot shows that its radius of constraint (RC) is lower in S- than in G1 phase. Cell-cycle specific constraint could arise from two sources. It might either reflect a general change in the nucleoplasm, such as NTP levels or viscosity of the internal nuclear milieu, or the action of DNA polymerases on
the chromosome, which restricted chromatin mobility due to an association with replication factories. To test if this were the case, we excised a ring of chromatin tagged with the lacGFP repressor, to see if its mobility would also drop in S phase (Figure 4.1.A; described in Chapter 2 and 3). First we note that in G1 phase the excised chromatin ring has a plateau corresponding to a $R_C$ value of 0.88 $\mu m^2$, which is considerably higher than that of the integrated LYS2 copy, which moves within 0.66 $\mu m^2$. Furthermore, the integrated locus has a significantly lower diffusion coefficient (Figure 4.1.B-D). Surprisingly, the mobility of the excised ring, which does not contain any origin of replication, drops in S phase, by almost two fold. The MSD plot of the excised LYS2 ring in S phase shows that a plateau level reached slower, but at longer time intervals (>150 s), the $R_C$ probably approaches the same levels than in G1 phase (Figure 4.1.D and E). Since it is very unlikely that we would “capture” the replication fork on the excised circle of 16 kb, a fragment that replicates in less than 5 min, we conclude that this reduction probably reflects cell-cycle associated changes in the nucleoplasm or in factors required for this movement, and not the association of the chromatin fiber with a replication focus. Figure 4.1 also illustrates the constraint imposed by the continuity of the chromosomal fiber which is greater than the constraint imposed by S phase.
Figure 4.1. Chromatin mobility varies between G1 and S phase.

Absolute mean square analysis was performed on time-lapse series as described in Chapter 5, based on 3D-time lapse movies that were captured on the LSM510 (Zeiss) confocal microscope or by maximal projection of 6 confocal sections. Every MSD graph represents a mean of 7-10 movies as indicated below, error bars correspond to the standard deviation of the mean. Strain number, phase of the cell cycle, number of movies and number of frames analyzed are indicated in brackets. A) Sketch of the lac operator integration in the \textit{LYS2} gene. Induction of a site-specific recombinase results in ring formation. B) MSD plot of chromosomal \textit{LYS2} locus in G1 and S phase (GA2627; G1 phase: 7, 2170 frames; S phase: 8 movies, 2480 frame) C) Table of characteristic statistical values for \textit{LYS2} locus. D) Changes in mobility affect excised domains. The movement of a 16 kb ring containing the excised \textit{LYS2} locus (\textit{LYS2} ring) is reduced in S phase as is the chromosomal locus (\textit{LYS2} ring (GA2628);
4.2.2 Do altered histone acetylation levels change subnuclear position or locus dynamics?

**Subnuclear localization of Rpd3**

As described above, late firing of internal origins correlates with their preferential peripheral localization in G1 phase (Heun et al., 2001a). Furthermore it is known that the timing of these origins is influenced by *RPD3*. Therefore one could hypothesize that deacetylation of histone tails in G1, possibly in a subcompartment at the nuclear periphery, may mark the origin for late firing. In order to test the sublocalization hypothesis, we immunolocalized myc-tagged Rpd3 in the nucleus. We could not detect any enrichment at the nuclear periphery, yet Rpd3 is clearly excluded from the nucleolus. As shown in figure 4.2 no colocalization with the nucleolar marker Nop1 could be detected (Figure 4.2.B) and in the cells stained with a nuclear porin antibody, some cells show lack of staining at the nuclear periphery, presumably at the position of the nucleolus (Figure 4.2.A). This suggests that Rpd3 does not act at the rDNA, which is consistent with the finding that *rpm3* disruption did not alter acetylation levels at the rDNA (Robyr et al., 2002). These findings do not exclude that late firing origins are marked at nuclear periphery, but simply show that Rpd3 is not trapped in a perinuclear compartment.
Rpd3-myc was localized using 9E10 monoclonal antibody, nuclear porin and Nop1 were detected by rabbit sera and a Cy5 coupled secondary antibody as described in (Gotta et al., 1997). Microscopy was performed on the LSM510 confocal microscope (Zeiss); the scale bar corresponds to 2 μm.

**Subnuclear localization of replication origins in rpd3Δ cells**

The question if hypoacetylated histones or the action of Rpd3 are required for peripheral positioning of late firing origins can be addressed by scoring subnuclear localization in rpd3Δ cells. Subnuclear position was scored for the following origins of replication located at internally on a chromosome. Their respective timing is indicated in brackets. ARS1413 (late), ARS603 (late), ARS607 (early) ARS c4-908 (early). Position was quantified by classification into three concentric zones of equal
surface as described in Chapter 5. Figure 4.3 shows only the percentage of loci localizing to the outermost zone. As described by Heun et al, a random localization of early replicating internal origins (ARSc4-908 and ARS607) as well as the weak peripheral localization of late ARS1413 in G1 could be reproduced (Figure 4.3 A, Heun et al., 2001a). While ARS1413 localization became random in S phase, position of another late internal origin (ARS603) showed a slightly peripheral subnuclear positioning both in G1 and in S phase (Figure 4.3.D). Disruption of RPD3 led to the following changes in localization patterns: Late firing ARS1413 became randomly distributed in G1 phase, consistent with the hypothesis that peripheral localization depends on modifications by Rpd3. For the late ARS603 however, the image is less clear: peripheral localization was specifically lost in S phase, but maintained during G1. The early replicating control origins even complicate the image more. While the subnuclear localization of ARSc4-908 did not change subnuclear localization in the mutant strains, ARS607 was generally more peripherally located (Figure 4.3.A and B). This may be due to the relative proximity to the telomere (70 kb), the anchoring of which is stronger in the rpd3 mutant (as described below). While RPD3 deletion has clear consequences on telomere physiology, the effects it has on internal sites are pleiotropic (Robyr et al., 2002). Furthermore, the contiguity of the chromosomal fiber may mask very local effects in subnuclear localization. Specific targeting of histone acetyl transferase activity may give a clearer result (see below).
Figure 4.3. Subnuclear localization of replication origins in \( rpd3\Delta \) cells in G1 and S phase of the cell cycle. Bars represent the percentage of localization to the outermost zone; a peripheral ring along the nuclear envelope, covering 33 % of the nuclear surface (see Chapter 5). The measured distribution was compared to a random distribution (red line at 33 %) by a proportional test (Taddei et al., 2004). Significant differences (\( p>0.05 \)) are indicated by asterisk (*). The corresponding p-values for G1 and S phase and the strain number are indicated in parentheses. A) early replicating ARS c4-908 (WT: GA-1325; \( p=0.36/0.63 \); \( rpd3\Delta \): GA-1465; \( p=0.58/0.22 \)) B) early replicating ARS607 (WT: GA-1461; \( p=0.47/0.20 \); \( rpd3\Delta \): GA-1462; \( p=5.7\times10^{-12}/51.5\times10^{-4} \)) C) late replicating ARS1413 (WT: GA-1323; \( p=0.05/0.21 \); \( rpd3\Delta \): GA-1466; \( p=0.29/0.06 \)) D) late replicating ARS603 (WT: GA-1467; \( p=4.1\times10^{-4}/1.6\times10^{-3} \); \( rpd3\Delta \): GA-1468; \( p=2.4\times10^{-4}/0.18 \).
Deletion of Rpd3 and Sir2 histone deacetylases and their effect on
telomeres

Subtelomeric origin ARS609 does not change the timing upon \textit{RPD3} deletion, but telomere position effect (TPE) is strongly increased (De Rubertis et al., 1996; Rundlett et al., 1996). Since increased TPE is due to more spreading of SIR proteins at telomeres, we would expect a reinforced anchoring of ARS609 (and telomere 6R) (Hediger et al., 2002; Taddei et al., 2004). Figure 4.4 clearly shows that this is indeed the case. Increased silencing or more precisely more Sir4 (as shown by Taddei et al., 2004) increases the peripheral localization. On the contrary, disruption of the NAD dependent deacetylases Sir2 abolishes TPE and greatly reduces anchoring. The residual anchoring is probably due to tethering via yKu proteins, as discussed in more detail in Chapter 3.
Figure 4.4. Local chromatin structure (i.e. the strength of TPE and SIR dependent anchoring to the nuclear envelope influences localization of Telomere 6R (ARS609). Localization to all three concentric zones of subnuclear position are shown for G1 phase (G1), early S phase (early S) and mid-late S phase (mlS). Cell cycle stages and subnuclear positions were classified as described in Chapter 5. Fold increase or fold depletion compared to a random distribution (grey line; obs/random) is marked below the corresponding bars. Distributions are shown for A) (wt, GA-1459), B) (sir2Δ, GA-1846) and C) (rpd3Δ, GA-1842) strains. Reproduced with changes from (Hediger et al., 2002).
Targeting histone acetyl transferase activity

Mutation of rpd3, which is the major histone deacetylase in yeast, broadly changes chromatin structure and expression levels. Therefore, the cause of changes in subnuclear position is difficult to predict. An elegant approach used by Vogelauer and coworkers was the local targeting of HAT activity to an origin of replication. Integration of binding sites for the transcription factor Gcn4 near ARS1412 specifically changed its timing. The change depended on the Gcn5 histone acetyl transferase which is recruited by Gcn4 (Vogelauer et al., 2002). Here, we wanted to investigate if local HAT recruitment to ARS1412 would also lead to loss of peripheral localization during G1 phase. As speculated, targeting of Gcn4 (and in turn Gcn5) led to a loss of peripheral localization in G1 phase an effect which could be suppressed by disruption of GCN5 (Figure 4.5 B). This raised the question if local changes on histone acetylation levels would change local chromatin mobility. We performed time lapse microscopy on all three strains described above which have a tagged ARS1413 and analyzed their mobility. When targeting Gcn4 (and Gcn5), no difference to the wt strain could be detected. Surprisingly, disruption of the HAT GCN5 did not caused any significant change in mobility, although a tendency towards a slight increase in local diffusion coefficient can be seen (Figure 4.5.C and D).

We conclude that altered acetylation levels, which lead to changes in the time of origin firing, can influence subnuclear localization. Their direct relation however remains elusive. Although acetylation levels influence anchoring to some degree, no significant mobility changes could be detected. This can be for several reasons: Either the lac operator tag is to far away from the site of targeting or, what is also supported by Chapter 2, histone acetylation levels per se do not influence chromatin mobility observed by fluorescence microscopy and single particle tracking.
Figure 4.5. Effects of Gcn5 targeting on subnuclear position and chromatin mobility.

A) Map of late firing origins ARS1412 and ARS1413 which are marked with an array of lac operators (green boxes, Heun et al., 2001a) and Gcn4 binding sites (red boxes, Vogelauer et al., 2002). B) Subnuclear localization of the ARS1413. Bars represent the percentage of localization to the outermost zone at the peripheral ring along the nuclear envelope covering 33% of the nuclear surface (see Chapter 5). The measured distribution was compared to a random distribution (red line at 33%) by proportional tests (Taddei et al., 2004) Strain number and corresponding p values for G1 phase (dark gray) and S phase (light gray) are indicated. (wt: GA-1323; n=101/62; p=0.05/0.21, Gcn4bs: GA-1810; n=183/114; p=0.16/0.43; Gcn4bs gcn5Δ: GA-1811; n=83/44; p=0.03/0.07). C) MSD plots of ARS1413 locus. Error bars correspond to the standard deviation of the mean and are shown for GA-1810 (Gcn4bs) and GA-1811 (Gcn4bs gcn5Δ). D) Statistical analysis of locus mobility as described in Chapter 2 and 5.

<table>
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<tr>
<th>ARS1413</th>
<th>D_{1.5-12s} [10^{-3}um^2/s]</th>
<th>R_{0} [um]</th>
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<td>-</td>
<td>2.15±0.18</td>
<td>0.72</td>
<td>22.8</td>
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<td>Gcn4bs gcn5Δ</td>
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</table>
5. FLUORESCENCE MICROSCOPY ANALYSIS

The following Chapter contains a short introduction about live fluorescence microscopy in yeast in section 1. The second contains a paper of the Cell Biology Laboratory Handbook edited by J.Celis, to be published in 2005. It summarizes the instrumentation and analyses we have used in subnuclear positioning and dynamics analysis of yeast chromatin loci. Sections 5.3 and 5.4 are supplements to this methods Chapter. They contain more recent or more detailed supplemental descriptions of live fluorescence microscopy on tagged chromosomal loci in yeast. These are by followed by a more detailed description of chromatin dynamics analysis using different software tools and establishing meaningful parameters (5.4). In the fifth section possible mathematical models for chromatin mobility and their limitations are discussed.

5.1 Time-lapse movies in yeast

The mechanisms by which proteins, nucleic acids or lipids govern the generation, maintenance and function of cellular organization has been investigated using biochemical and genetic experiments that span diverse approaches from in vitro reconstitutions to atomic resolution structure determinations. Even though these techniques allow an incredibly detailed view on biological processes, they remain population based and show a statistical average. To these data, microscopy based techniques add another dimension to our understanding of cell function by their capability of distinguishing features in single cells on a subcellular level. For a long time cellular structures have been microscopically investigated using fixed specimen, e.g. by the use of immunocytochemistry or fluorescence in situ hybridization. But
with the cloning of green fluorescent protein (GFP), this has changed dramatically (Chalfie et al., 1994). GFP can theoretically be fused to any protein and thereby, any cellular structure can be marked in the environment of the living cell. Furthermore, the development of spectral variants of GFP (Matz et al., 2002) and the discovery of the unrelated red fluorescent protein (Fradkov et al., 2000) make it possible to perform multicolor live imaging. Having a tool at hand that allows microscopic in vivo imaging of proteins, it’s only a question of adding the time dimension to observe the real dynamic nature of biological processes. Such time-lapse microscopy experiments have gained great popularity over the course of the last years and even complex functions such as gene activation can be followed microscopically from the moment of the activation of a gene to the translation of the corresponding protein (Janicki et al., 2004).

5.1.1 Confocal and wide-field microscopy

Yeast cells and especially the nucleus, are small (~2 μm in diameter) and the concentration of fluorescent molecules is generally low. Therefore, microscopic experiments in yeast require both, considerable magnification (up to the physical limits of light microscopy, as discussed in section 5.6.1) and light sensitivity, which are counteracting each other. Microscopes and detection techniques improved considerably over the last decades, and several new approaches were developed. Two main principles of image acquisition can be distinguished: Wide-field and confocal microscopy. In (Stephens and Allan, 2003) three basic techniques for live fluorescence microscopy are discussed:

Classical confocal microscopes use lasers to scan the sample and illuminate each point/pixel successively. Photomultiplier tubes (PMT) enhance the incoming light and
convert the photons to electrons pixel by pixel. A pinhole eliminates the out-of-focus light, and the result is an image that shows the in-focus light only (a confocal image). PMT exhibit a lower dynamic range but less background than a CCD camera. Because confocal microscopes are scanning microscopes, excitation wavelengths are limited to existing laser lines (typically 458, 488, 514, 543, 568, and 633 nm) making it hard or very inefficient to excite some specific fluorophores (e.g. CFP). Recently lasers in the blue shifted excitation spectrum were developed although in this range phototoxicity is strong (405 nm laser diode). The other draw-back of a scanning microscope is the speed of acquisition. Only very small regions of interests (ROI; in our case 35 x 40 pixel) allow rapid enough sampling intervals (1.5 s/6 sections).

On the other hand, wide-field illumination, where the entire field of view is illuminated by a standard mercury or xenon lamp, combined with a charge-coupled device (CCD) camera that records the emitted light, results in an image that includes the out-of-focus light. In comparison to PMTs CCD cameras are less sensitive. The dynamic range of the image is much better, although also the background is elevated. Recent developments in camera design ameliorate this. If desired, the smearing imposed by the microscope optics in the later method can be computationally corrected by the use of deconvolution methods (Rines et al., 2002; Schaefer et al., 2001).

The third approach, the Nipkow disk confocal microscopy (a.k.a. spinning disc confocal microscopy) combines the advantages of confocal and wide-field microscopy to a certain extent. Nipkow disk systems directly deliver confocal images, but do not require laser-scanning of the sample because the image is directly recorded by a CCD camera. It was not used in these studies.
The choice method that provides the best results depends on the kind of specimen observed and on the kind of images required (2D, 3D, rapid time-lapse). Specific advantages of these systems in time-lapse microscopy on tagged chromosomal loci are discussed below. Subsequently, Chapter 5.6.1 will give a perspective on more recent developments of microscopes.

5.1.2 Light sensitivity

In order to prevent cell damage which can influence physiology and growth of the cell, it is important to limit the duration and intensity of illumination. In particular, exposure of yeast cells to excitation light of a wavelength shorter than 480 nm must be kept at a minimum to avoid GFP photobleaching and phototoxic effects on cells (Shaw et al., 1997).

For bleaching of the fluorophore but also for phototoxicity, the amount and wavelength of the light which gets exposed to the cell is important. It mainly depends on the excitation light source. This usually is a mercury (HBO) or xenon (XBO) arc lamp in wide-field or a laser as required for confocal microscopy. These light sources differ in their emission properties and therefore have advantages and disadvantages for image acquisition and phototoxicity. Arc-discharge fluorescent lamps emit light over a broad spectrum. Whereas the HBO emit strong light at variable intensities and several peaks of emission, the XBO shows a more steady and continuous emission spectrum and is better suited for sensitive life fluorescence microscopy. The use of a monochromator helps to precisely choose the excitation wavelength and to reduce illumination by unwanted wavelength. Details can be found at http://micro.magnet.fsu.edu/. It is equally important to shut off illumination light when it is not needed. In the confocal microscope this is inherent, but in the wide-
field microscope residual light from the fluorescence excitation light source has to be blocked by shutters. They provide more complete blocking than excitation filters or the “parking position” of the monochromator. Furthermore, infrared light from the halogen light source for transmission images can affect growth of yeast cells. This impact can be reduced by placing infrared filters in the light path (Wiget P, personal communication).

Using a confocal microscope, and minimizing both the laser intensity and the number of scans averaged as well as proper adjustments of the pinholes, are all essential for efficient live microscopy on extended time intervals.

Photobleaching and phototoxicity impose a trade-off between maximal image brightness and the total number of images that can be acquired on a single sample. In some cases, attenuating the illumination intensity and partially compensating by using longer exposure times can optimize image quality.

In conclusion it can be said that it is important to test the impact on cell growth for every new microscopy condition established. If the phototoxic effect is too strong, cells often get blocked in mitosis. Therefore, for any condition, proper growth (and passage through mitosis) has to be assured after image acquisition for at least one entire cell cycle.
5.1.3 Microscopy within the yeast nucleus

Due to the small size of yeast, a major concern for extended studies is focus stability. Loss of focus during time-lapse image acquisition has two main causes: First, instability of the microscope itself due to temperature changes and mechanical drift. Second, focus instability can also be due to changes in the sample preparation. A standardized handling procedure for samples is therefore another crucial aspect to stable long-term observations and is further discussed in section 5.2 and 5.3.

Intrinsically, yeast cells do not seem to be very well-suited for time-lapse studies because they are very small and, GFP signals are usually weaker than in other organisms, due to the lower protein amounts and smaller structures. However, cell cycle progression is easily followed, since budding is a marker of cell cycle entry and septation is a clear marker of cytokinesis. Further, bud size and shape offer valuable cues to cell cycle position as described in part 4.2. Therefore, microscopic cell cycle related studies clearly benefit from observing individual yeast cells. Questions like mitotic spindle behavior (Kusch et al., 2003) functioning of cohesins (reviewed in Nasmyth, 1999) or the positioning and dynamics of chromosome loci such as replication origins and telomeres (Hediger et al., 2002; Heun et al., 2001a) are also well studied by time-lapse microscopy. The possibility to easily tag proteins with fluorescent markers such as GFP allows tracking abundance and/or localization of proteins throughout the cell cycle and thereby makes yeast a valuable model to obtain more detailed information on cellular processes and powerful insight into cell autonomous behavior.
5.2 Quantifying mobility of individual chromosomal loci

The manuscript to be published in the Cell Biology Laboratory Handbook edited by Celis et al. 2005 summarizes the instrumentation and analysis used in subnuclear positioning and dynamics analysis of yeast chromatin loci.

Tracking Individual Chromosomes with Integrated Arrays of \( lac^{op} \) Sites and GFP-\( lac^d \) Repressor: Analysing Position and Dynamics of Chromosomal Loci in \textit{Saccharomyces cerevisiae}

Frank R. Neumann, Florence Hediger, Angela Taddei, and Susan M. Gasser

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I. INTRODUCTION

The visualisation of specific DNA sequences in living cells, achieved through the integration of \( lac \) operator arrays (\( lac^{op} \)) and expression of a GFP-\( lac \) repressor fusion, has provided new tools to examine how the nucleus is organised and how basic events such as sister chromatid separation occur (Straight et al., 1996; Belmont, 2001). In contrast to other methods, such as fluorescence in situ hybridisation, the \( lac^{op}/ \) GFP-\( lac \) repressor (GFP-\( lac^{op} \)) technique is noninvasive and therefore interferes minimally with nuclear structure and function. In addition, it facilitates analysis of the rapid dynamics of specific DNA loci (Gasser, 2002). Although this technique has been adapted to organisms from bacteria to humans, the ease with which GFP fusions can be targeted to specific chromosomal sites depends on the ability of the organism to carry out homologous recombination. This process is very efficient in budding yeast, allowing pairs of chromosomal loci to be analysed at the same time through the use of two bacterial repressors \( lac^d \) and \( tet^R \) fused to different GFP variants. Given the relatively advanced state of the art in budding yeast, this article presents protocols optimised for this organism. These provide a starting point for adapting multilocus tagging to other species. Moreover, the techniques described here for the quantitative analyses of locus dynamics are universally applicable.

II. MATERIALS AND INSTRUMENTATION

Yeast minimal and rich media (SD, YPD) are described in Guthrie et al. (1991). Cells can be mounted on a depression slide (Milmia SA, Cat. No. CAV-1, Fig. 2A) upon 1.4% agarose (Eurobio Cat. No. 018845) containing SD medium with 4% glucose (Fluka). Aliquots of this can be kept at 4°C for months. Alternatively, cells can be immobilised on a 18-mm coverslip treated with concanavalin A (Con A, Sigma, Cat. No. C-0412) in a cell observation chamber (Ludin chamber, Life Imaging Services, Fig. 2B). Con A dissolved to 1 mg/ml in H₂O is stable at −20°C for months. Wide-field microscopy is performed on a Metamorph-driven Olympus IX 70 inverted microscope with Olympus Planapo 60x/NA=1.4 or Zeiss Planapo 100x/NA=1.4 objectives on a piezoelectric translator (PIFOC; Physik Instrumente), illuminating with a PolychromeII monochromator (T.L.L. Photonics). Also needed is a CoolSNAP-HQ digital camera (Roper Scientific) or equivalent, and both the FITC filter set for detecting
III. PROCEDURES

A. Preparations

1. Plasmids and Strains

   Yeast transformation and growth are as described (Guthrie et al., 1991). The lac<sup>op</sup>/GFP-lac<sup>op</sup> system for site recognition exploits the high affinity and specificity of the bacterial lac repressor for its recognition sequence (lac<sup>op</sup>). All procedures are performed analogously for the tetR/tet<sup>r</sup> system (Michaelis et al., 1997).

   1. Plasmids or integrations of repetitive arrays are difficult to propagate in both bacteria and yeast due to recombination induced excision events. To avoid this, bacteria should be grown at 30°C in a recombination-deficient strain [STBL2 (Invitrogen Life Technologies) or SURE (Stratagene)].

   2. Integrate a copy of lac repressor fused in frame to sequences encoding the S65T V163A, S175G derivative of GFP and a nuclear localisation signal, e.g., pAFL144 into the yeast strain. This red-shifted GFP derivative has a higher emission intensity and longer fluorescence time than natural GFP (Straight et al., 1998). The lac<sup>op</sup> later helps to stabilise the lac<sup>op</sup> array in yeast.

   3. Insert a multimerised lac<sup>op</sup> array (usually 256 copies or ~10kb) into the chromosome by standard transformation using a linearised construct that integrates by homologous recombination. Integration is directed to a genomic locus by a unique cleavage within a polymerase chain reaction (PCR)-generated genomic sequence >200kb inserted into the host plasmid (e.g., pAFL52 integration is selected by growth on 5-Drp; Straight et al., 1996; Heun et al., 2001a; Hediger et al., 2002). In yeast as few as 24 contiguous lac<sup>op</sup> sites can be detected readily.

   4. Check the proper insertion by standard colony PCR and/or Southern blotting (Guthrie et al., 1991). Binding of lac<sup>op</sup>-GFP to the lac<sup>op</sup> array results in a bright focal spot, detected readily by fluorescence microscopy within the nucleoplasm. Confirmed transformants with bright signals should be frozen and stored immediately as individual colony isolates. When strains are recovered from frozen stocks, they should be grown on selective medium to avoid further excision events.

   Note: Other GFP fusions, optimised forms of CFP or YFP (or ECFP and EYFP), have also been used successfully in yeast (Lisby et al., 2003). The lac repressor used is also modified to prevent tetramerisation, thus minimising artefactual higher order interactions between lac<sup>op</sup> sites (Straight et al., 1996).

   5. Double tagging. If the position or mobility of two genomic loci is to be compared, one should avoid tagging both with the same repeat. It has been shown that identical arrays can undergo a pairing event that, at least in the case of the tet system, depends on the expression of the repressor (tetR; Fuchs et al., 2002). By using tet<sup>r</sup> for one site, and lac<sup>op</sup> for the second, the risk of spurious pairing is eliminated. Useful pairs of GFP derivatives are CFP and YFP, or GFP and the new monomeric mRFP (Campbell et al., 2002).

   6. In contrast to the lac<sup>op</sup>-GFP fusion (Figs. 1A and 1B), the tetR-GFP gives a high and generally diffuse nucleoplasmic background in yeast, both in the presence and in the absence of tet<sup>r</sup> repeats (Figs. 1C and 1D).

   7. Dynamics. If movement analysis is to be pursued, it is important to differentiate the movement of the nucleus itself or that induced by mechanical vibrations from the dynamics of the chromosome. Nuclear movement can be subtracted from that of a specifically tagged site by any of the following three methods.

   a. Visualisation of the nuclear envelope with Nup49-GFP (Belgareh et al., 1997; Heun et al., 2001a). In this case the nuclear centre can be interpolated from the oval or circular pore signal in an automated fashion by software such as ImageJ or Metamorph (Figs. 1A and 1B). The DNA locus position is then determined relative to the nuclear centre for each frame.

   b. Diffuse nucleoplasmic signal of tetR-GFP (Figs. 1C and 1D). The centre of the nucleus is defined by interpolation frame by frame and locus movement is calculated relative to this.

   c. By comparing the motion of two tagged loci, one can calculate average movement without concern for nuclear drift. The fact that both
Chapter 5

TRACING INDIVIDUAL CHROMOSOMES

FIGURE 1  (A and C) An overlay of the phase image and the fluorescence image of a GFP-tagged yeast cell in G1 phase. (B and D) The corresponding fluorescence image. The lacI array is integrated at the LYS2 locus; the nucleus is visualised by the tagged nuclear pore component Nup62-GFP (A,B) or by using the diffuse staining of nucleoplasm by lacI-GFP (CD). Bar: 1 μm.

loci are moving has to be taken into account for movement quantification (see later).

2. Growth and Cell Preparation

1. All yeast strains to be analysed should be cultured identically and preferably to an early exponential phase of growth (<0.5 x 10^7 cells/ml) in synthetic or YPD medium, starting from a fresh overnight culture. Wash cells once before observation to avoid YPD autofluorescence. We recommend two mounting techniques for living cell visualisation.

2a. SD-agarose-filled slides (Fig. 2A): Immobilised cells between an agarose patch on a depression slide and a coverslip to avoid flattening or distortion of the yeast by coverslip pressure on a normal glass slide. Cells sealed in this way are in a closed environment in which the depletion of O_2 and production of CO_2 bubbles can influence growth and impair visualisation. Optimally this technique is used for imaging periods limited to <60 min.

i. Melt an aliquot of SD/agarose at 95°C until the agarose has completely melted, but not longer.

ii. Vortex briefly and transfer 150 μl into the well of a depression slide that is preheated either by a heating block or by passage through the flame of a Bunsen burner.

iii. Immediately pass a normal microscope slide over the depression to remove excess agarose as depicted in Fig. 2A.

iv. While the agarose solidifies, recover the cells from 1 ml of culture by centrifugation for 1 min at <10,000 g.

v. Resuspend the cells in ~20 μl of appropriate medium.

vi. Once the agarose has solidified, remove the upper slide by sliding along the depression slide surface and place ~5 μl of concentrated cells on the agarose patch.

vii. Close with a coverslip, eliminate eventual air bubbles, and seal with nail polish.

Note: Monitor bud emergence and cell division carefully, as some brands of nail polish contain solvents that influence yeast cell physiology negatively.

2b. Cell observation chamber (Ludin chamber, Fig. 2B): The second technique uses a Ludin chamber in which cells are attached noncovalently to a coverslip by a lectin. The medium-filled chamber is assembled as shown in Fig. 2B. A flow of fresh medium can be applied.

i. Coat 18-mm coverslips with 10 μl Con A (1 mg/ml in H_2O) and let them air dry for >20 min. Coated slides can be kept for weeks at room temperature.

ii. Adhere cells to the Con A-coated coverslip by sedimenting 1 ml of the culture at 1 g for 3 min at room temperature.

iii. Remove excess culture and add ~1 ml fresh preheated medium before closing the chamber.

3. Temperature Control

In order to have a stable condition for microscopic observation, the temperature of the microscope and
room should be controlled carefully (±2°C). Two mechanisms are used standardly. The first is to enclose the entire imaging part of the microscope in a commercially available temperature-regulated box (e.g., Life Imaging Services or Zeiss). A second, less precise method is to regulate the temperature of the slide through a heated stage.

B. Image Acquisition

1. General

The choice of imaging technique depends on the question being asked. To derive quantitative information on the position of a given locus relative to a fixed structure (e.g., the spindle pole body, nucleolus, or nuclear envelope), three-dimensional (3D) stacks and detection of different wavelengths may be necessary. An analysis of fine movement and chromatin dynamics, however, requires the rapid and extended capture of one or more fluorochromes. Bleaching of the signal is often a major limiting factor in time-lapse imaging. One should note that chromatin movement is very fast [movements >0.5µm in less than 10s (Heun et al., 2001a)], making it necessary to have rapid image acquisition with a minimal interval between sequential images. To optimise acquisition, parameters such as image resolution, the number of z frames, intervals between frames, light intensity, and exposure time can be varied. In all cases, it is of utmost importance to minimise and monitor laser- or light-induced damage to the organism during imaging, in part by determining the time required for one division cycle in imaged and nonimaged cells.

Cell Cycle Determination

As position and mobility of a chromosomal locus can vary with stages of the cell cycle, it is crucial to determine precisely what stage each imaged cell is in. This is done by monitoring bud presence and bud size, as well as the shape and position of the nucleus, as visualised by the Nup99-GFP fusion and a transmission or phase image. Figure 3 summarises the morphologies that characterise each stage of the cell cycle.

2. Wide-Field Microscopy and Deconvolution

For the imaging of large fields of cells, best results are obtained with a wide-field microscope equipped with a PIFOC, Xenon light source, and monochromator that allows a broad and continuous range of incident light wavelengths, as well as rapid switching between these values. Images are acquired by a high-speed monochrome CCD camera run by a rapid imaging software, such as Metamorph. The limiting step is often the speed of signal transfer from the CCD chip to the RAM and/or hard disk of your computer.

z-Stacks

Wide-field microscopy is well adapted to experiments in which a large number of cells (200–300) need to be scored, e.g., when determining the subnuclear position of a given locus relative to the nuclear envelope or another tagged locus or landmark (e.g., spindle pole body or nucleolus). The reference point should optimally be tagged with a different fluorescent protein. If two loci bind the same fluorescent fusion proteins, then their intensities should be significantly different. Rapid through-focus stacks of images using the full chip capacity of the camera are taken of cells growing on agar or in a Ludin chamber (such that 20–30 individual cells are resolved per field). Optimal parameters for GFP are as follows: exposure time, 100–200 ms; z spacing of 200 nm for 18 focal planes, excitation wavelength 475 nm. For dual-wavelength capture, images of both wavelengths (GFP, 432 nm; YFP, 514 nm, ~500 ms) must be acquired before the focal plane changes. A phase image is taken after every stack of fluorescence images. Wide-field images have out-of-focus haze and deconvolution of the z stack is often necessary to reassess blurred intensities back to their
original source. Use Metamorph software or other available deconvolution packages.

Three-Dimensional Time Lapse

The conditions for capturing 3D time-lapse series are as follows: 5–11 optical z slices taken every 1 to 4 min, z sections are 200 to 400 nm in depth, and the exposure time is ~50 ms. Using these settings, up to 300 stacks of five sections each (1500 frames) at 1-min intervals can be captured without affecting cell cycle progression. More rapid sampling with this system, however, leads to bleaching and potential cellular damage. Until this can be remedied by more rapid and more sensitive CCD cameras, wide-field microscopy is recommended for less rapid time-lapse imaging (intervals ≥60 s) on larger fields and confocal microscopy (see later) for very rapid time-lapse imaging (intervals ≤2 s) on small regions of interest (typically one yeast nucleus).

For very long imaging times (>1 h), stray light should be suppressed by inserting an additional shutter. Deconvolution is performed using the Metamorph fast algorithm with five iterations, a sigma parameter of 0.7, and a frequency of 4.

3. Confocal Microscopy

To follow chromatin dynamics in individual cells with rapid time-lapse microscopy, the Zeiss LSM510 scanning confocal microscope is particularly well adapted, although the laser and acousto-optic tunable filter (AOTF) system is limited in activation wavelengths. Its positive attributes are an ability to limit scanhead motion to a minimal region of interest (ROI), rapid and well-regulated scanning speeds, and the possibility to adjust pinhole aperture and laser intensities to very low levels, while maintaining maximal sensitivity.

General Settings

To reduce the risk of damage by illumination, the laser transmission is kept as low as possible, and the cells are imaged as rapidly as possible within a minimal ROI. Useful settings for the Zeiss LSM510 are as follows.

Laser: argon/2 458, 488, or 514 nm tube current 4.7 amp. Output 25%.
Filters: Channel 1: Lp 505 for GFP alone; channel 1 Lp 530, channel 3 Bp 470-500 for YFP/CFP single track acquisition.
Channel setting: Pinhole 1–1.2 airy unit (corresponding to optical slice of 700 to 900 nm); detector gain: 930 to 999; amplifier gain: 1–1.5; amplifier offset: 0.2–0.1 V; laser transmission AOTF=0.1–1% for GFP alone, 1–15% for YFP, and 10–50% for CFP in single track acquisition. In order to use minimal laser transmission the pinhole must be aligned regularly.
Scan setting: Speed 10 (0.88 µs/pixel); 8 bits image; average/median; line; zoom 1.8 (pixel size: 100 × 100 nm).
Imaging intervals: 1.5 s

Note: If CFP and YFP signals are very weak, images can be acquired sequentially using the more sensitive LSM 510 channel 1 in multitrack mode. This allows the use of broader filters: long-pass filter Lp 475 for CFP and Lp 530 for YFP. Alternatively, and to avoid any cross talk, recover the YFP signal as before and use Bp 470-500 on channel 3 for CFP. These latter parameters will slow the imaging process.

Two- or Three-Dimensional Time Lapse

If maximal capture speed is desired, only one image per time point can be taken, as long as the GFP spot stays in the imaged plane of focus (called 2D time lapse). Often the plane of focus has to be changed manually to follow the spot. Image acquisition in 3D has two main advantages. (1) The GFP spot does not have to be followed manually as it is always present in one of the focal planes. A subsequent maximal projection along the z axis produces a complete 2D time sequence without loss of focus on the GFP spot. (2) After image reconstruction, one can visualise the nucleus and calculate distances in 3D. Such measurements are nonetheless compromised by the reduced optical resolution in z (20.5 µm for 488-nm light).

Specific 3D time-lapse settings are as follows: six to eight optical slices in z, 300–450 nm spacing in z with Hyperfine HRZ 200 motor using a ROI of 3 × 3 × 4.5 µm and time intervals of 1.5 s. A 12-min time-lapse series at 0.2% laser transmission did not influence cell cycle progression.

C. Image Analysis

1. z Stacks

Determination of the subnuclear position of a GFP-tagged locus is monitored relative to the centre of the Nup49-GFP ring. Nuclei in which the tagged locus is at the very top or bottom of the nucleus are not scored because the pore signal no longer forms a ring but a surface and a peripheral spot will appear internal.

1. Measure the distance from the centre of intensity of the GFP spot to the nearest pore signal along the nuclear diameter, as well as the nuclear diameter itself, using the middle of the GFP-Nup49 ring as the periphery (Fig. 4). Several programs can export coordinates
of points of interest, and the publicly available point picker plug-in for ImageJ (Rasband) is useful.

2. Calculate the distances/diameter ratio, e.g., using Excel. Determine the precise relative radial position by dividing the distance between pore and the spot by half of the calculated diameter, thus normalising distances.

3. Classify the position of each spot with respect to three concentric zones of equal surface (Fig. 4). The peripheral zone (zone I) is a ring of width $0.184 \times$ the nuclear radius ($r$). Zone II lies between $0.184$ and $0.422r$, and zone III is the centre of the nucleus with radius $= 0.578r$. In a predicted random distribution every group would contain one-third of the cells.

4. Compare the measured distribution to another (e.g., other cell cycle phase, another condition or a random distribution) with a $\chi^2$ analysis. If only percentages of one zone (e.g., the outermost zone) are compared for different conditions (or to a random distribution), a proportional test should be applied. Statistical significance is determined using a 95% confidence interval.

2. Three-Dimensional Time Lapse Locus Tracking

A prerequisite for the precise description of chromatin movement is the knowledge of the coordinates of the locus and of the nuclear centre for each frame of a time-lapse movie. In collaboration with D. Sage and M. Unser (Swiss Federal Institute of Technology, Lausanne), a best-fit algorithm has been developed that reliably tracks a moving spot in 2D time-lapse movies or in maximal projections of $z$ stacks in 3D time lapse using nuclei carrying Nup49-GFP or expressing tetR-GFP to detect the nucleoplasmic signal. This system is complete and dramatically improves reproducibility and the speed of analysis, while allowing user intervention at several stages. The algorithm has been implemented as a Java plug-in for the public domain ImageJ software (Rasband; Sage et al., 2003). The spatiotemporal trajectory is exported as $x,y$ coordinates for each time point in a spreadsheet. An implementation for 3D image stacks over time will soon be available. Automated image analysis requires three steps.

a. Alignment phase. The first step is an alignment module that compensates for the translational movement of the nucleus, cell, or microscope stage. This is achieved by a modifiable threshold on the image. The extracted points are then fitted within an ellipse using the least-squares method. Finally, each image is realigned automatically with respect to the centre of the ellipse.

b. Preprocessing phase. To facilitate the detection of the tagged locus, the images are convolved with a Mexican-hat filter. This preprocessing compensates for background variations and enhances small spot-like structures.

c. Tracking phase. The final step is the tracking algorithm. Using dynamic programming, which takes advantage of the strong dependency of the spot position in one frame on its position in the next, the optimal trajectory over the entire period of the movie is determined. The following three criteria influence spot recognition: (1) maximum intensity (i.e., the tagged DNA is usually brighter than the pore signal), (2) smoothness of trajectory, and (3) position relative to the nuclear centre. This latter criterion is necessary because Nup49-GFP staining can be confused with a weak perinuclear locus. All three parameters can be modulated individually in order to optimise the tracking for different situations (loci that are more mobile, more peripheral, of variable intensity, etc.). Most importantly, the program has the option of further constraining the optimisation by forcing the trajectory to pass through a manually defined pixel. In that way mistracked spots can again be added manually to the correct trajectory, which is recalculated quasi-instantaneously. This tracking method proves to be extremely robust and reproducible due to its global approach.

Note: Some commercially available software are also able to track objects [e.g., Imaris (Bitplane), Velocity (Improvision)], although tracking efficiency is variable and usually requires uniformly high-quality images. The algorithms are mostly based on threshold principles, which are rarely modifiable or interactive.
Characterisation of Movement

Because each time-lapse series represents a single cell, it is indispensable to average 8–10 movies over a total time >40 min for a given strain or condition. Subtle differences require a larger data source. Useful parameters for quantitative analysis include the following.

a. Track length. The projected track of the tagged locus can be visualised using LSM software, ImageJ, Excel, or other programs (Fig. 5A). The sum of all 1.5-s step lengths within a time-lapse series yields the total track length of that movie. From this, average track length and velocity (μm/min) can be calculated, but often this parameter is not very revealing.

b. Step size. A histogram of step size distribution describes the nature of the movement more precisely. Statistical parameters such as mean, median, and standard deviation of individual and groups of movies can be calculated and compared with statistical tests (e.g., ANOVA). Even small but reproducible significant differences can be documented due to the large number of measurements.

c. Large movements. Often differences in mobility are not obvious by comparing average speed, yet the frequency of large steps >500 nm will vary significantly. These indicate transient high velocity movements. We generally score for steps larger than 500 nm during seven frames (10.5 s), an interval that has proven useful for distinguishing patterns of mobility between different physiological states and stages of the cell cycle (Heun et al., 2001b). These are reported as the number of large steps per 10 min, averaged over at least 50 min of time-lapse imaging. Although a 500 nm is a meaningful cutoff, any threshold over 300 nm can be used.

d. Mean square displacement (MSD). Observing the movement of a DNA locus over time not only gives information about its velocity, but also about the subvolume of the nucleus that it occupies during a given period of time. It has been shown for several chromosomal loci that chromosomal domains are able to move apparently randomly in a given subvolume (Gasser, 2002). This constraint can be quantified by MSD analysis, assuming that the movement of the spot follows a random walk. Ideally it describes a linear relationship between different time intervals and the square of the distance travelled by a particle during this period of time (MSD or Δd²), where Δd² = d(t) - d(t + Δt)² (Berg, 1993; Marshall et al., 1997; Vazquez et al., 2001). In order to get the numbers, one must calculate the distances travelled by the spot for each time interval (1.5, 3, 4.5 s...) and plot the square of the mean against increasing time intervals. These calculations and the corresponding graphs can be performed easily in Excel (Microsoft) or Mathematica (Wolfram Research). A representative MSD graph is shown in Fig. 5B. In these curves, the slope reflects the diffusion coefficient of the particle, and the linearity of the curve is usually lost at larger time intervals due to spatial constraint on the freedom of movement of the locus, i.e., the random walk of the particle is obstructed by the nuclear envelope or other subnuclear constraints, leading to a plateau (horizontal dashed line in Fig. 5B). The height of this plateau is related to the volume in which the particle is restricted. The slope of the MSD relation is directly correlated with diffusion coefficient. As
explained earlier, in enclosed systems, the diffusion coefficient decreases with increasing $\Delta t$ due to space constraints exerted on the particle dynamics. Nevertheless, the maximal diffusion coefficient can be calculated for very short time intervals and reflects the intrinsic mobility of particles (see sloping dashed line in Fig. 5B). For chromosomal loci in yeast, we observed a maximal diffusion coefficient in the range of $1 \times 10^{-10}$ to $1 \times 10^{-14}$ m$^2$/s based on short time intervals. If distances are measured between two separate moving loci, $(\Delta x)^2$ reflects two times the MSD of an individual spot or focus moving relative to a fixed point (Vazquez et al., 2001). A more theoretical discussion of these parameters is found in Berg (1993).

IV. COMMENTS

It is very difficult to accurately quantify the intensity of a small, mobile GFP-lac focus. Even in deconvolved images it can differ by twofold in sequential images.

This protocol shows the optimal method for the described microscope setups. For different microscopes, the values and methods of this protocol are simply a starting point for further optimisation. As improvements in technology (e.g., more sensitive and rapid CCD cameras) and reagents (e.g., more stable or more intense GFP variants) evolve, future adjustments of this protocol will be indispensable.

The method described here can also be applied to Schizosaccharomyces pombe with a few changes, one being immobilisation on a coverslip with isoelectric B (1 mg/ml) (Williams et al., 2002) or lectin from Bandeiraea simplicifolia (Iyophilized powder, Sigma Cat. No. L2380).

V. PITFALLS

1. To ensure that DNA movements are not the result of nuclear rotation, fluorescence recovery after photobleaching on GFP nuclear pore components should be performed over the same time intervals used to monitor DNA movement.

2. To increase oxygen concentration and to prevent massive production of CO$_2$ under the cover slide, vortex the agarose/medium before making the patch.

3. Growth conditions must be standardised thoroughly, because both choice of carbon source and its concentration significantly influence subnuclear position and dynamics of tagged loci.

4. Cells grown in minimal medium may not pellet as well as cells grown in YPD. Concentrate cells by centrifuging 2 volumes of culture in the same 1.5-ml tube.

5. In the Ludin chamber yeast cells often bud upwards into the medium (i.e., parallel to the optical axis). Thus it is important to scan the entire cell in transmission mode not to miss the presence of a bud.

6. Observations made on individual cells are often not representative of entire populations. It is crucial to verify observed differences with the appropriate statistical tests.

References


Rashband, W. Imagej, National Institute of Health, Bethesda, MD.


Chapter 5

5.3 Acquisition and yeast growth

The general methods and considerations to monitor tagged chromosomal loci are described in part 5.2. This section complements the general protocol and points out possible pitfalls. Furthermore, some procedures optimizing sample preparation and image acquisition are discussed.

5.3.1 Cell growth and sample preparation

Growth media

For time-lapse studies, yeast cells are pre-grown overnight in synthetic medium containing 2% of glucose. Optionally, other carbon sources such as raffinose for later induction of the \textit{GAL1} promoter can be used at the same sugar concentrations. In the morning of the experiment day, cultures were first diluted into fresh synthetic medium to an OD_{600} of maximally 0.1 in order to ensure exponential growth.

Critical parameters for GFP stability are the carbon source and the pH of the growth medium. We have observed that cells grown in galactose containing minimal media are experiencing much stronger and faster bleaching than cells grown in glucose containing minimal media. The cause of this is unknown but could be connected to the general physiological changes upon change of the carbon source. It is further known that GFP is sensitive to changes in pH (Patterson et al., 1997). We observed
more fading in acid media. It is therefore recommended to grow cells in media with a pH ≥ 6.

(Straight et al., 1996) use growth medium which is supplemented with 6.5 g/l Na citrate, which is supposed to minimize bleaching of GFP. We tested several concentrations of citrate supplemented to the growth medium. Snapshot images taken on the wide-field fluorescence microscope did not show any increase the photostability of Nup49-GFP and on lac9p-GFP marked chromosome loci on. When supplementing the growth medium with 22 mM citrate in time-lapse imaging using a the LSM510 confocal microscope, we observed faster bleaching in the citrate containing samples, although the initial signal intensity was approximately 35% above the one of the mock situation.

**Agarose pad preparations**

Before mounting the cells on agarose pad containing microscope slides cells were concentrated in the same medium in a table-top centrifuge for 30 seconds at 10000 rpm. In minimal medium cells aggregate less well and tend to stick to the wall of the tube. Therefore several rounds of centrifugation with a new aliquot of cells may be required in order to get a small but visible pellet. The ease of pelleting also differs between different brands of 1.5 ml tubes (best worked those from Eppendorf AG, Hamburg, Germany, # 0030125.150). The pellet was then resuspended in ~30 μl of synthetic medium by pipetting thoroughly. This resolves aggregates of cells which help during image acquisition and analysis because it is difficult to determine the budding pattern of cells that are too close one to another.

The agarose-medium was prepared as illustrated in Chapter 5.2. Pads were prepared by placing a drop of 180 μl hot agarose medium onto ground well microscopy slides that was pre-heated in the flame for a few seconds (alternatively they can be placed on
a 95°C heating block ~15 s before adding the agarose). One has to ensure that the slide is warm enough in order to prevent rapid solidification, but not too hot to cause evaporation of the agarose medium when it is placed onto the slide. The cavity slide is then covered with a standard microscope slide that the entire cavity is filled with agarose without air-bubbles. Slides were then left on the bench for at least 15 minutes in order to solidify. Slides having air-bubbles should not be used for extended acquisition, because preparations containing air-bubbles usually lose focus more rapidly than those without. Although we recommend the use of a Ludin chamber (see section 5.2) for extended time-lapse studies this can also be done on agarose pads. Generally, it is recommended to prepare multiple slides at a time, although they cannot be kept longer than a few hours, since the agarose begins to dry out. When removing the upper slide from the one with the solidified agarose pad, it can eventually be quickly warmed up the by passing once through a flame. 6 μl of concentrated cells are then placed onto the agarose containing well and covered with a coverslip, again carefully avoiding the formation of air bubbles. Slight shifting back and forth of the cover slide prevented excess liquid on the agarose and helps distributing the cells. The coverslip was sealed with nail polish to allow for long investigation times without the loss of any liquid. “Cover-Girl” (Migros, Switzerland) nail polish has turned out to seal better than “Belle-Dame” (Coop, Switzerland). The latter inhibited growth in overnight experiments (personal communication P. Wiget). To achieve focus stability, such sealing is very important. But this also means that cells are growing anaerobically. When growing in a medium rich in sugar, rapid growth is fueled by fermentation, with the production of ethanol. When the fermentable sugar is exhausted, the yeast cells turn to ethanol as a carbon source for aerobic growth. This switch from anaerobic growth to aerobic respiration upon
depletion of glucose, referred to as the diauxic shift, is correlated with widespread changes in the expression of genes involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage. To avoid this, (Hoepfner et al., 2000) suggested to vortex the agarose mixture prior to slide preparation in order to saturate it with oxygen. In our hands we use 4% glucose to help maintain fermentation. This may also contribute to the fact that no delay in cell-cycle time was observed over the course of the time-lapse studies.

5.3.2 Image acquisition

Two different microscopy setups were used for image acquisition. All rapid time lapse movies were acquired on a Zeiss LSM510 confocal microscope.

The wide-field microscope (Olympus IX-70) was used for 3D stacks and for conditions where several cells were imaged over longer time intervals. It was equipped with a monochromator (Polychrome II, TILL Photonics, Graefelfing, Germany), a piezoelectric z-drive (PIFOC; Physik Instrumente, Karlsruhe, Germany) and a fast and sensitive charge-coupled device (CCD) camera (CoolSNAP HQ; Roper Scientific Inc., Tucson, USA) as described in Chapter 5.2. For GFP microscopy a Chroma filter for FITC containing a 515 nm longpass emission filter was used. Both microscopes were equipped with Zeiss Plan-Apochromat 63x or 100x/1.4 Oil lenses. The aim of every movie recording is to capture as much detailed information and signal as possible. Therefore, at every time-point a stack of images is taken in the focus direction (Z-axis), resulting in a 4-dimensional dataset (x, y, z, time) that covers the entire nuclear volume over time. In order to improve focus stability for long experiments, it was important to preheat the microscopes by turning them on in
advance, ideally in a temperature controlled box (*e.g.* The Box by Life Imaging Services, Reinach BL, Switzerland).

GFP fusions were made using the S65T variant of green fluorescent protein. For a more detailed description of time-lapse microscopy techniques used see also Chapter 5.2.

**GFP photostability and photodamage**

General phenomena caused by sample illumination have been discussed in section 5.1.2. It concluded that in yeast and especially in the yeast nucleus, GFP signals are much lower than for example in cell culture systems. Often the weak signal requires stronger light or longer exposure times which results in faster fading of the fluorophore and possible damage to the cell known as photodamage or phototoxicity. It can be said that during imaging, we have to make a trade off between sensitivity, sampling rate (in the z-axis and in time), and bleaching or phototoxicity. Only thorough analysis will lead to the optimal parameters and will allow the choice of the optimal microscope. We optimized the parameters for rapid 3D microscopy of marked yeast chromosomes. Interestingly we observed much less fading using the LSM510 confocal microscope than the wide-field microscope. Typical values for 3D time-lapse of one nucleus with a GFP-marked chromosome locus after which cells continued to grow are: 450 nm spacing, 6 images of 36 x 42 pixels at intervals of 1.5 s at a laser transmission below 2% and an averaging of 4. When monitoring several cells with a lower time resolution, stacks of 16 images with a spacing of 200 nm were made using the Olympus fluorescence microscope. For microscopy using two tagged sites and the fluorophores CFP and YFP the detailed imaging conditions for marked chromatin loci are given by (Bystricky et al., 2005).

**Focus stability**
Due to the high numerical aperture ($N_A$) and large magnification of the lenses used, the depth of focus is very shallow and any change of the specimen position along the optical axis ($z$-direction) becomes immediately visible. For long time-lapse observation either permanent manual focus adjustments and/or a high degree of focus stability is absolutely necessary. Two main causes for focus changes and instability were observed: Changes in the focus position of the microscope and on the basis of instability in the preparation. The stability and focus position of the microscope hardware is strongly influenced by temperature. Therefore, having a stable environment is crucial for long-term investigations. In our case, the microscopes are placed in rooms that have a sufficiently constant temperature ($\sim 20^\circ$C). Additionally, most of the experiments were carried out in a temperature controlled box that maintains the stage, optics and stand of the microscope at $30^\circ$C (LIS, Reinach BL, Switzerland). Another source for temperature changes becomes obvious when comparing the Olympus IX-70 microscope based system (TILL Photonics, Germany) to the Zeiss LSM510 which is based on an Axiovert 200 (Zeiss, Germany): Whereas the Olympus microscope is a mostly mechanical device and the focus was driven by a piezoelectric crystal, the LSM510 has many more motorized parts with complex electronics and a power transformer within the base of the microscope. As a consequence, this microscope requires longer to heat up to a stable temperature. This is achieved by turning it on $> 3$ hours before the start of the time-lapse recording and by switching on the temperature controlled box. This helps stabilize the sample and the microscope. Olympus microscopes have screws to fix the focus drive and they should be tightened in order to reduce focus hardware driven focus drifts. The other source of focus instability observed was the specimen itself. The use of a liquid perfusion chamber is recommended (Ludin Chamber Type I, LIS, Reinach BL,
Switzerland) and could circumvent many problems of agarose pad preparations. They have the advantage of a relatively large and more stable volume of growth medium which can be easily exchanged (even during image acquisition). Best results were obtained by medium exchange through gravity because all peristaltic pumps assayed caused pressure changes within the Ludin chamber, and ensuing focus shifts. Only a qualitatively well-prepared agarose pad stays stable for several hours. Liquid loss due to insufficient sealing or too little liquid for the amount of agarose used lead to shrinking of the agarose pad and thereby to a change of the optimal focus position in the according direction. Conversely, excessive liquid or overly dry agarose leads to swelling. Production of gas and local heating of the sample can also cause focus shifts.

A frequent source rapid focus shifts but also of changes in signal quality common to all preparations are small air bubbles in the immersion oil. It is thus important that the small drop of immersion oil placed on the objective or the cover slide does contain absolutely no air bubble.

Precise preparation of the samples is equally important as the microscope stability and only an optimal combination of the two is sufficient for time-lapse recording over long time periods.

5.4 Quantification of locus mobility

5.4.1 Image analysis software

As described in figure 5.1, the workflow from image acquisition to the final analysis of mobility comprises several steps which are performed in the LSM software (Zeiss, Germany), ImageJ (Rasband, 1997-2005), Mathematica (Wolfram Research) and Excel (Microsoft). In selected cases, Imaris (Bitplane, Switzerland), Photoshop
(Adobe) and Metamorph (Universal Imaging) were used for deconvolution and further visualization. This workflow to analyze locus mobility still can be optimized. Using more complete Mathematica notebooks generating one large Excel-compatible output file or further automating steps in the ImageJ could improve the time of analysis significantly.

**ImageJ**

The public domain ImageJ software (Rasband, 1997-2005) provides a simple but powerful assembly of software functions for 2D image treatment and analysis. It is publicly available easily expandable or adaptable for specific functions by the use of custom made plug-ins. This made it an ideal environment to implement the algorithms developed by Daniel Sage and Michael Unser (EPFL, Lausanne, Switzerland). In a collaborative effort, they developed a powerful software tool to reliably track fluorescent spots within the nucleus was developed and made publicly available as a Java Plug-in. The position of the fluorescent spot is determined using a best-fit algorithm using several parameters as spot intensity, shape, position of adjacent signals, or mobility it can be reliably tracked. In order to correct for drift of the nucleus or the cell, the center of the nucleus is calculated for each frame. Normalization of the spot coordinates by the coordinates of the nucleus results in the precise absolute coordinates of the fluorescent spot for every time point. Nuclear rotation is minimal and can be neglected for the timescale of image acquisition, as shown by photobleaching studies (Bystricky et al., 2004). The Java Plug-in is available under (http://bigwww.epfl.ch/sage/software/), the principle is described in Chapter 5.2 and all technical details are explained in (Sage et al., 2003).

Practical considerations for tracking with Spottracker:
Different growth conditions importantly influence the GFP signal strength and stability significantly. For example, cells growing on galactose containing minimal media show faster fading and weaker signals. Most mutant strains exhibiting growth defects also have weaker GFP signals: as an example Chapter 2 one notes that the slightly toxic effect of the expression of transcriptional activators (VP16 and Gal4 activation domain) reduced signal/noise ratio. Weaker signals also decrease the tracking fidelity of the Spottracker ImageJ Plug-in described above. Routine frame by frame validation of the tracking result that becomes more time consuming in these cases and leads to more user interventions during the tracking process. Generally, bad quality movies can cause slightly longer path lengths and an up to 10 % higher for the first time interval, although we noted no impact on other parameters. Different tracking parameters can be adjusted as described in (Sage et al., 2003). In our studies they were set as follows: cone aperture: 6, normalization factor: 60; center constraint: 20; movement constraint: 20. (On the other hand, when working with a strain lacking nuclear pore-GFP, where the center is determined by the tetR-GFP signal, the center constraint slider was set to 0).

Two parameters are particular crucial to maintain constant when comparing different experiments. The first is the movement constraint parameter which can directly influence step size since it is a measure of spot mobility, the second the center constraint value which can have a similar, though less pronounced effect.

If fluorescent signals from two nuclei are present in the same field, the nucleus to be analyzed can be delimited by selecting it with a box or circle. Hence the quantification will only take into account those pixels and center tracking errors are prevented.
Moreover, it is important to note that output format has changed with earlier versions of Spottracker module. It is therefore critical to check the content of each column and adjust it correspondingly in the Mathematica notebook.

Mathematica

Mathematica (Wolfram Research, Inc., Champaign, IL, USA) was used to do most calculations that are based on the absolute coordinates of the fluorescent spot. A notebook programmed by Marek Blasczcyk (University of Lausanne, Switzerland) was used to record and compute a range of parameters discussed below.

The notebook standardized basic calculations, but the following parameters have to be changed by hand for every notebook.

1. The name of the series of movies (header and file name).
2. The path (directory) and the individual file names of the Spottracker output files containing the coordinates of the nucleus and the fluorescent spot.
3. Check that the X and Y coordinates marked in the notebook correspond to the output file format. There are different formats, depending on the version of Spottracker used.
4. Adjust the time scale or pixel size if different from 1.5 s or 100 nm.

The notebook calculates all relevant parameters (discussed below) and presents them graphically. A subset of them is exported into different output files which can be read by other programs, such as Excel. These files comprise statistical values such all step size parameters (see above) or the MSD plots, all are subsequently compiled in Excel. This complicated procedure has the following possible pitfalls:

1. There are different formats of input files resulting from different versions of the Spottracker Plug-in. Caution must be taken because they differ in the order
of the input data. Therefore they can not be mixed as this leads to false
description of movement by the notebook.

2. Many auxiliary functions for the analysis notebook are assembled in a
Mathematica Add-on (*microscopy.m* written by Marek Blaszczyk). It has to be
placed in the Mathematica Add-on folder.

**Excel**

Excel is used to compile, compare and summarize all different output data, *i.e.* the
different MSD curves and the numbers resulting from the quantitative analysis of the
movies.

**Imaris**

This software was solely used to visualize locus mobility in 3 dimensions over time.
The MeasurementPro and Tracking modules allows volume and surface rendering
over time and were instrumental for creating the 3D tracks (see Chapter 3). Due to the
large z spacing of the original images, additional z-slices were interpolated which
gives a better impression of the actual 3D trace. Quantitative analysis turns out to be
extremely difficult and time consuming since the spot-tracking and can solely be done
by applying an intensity threshold. This makes it impossible to reliably track larger
amounts of data but allows very intuitive visualization of single movies of outstanding
quality.
5.4.2 Tracking of marked chromatin loci

The tracking process is described in Chapter 5.2, 5.4.1, and in (Sage et al., 2003). As noted above, tracking efficiency and accuracy depends on movie quality, which again depends on ideal growth conditions. It has to be noted that within different cells of the same strain we observe natural variations are in spot intensity but also in locus mobility.
5.4.3 Analyses based on locus coordinates

The main parameters are explained in section 5.2 but here, we introduce additional or modified parameters, based on the coordinates of the locus over time. These relate to the analysis of the 1.5 s step size or to the mean square displacement (MSD) analysis. At the end of this section, one general parameter resulting from the MSD analysis, the diffusion coefficient is discussed in more detail and is compared with other methods of quantification.

Step size analysis

The normalized coordinates allow direct calculation of the step size for different time intervals. The most detailed analysis of single step size is the box plot (a.k.a. Box-and-Whisker plot, Figure 5.2.A). It is a histogram-like method of displaying data (Tukey, 1977). The central box is delimited by the quartiles Q1 and Q3 and the statistical median M forms a horizontal line in the box. From this box, the "whiskers" extend to the farthest points that are not outliers (i.e., the upper and lower percentile). Outliers are marked with a dot at the corresponding position. A typical box plot is shown in figure 5.2.A. Since we sought to find differences in the mobility of tagged telomeric regions, Tel6R, and Tel3R/HMR, a detailed step size analysis was performed, analyzing step sizes of all 1.5 s intervals. Since we know from the position analysis that both loci are enriched at the nuclear periphery (chapter 3 and Hediger et al., 2002) analysis was also performed on differences in displacements along the radius. The box plots show two things: 1) there are no significant differences in mobility between both loci and 2) in both cases there radial component on mobility is below the expected value lower than expected for a free projected random walker,
implying more movements along the nuclear envelope. More details on the comparison between these two loci are given at the end of section 5.5.3.

Since in most cases all parameters represented by the Box-plot change in the same manner, it is sufficient to compare the median of the step sizes. Because the tracking in general can be influenced by image quality, it is important to assure that differences observed in the Box-plots do not simply reflect image quality.

Additionally, we have also reported value of the mean step size. But empirically the mean was shown to be less conclusive than the median and was therefore not reported in recent studies.

Finally, step size cannot only be compared for 1.5 s intervals but can also be assessed for larger time intervals. A value which has turned out to be discriminative is the number of steps which are larger than 0.5 μm in a 10.5 s interval. The frequency of these large steps is given for 5 min. As shown in figure 5.1.B large movements occur much more frequently at more mobile telomeres and maximally for transcribed internal sequences. This value not only increases with higher mobility and less constraint but it may also reflect directed movements as speculated by (Heun et al., 2001b).

Although we can detect several steps of movement in the same direction, we could not find a preferential orientation (e.g. along the radius). For geometrical reasons this was not likely. Locus movement was tracked on 3D images projected 2 dimensions, since resolution is much better in the x- and y-dimension (discussed above). The drawback of the method is that during the projection process, 3D information is lost – making it difficult to address questions of directionality. In order to address this question reliably, z-stacks with smaller spacing followed by deconvolution have to be taken - at the cost of imaging speed.
Mean square displacement analysis

The mean square displacement is a measure of the average distance a molecule travels over given time intervals. It is based on the random walk model, for which the MSD increases only with time. The rate of growth of the MSD depends on how often the molecule suffers collisions, i.e. at higher density; it will take longer to diffuse a given distance, as other molecules continually impede its progress. This linear slope directly reflects the diffusion coefficient of the molecule as discussed below. In the yeast nucleus however, DNA is not free to move in an infinite space. Its mobility is spatially restrained by 1) nuclear volume, 2) nuclear compartmentalization, 3) specific anchoring sites on DNA, and 4) the contiguity of the chromosome fiber. Therefore at longer time intervals, the MSD graph is no more linear and converges to a plateau value which characterizes the confinement a locus observes. In locus mobility analysis in yeast, this saturation value of the MSD at long time intervals \(<d^2(\infty)>\) is reached in many though not all cases within 150 s (which is the largest time interval measured as discussed below). In our quantifications, we define the radius of constraint based on the highest MSD value even if no visible plateau is reached yet. Theoretical aspects of the ratio between \(<d^2(\infty)>\) and the radius of constraint (\(R_C\); equation 5.2) or when a plateau is reached are discussed in section 5.5.

Initial MSD \(<d^2>\) analyses were performed in one dimension only and the distance to the locus was measured from the periphery along the radius. Plotting the radial \(<d^2>\) over time intervals results in what we refer to as radial MSD analysis (Heun et al., 2001b). It shows to which extent a locus is constrained by the periphery, but cannot discriminate between different movements along the nuclear periphery, i.e. if a given locus always stays at the same spot or moves along the nuclear envelope. Analysis of subnuclear position can be more precisely be determined by
quantification of a large number of cells (described in 5.2) and information about mobility is more accurately given by the “absolute” MSD analysis which is based on the coordinates from 3D projections as described above.

The diffusion coefficient is proportional the slope of the MSD graph (for details see Chapter 5.4.4). Due to variations of shortest time interval we found that D of an interval from 1.5 to 12 s gave more robust and distinctive results than the slope defined by the origin and the 1.5 s time point. Nevertheless, the initial points of the MSD graph are mainly linear. Some plots show a second linear section with a lower slope at larger time intervals, characteristically at 50 to 100 s intervals. Nevertheless, in our hands, none of these turned out to give conclusive results.

Generally it can be said that the maximal time interval used in the MSD analysis should not exceed one third of the length of the entire movie after this the error becomes larger due to less averaged squared displacement values. Furthermore, there are variations between MSD analyses of individual movies (see figure 5.3 and section 5.5). We therefore recommend averaging the MSD graphs from > 8 individual cells over a total time of > 1 h to get more robust MSD curves.

MSD graphs resulting from simulated constrained random walks gave further insights to the behavior of MSD graphs. They are discussed in section 5.5 together with possible refinements of the model as well as in Chapter 2 (Figures 5.3, 2.1 and section 5.5).

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Figure 5.2. Analysis of Tel3R and Tel6R mobility based on 11 and 10 movies of 300 frames respectively. Data were acquired as described in this Chapter and cells were grown in galactose containing minimal media. A) Whisker-and-Box plot showing the distribution of 1.5 s step sizes. B) Frequency of large steps (>500 nm / 10.5 s) in 5 min, for projected 3D and radial distances. C) The MSD plot for Tel3R and Tel6R.
(curves averaged over). The Box indicates a close-up of the initial 12 s of the curves. The red lines indicate the linear regressions leading used for the calculation of diffusion coefficients. **D)** Representative traces of 5min movies of Tel6R and Tel3R. **E)** Schematic representation of confinement of a tagged chromosomal site (yellow dot) to a simple spherical cap on the nuclear membrane. Due to diffusive motion the dot occupies different locations at different data acquisition times. Projections onto the xy plane are indicated. **F)** The dots are calculated values of $\langle d^2 \rangle(\infty)$ for spherical caps of increasing surface area. The intersection with the experimental observed values of $\langle d^2 \rangle(\infty)$ suggest that Tel3R explores a spherical cap that covers about 10% of nucleus, while the cap for Tel6R is nearly twice as large.

### 5.4.4 Diffusion of chromatin loci

There are three main approaches to measure diffusion coefficients by fluorescence microscopy *in vivo*: a) Various techniques using photobleaching (FRAP, FLIP or CP), b) fluorescence correlation spectroscopy (FCS) and c) techniques based on Single Particle Tracking (SPT). They differ in the methods used but also the time scale of the experiment and are hence complementing each other. A good comparison of the methods is found in (Wachsmuth et al., 2003). Chromatin mobility was measured using all three approaches which are described below. Finally, diffusion coefficients can be calculated based on the Stokes-Einstein equation (5.3). An overview of the diffusion coefficients resulting from the different methods is given in table 5.1 and is discussed at the end of this section.
Fluorescence correlation spectroscopy (FCS)

The most rapid method is Fluorescence Correlation Spectroscopy. FCS measures the steady-state concentration fluctuations in a microscopic observation volume. Intensity changes due to diffusion of fluorescent molecules into and out of the observation volume allow quantification of the concentration and the diffusion coefficient of the fluorescent molecule. FCS in living matter has a time resolution of milliseconds. In vivo FCS measurements on marked yeast chromosomes, point to diffusion coefficients for tagged loci of $0.139 \pm 0.011 \mu m^2/s$ which corresponds to much faster diffusion than estimated by SPT (L. Gehlen and J. Langowski, personal communication).

Techniques using photobleaching

These techniques are based on the principle that light can irreversibly shift a fluorophore to its non-fluorescent state. A very popular method is fluorescence recovery or redistribution after photobleaching (FRAP). Bleaching a defined portion of a fluorescent signal leads to the loss of fluorescence in this region. The kinetics at which fluorescence in this region recovers or redistributes in the bleached region allows calculating its diffusive properties. Related methods measure the decrease in fluorescence intensity of the unbleached region (FLIP) or the gradual decrease in intensity upon continuous photobleaching (CP) with much lower intensity. The latter is advantageous for very rapidly diffusing molecules. Using FRAP and related methods allows determining the mobility of fluorescently tagged molecules in living cells. The following parameters can be quantified: diffusion coefficient, immobile fraction and binding or residence time of the tagged molecule of interest (Axelrod et al., 1976; Carrero et al., 2003; Houtsmuller et al., 1999). Chromatin mobility has been
measured by a specific FRAP approach (two-photon counterpropagating fluorescence recovery after patterned photobleaching (2P-c-FRAPP) developed by Davis and Bardeen. Measuring Hoechst stained DNA in vivo results in an mean diffusion coefficient of $5 \times 10^{-4} \, \mu m^2/s$ (Davis and Bardeen, 2004). This is in agreement with SPT data in other cell culture systems (see table 5.1 and below).

**Single particle tracking (SPT)**

Diffusion coefficients for two dimensional Brownian motion of chromosomal loci can be calculated by averaging the square displacements as done above and based on equation 5.1 (Berg, 1993; Saxton, 1997).

\[
<d^2>_n = 2n \, Dt
\]  

(5.1)

Where $<d^2>$ is the mean square displacement over time ($t$), and ($n$) is the number of dimensions. The diffusion coefficient ($D$) at a time point is therefore proportional to the slope of the MSD graph at the corresponding time interval. For a random walk in 3D, the diffusion coefficient ($D$) equals $<d^2>_{3D}/6t$. In order to calculate $D$ in 3 dimensions, the MSD values measured on images resulting from 3D projections ($<d^2>_{3Dproj}$) have to be multiplied by a factor of 1.5 (see paragraph 5.5.2). Therefore the equation for 2D random walks is equal to the value for projected 3D random walks (see equation 5.2).

\[
D = <d^2>_{3Dproj}/4\Delta t
\]  

(5.2)

Interestingly, a linear regression of the initial points in the MSD analysis does not pass by the origin (0) but crosses the y-axis in the positive range (see blow up and red lines in figure 5.2.C). Therefore diffusion coefficients for time intervals shorter than 1.5 s must yet be higher for which evidence comes also from FCS measurements (see
above). The hypothesis is further supported by measurements based on SPT on isolated bacterial nucleoids containing a lac operator marked chromosome at a time-resolution of 48 ms, although the artificial situation has to be pointed out (Cunha et al., 2005).

**Calculation using the Stokes-Einstein equation**

Molecules in solutions are undergoing constant diffusion. This diffusion is the result of thermal fluctuations in the suspension and is often referred to as “Brownian motion”. Therefore, theoretical diffusion coefficients can be calculated based on the hydrodynamic radius of the particle (R_H) and the viscosity of the medium (η) using the Stokes-Einstein equation (5.3). Consequently, experiments on the excised chromatin ring containing lac operators (as described in Chapter 3) allow the calculation of a theoretical diffusion coefficient.

\[
D = \frac{kT}{6\pi\eta R_H}
\]

(5.3)

- \( k \) = Boltzman’s constant \( (1.38 \times 10^{-23} \text{ Nm/K}) \)
- \( T \) = absolute temperature \( (273.15 \text{ (0°C)} + 30\text{°C} = 303\text{K}) \)
- \( \eta \) = viscosity of the medium (\( \eta_{\text{nucleoplasm}} = 4 - 6.6 \text{ ePoise (Ns/m}^2) \))
- \( R_H \) = hydrodynamic radius of the particle (\( R_{H_{\text{chromatin ring}}} \) see text)

The nucleoplasmic viscosity has not yet been estimated for yeast. For mammalian cells, range between 4 and 6.6 ePoise have been reported (Lang et al., 1986; Lukacs et al., 2000). Indeed, \( R_H \) is the most difficult parameter to estimate. \( R_H \) reflects the radius of a particle or polymer in solution. For double helical DNA, 1 bp = 0.34 nm, so for a 16.5 kb stretch (the size of the ring resulting from excision of HMR and the lac operators), the total length is 5.61 µm. Bystricky and coworkers describe the average
compaction ratio of yeast chromatin as ~40 fold (Bystricky et al., 2004). Calculating the radius for a 40 fold compacted circular unit, with a circumference of \(2\pi r\), gives \(r = 22\) nm. This value corresponds to the physical radius of the 16.5 kb chromatin cycle which is calculated based on its molecular weight and its density described below. However, since the chromatin ring not only consists of DNA but also associated proteins (for calculation of the molecular weight see below) and since a strict globular shape of the ring is unlikely, the hydrodynamic radius of the complex must be actually larger. Similarly, the radius of the mRNP of poly(A)RNA could be estimated four times larger than the poly(A)RNA alone (Politz et al., 1998)). A very rough estimation for the \(R_H\) would result in a value between 22 and 100 nm, resulting in a diffusion coefficient between 0.6 and 2.6 \(\mu m^2/s\). We notice that \(R_H\) is smaller than the size of the fluorescent spot which is ~200 nm.

The minimal \(R_H\) can further be calculated based on its density and its molecular weight. This can be estimated based on the following assumptions: DNA size: 16.5 kb; MW\(_{DNA}\): 610 kDa /kb; MW\(_{nucleosome}\): 110 kDa; nucleosome spacing: 165 bp / nucleosome, MW\(_{lac\text{-GFP}}\): 66kDa (lac\text{3}-GFP) \(\times\)256 (lacOp); (MW\(_{HMR\text{-proteins}}\) = 330 kDa; size HMR: 12 nucleosomes, SIR occupancy: 4 complexes / nucleosome). Based on these assumptions, the silent ring has a molecular weight between 43 and 54 MDa and the excised ring from the \(LYS2\) locus has an estimated molecular weight between 31 and 40 MDa. Given the fact that densities of proteins can vary a lot, HMR rings are estimated to have a similar density as ribosomes because they co-sediment in biochemical ring purification (personal communication, M.Gartenberg). The ribosome has a molecular weight of ~2.5 MDa and an estimated volume of 2.68\(\times\)10\(^3\) nm\(^3\). Based on this we can estimate the volume of the ring as 3.9\(\times\)10\(^6\) nm\(^3\) and its radius as 21 nm (which is the minimal radius for a HMR ring of strictly globular shape).
Interestingly, based in MSD analysis, we cannot distinguish between silent and non-silent chromatin rings (see Chapter 3), indicating that their \( R_H \) must be in the same order of magnitude.

Further indications for the size of \( R_H \) can be taken from the radius of gyration \( (R_G) \) which is smaller but linked to the \( R_H \). It has been described for isolated DNA fragments and plasmids of different size and is a measure of the size of a polymer molecule. \( R_G \) can be defined in terms of the distribution of distances (in any direction) of each monomer in the molecule from the centre of gravity of the molecule, \textit{e.g.} for a 10.5 kb (plasmid) \( R_G = 220 \) nm (Goodman et al., 2002). Furthermore, \( R_G \) can be calculated based on the persistence length \( (L_P) \) which is a measure for the stiffness of the fiber and the contour length of the molecule. Assuming that the ring has the same conformation as determined in (Bystricky et al., 2004), \( R_G \) can be calculated using equation 5.4.

\[
R_G = \sqrt{\frac{L \times L_P}{3}} \quad (5.4)
\]

This results in a radius of gyration \( (R_G) \) of the 16.5 kb chromatin ring between 79 nm and 105 nm.

\( R_G \) and \( D \) have also been determined \textit{in vitro} in aqueous solution (Smith et al., 1996). Not surprisingly, for linear DNA molecules, \( D \) lies above the values described for chromatin by FCS. Values from different methods are summarized in Table 5.1.

When comparing the data we quantified by MSD analysis with other data obtained in with similar methods (Bornfleth et al., 1999; Chubb et al., 2002; Marshall et al., 1996; Marshall et al., 1997) we find slightly larger diffusion coefficients (see table 5.1). This may result from several parameters: In yeast, centromeres tend to be tethered near the spindle pole body (SPB Bystricky et al., 2005; Jin et al., 2000). Therefore not surprisingly, the LEU2 locus which lies close to a centromere is more constrained in
movement than loci which are located along the arms of chromosomes. Differences to mammalian cell culture systems are more speculative, but either tracking quality or different chromatin compaction may account for the slight differences observed. Fast measurements and calculation using the Stokes-Einstein equation (5.3) shows much larger D than all SPT measurements described above. We therefore speculate that in the ms timescale there are fast movements which we cannot observe due to insufficient time resolution. This hypothesis is supported by the finding that a linear regression through the first time points does not pass through the origin but intersects the y axis. Only faster microscopy will help to prove this hypothesis.

**Conclusion on the calculation of diffusion coefficients for chromosome loci**

It can be said that the diffusion coefficients measured with SPT lie in the same range than published previously for longer time intervals (see table 5.1). We hypothesize that time intervals shorter than 1.5 s diffusion coefficients are larger. This increased diffusion has indeed been measured by alternative methods such as FCS or calculations based on the Stokes-Einstein equation (5.3). Very rapid SPT also points in this direction and will test the above hypothesis (personal communication John Sedat UCSF, USA).

In future, also the models used to quantify or calculate different parameters of mobility have to be refined. Notably, aspects of diffusion in non-homogenous media have to be included since it is known that nucleoplasmic viscosity can be variable. This can be due to macromolecular crowding (in more dense parts such as heterochromatic regions or nuclear bodies) and due to the fact that molecules of different sizes experience different local viscosity due to the sieve-like structure of
nucleoplasm (Braga et al., 2004; Weiss et al., 2004). Models refining the basic Brownian motion include reptation, random walks through fractal-like environments, effects of volume exclusion, or non linear random walks describing the interplay between diffusive motion, various binding events and active processes will give more insight into the diffusive behavior of tagged DNA loci.
<table>
<thead>
<tr>
<th>Method</th>
<th>Time scale</th>
<th>Diffusion coefficient [μm²/s]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS: <em>in vivo</em>, yeast Lac⁰ᵖ / lac⁰ˡ</td>
<td>1 ms</td>
<td>0.14</td>
<td>L.Gehlen, J.Langowski pers. communication</td>
</tr>
<tr>
<td>Lac⁰ˡ-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPT (of Lac⁰ᵖ / Lac⁰ˡ-GFP) in bacterial nucleoids</td>
<td>48 ms</td>
<td>0.15</td>
<td>(Cunha et al., 2005)</td>
</tr>
<tr>
<td>§ SPT (<em>in vivo</em>, yeast Lac⁰ᵖ / Lac⁰ˡ-GFP) LYS2 locus</td>
<td>1.5-12s*</td>
<td>1.45×10⁻³ / 5.00×10⁻³</td>
<td>This study</td>
</tr>
<tr>
<td>chromosomal / ring</td>
<td>24 s#</td>
<td>1.81×10⁻³ / 4.04×10⁻³</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 s#</td>
<td>1.56×10⁻³ / 3.63×10⁻³</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 s#</td>
<td>1.24×10⁻³ / 2.20×10⁻³</td>
<td></td>
</tr>
<tr>
<td>SPT (yeast) LEU2 locus</td>
<td>24 s</td>
<td>3×10⁻⁴</td>
<td>(Marshall et al., 1997)</td>
</tr>
<tr>
<td>SPT (neuroblastoma nuclei, BrDU injection)</td>
<td>20 min</td>
<td>1.94 × 10⁻³</td>
<td>(Bornfleth et al., 1999)</td>
</tr>
<tr>
<td>SPT human cells 5p14 locus</td>
<td>60 s</td>
<td>1.25×10⁻⁴</td>
<td>(Chubb et al., 2002)</td>
</tr>
<tr>
<td>SPT Free DNA in dilute solution</td>
<td>n.s.</td>
<td></td>
<td>(Smith et al., 1996)</td>
</tr>
<tr>
<td>23 kb (linear) R_G = 520 nm</td>
<td></td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>9.4 kb (linear) R_G = 310 nm</td>
<td></td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td>°FRAP (Hoechst stained chromatin in <em>Xenopus</em> cells)</td>
<td>n.a.</td>
<td>5×10⁻⁴</td>
<td>(Davis and Bardeen, 2004)</td>
</tr>
<tr>
<td>FRAP eGFP (nucleoplasmic)</td>
<td>n.a.</td>
<td>58 ± 9 μm²/s</td>
<td>(Houtsmuller et al., 1999)</td>
</tr>
<tr>
<td>Stokes-Einstein equation</td>
<td>n.a.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R_H = 22/ 100/ 200/ 400 nm</td>
<td></td>
<td>2.64/ 0.55/ 0.28/ 0.14</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.1.** Comparison of diffusion coefficients measured by different methods. Details are marked in the text. * Calculated using the slope of the 1.5-12s time interval; # calculated for comparison with other data based on the slope between the origin (0); § since in an obstructed random walk D changes with time, values are given for different time intervals; ° using two-photon counterpropagating fluorescence recovery after patterned photobleaching at a 100 nm resolution; n.s.: not specified in the literature; n.a.: not applicable.
Chapter 5

5.5 Modeling of yeast chromatin mobility

This section is a summary of collaborative efforts to characterize chromatin mobility by modeling. Initial work with to simulate constraint random walks in 2D was done with Marek Blaszczyk (University of Lausanne, Switzerland). Jonas Dorn (Scripps Research Institute, La Jolla, USA) has resolved the integral to get a direct correlation between the plateau of the MSD analysis and the radius of constraint. Angelo Rosa and Andrezj Stasiak (EPFL and University of Lausanne, Switzerland) did further calculations on diffusion constrained by the nuclear envelope. Lutz Gehlen (Gasser group FMI, Basel, Switzerland) developed the equations describing the progression of the MSD curve for a constrained random walk and calculated the theoretical correlation the time a given MSD curve reaches its saturation level for different modes radii of constraint and diffusion coefficients.

5.5.1 The random walk model and its limitations

All calculations below are based on the model of a random walk in a confined volume. They were done in the aim to better understand the measured locus movements and to evaluate if these measured movements could be approximated by simple random walks. Some simplifications are made which have to be reconsidered in, more elaborate models in the future:

1. The viscosity of the nucleus is not uniform, i.e. different particle sizes do experience different viscosities. This can be exemplified by a sieve model through which small molecules can pass but larger ones get entangled.
2. The distribution of step sizes in basic random walk model the step sizes should follow a Gaussian distribution which is clearly not the case.
3. The radius of confinement deduced from the MSD plot defined a spherical volume of confinement. We are aware of the fact that this model simplifies the actual movements and regions of confinement. Models have to be developed to describe chromatin movements more accurately. Furthermore chromatin dynamics are locus dependent, e.g. telomeres which are partially attachment at the nuclear envelope – suggesting that different loci may require different models.

5.5.2 Geometrical considerations:

Random walks in 2D, 3D and 3D projections

The random walk of chromatin in yeast was simulated in several ways. First, we simulated this in 2D, confined within a circle, using a step size distribution similar to our measurements and uniformly distributed angles. To a limited extent this corresponds to the movies described in the previous Chapters. Thereafter, we expanded the model to 3 dimensions, creating a randomly generated positions walk in a sphere. Here again the direction was determined (by choosing among uniformly distributed points on a sphere) and the corresponding step size was added. This allowed to directly correlate our measurements in 3D and on z-projections in 3D to simulated data, since all microscopic data are either acquired in 3D and projected or alternatively, the optical section is widened (e.g. by opening the pinhole) resulting in a “optical projection” in the z dimension.

MSD-curves from 3D projections can be easily converted to absolute 3D curves by multiplication with a factor of 1.5 (Lutz Gehlen, personal communication). This justifies the use of MSD-curves resulting from analysis of 3D time lapse movies projected in the z dimension. Since tracking coordinates in x and y dimension is more
accurate and since there is no preference in special orientation the z dimension can be extrapolated if needed.

**Step size and border effects**

In order to get a random walk in a confined volume the direction of movement is determined at every step based on a uniform distribution of spots on a sphere. Both fixed and variable step sizes can be used. If a movement exceeds the limits of the confinement sphere (or circle), the step is rejected and a new one is determined. Once a spot is close to the nuclear periphery, the probability to be rejected is higher for large step sizes than for small ones, what is known as border effects. As mentioned, the effect gets more pronounced for large step sizes (typically, if they are >10% of the radius of constraint). Therefore, simulations were performed with sub-steps of 10 nm in size. 120 such steps correspond to one 100 nm step on the 3D-projection, which the value determined experimentally and which was used in previous simulations. For calculations of D according to formulas 5.1 and 5.2, one has to state that they only match exactly free random walks, even though the deviation at short time intervals is minimal.

**5.5.3 Random walk simulations**

The actual calculations were programmed either in Mathematica (Wolfram Research) or in Perl (www.perl.com).

When performing a random walk in 3D we had two modes of step size generation were evaluated. Either a Gaussian distribution was used; else one 1 s step was decomposed in 120 10 nm sub-steps. The latter is advantageous because of minimal border effects.
As mentioned above (equation 5.1), the diffusion coefficient of a free random walk is proportional to the linear slope of the MSD plot. Contrary to a free random walk, the slope of the MSD plot of a confined random walk is not linear, i.e. it depends on the time interval observed. For short time intervals non-linearity is barely detected, but it gradually increases with time until it ultimately will approximate a plateau (with a slope of 0). In order to determine diffusion coefficient (which depends on the slope of the plot), it is therefore important to indicate the time interval it was measured in (see above). Due to experimental constraints we define diffusion coefficients for the 1.5-12 s intervals. Since the “linear” part of the MSD curve, decreases with small radii of constraint, the simulations could confirm linearity even for constrained loci.

In order to properly interpret the MSD graphs it is important to know at what time point the radius of constraint is reached. Again random walk simulations can indicate the range when this plateau level is reached for varying radii of constraint. It depends on the $R_c$ on one hand and on the step size (i.e. on the slope of the curve) on the other.

We set the step size to a fixed value of 100 nm which corresponds to the value measured for an internal locus ($ATG2$). We consider the plateau to be reached, when the value is larger than 95 % of the maximal value. Empirically the value shows quadratic dependence and corresponds to equation (5.5).

$$\Delta t_{\text{max MSD}} = 0.00033 \ R_c^2 \quad (5.5)$$

We can conclude that for an $R_c$ of 600 nm the plateau is reached after 115 s, for the maximal $R_c$ measured (850 nm) the plateau would be reached after time intervals of 230 s only. Nevertheless, in the experiments the plateau is reached at much shorter time intervals since the step size is twice as large as assumed for this calculation ($LYS2$ ring; Figure 2.1). This Justifies the movie length of 7.5 min and the maximal time interval in the MSD analysis of 2.5 min from a theoretical point of view.
Calculation of the radius of constraint

Because MSD graphs are the most robust form of evaluation for our time lapse movies, it is important to determine the radius of constraint corresponds to a specific plateau value in the MSD analysis. When basing the simulation on a radius of constraint we can precisely correlate the MSD-plateau with a corresponding $R_c$. However, since border effects can never absolutely be excluded and since estimations from the above simulations do not give a precise relationship, it is more accurate to solve the problem analytically. Based on a random walk model (i.e. equal distribution of points within a sphere and projections on one equatorial plane) Jonas Dorn (Scripps Research Institute, La Jolla, USA), set up an integral equation to calculate the radius of constraint (Equation 5.6). This is based on the mean square distance between two points with the spherical coordinates $(\rho_1, \tau_1, \varphi_1)$ and $(\rho_2, \tau_2, \varphi_2)$ which lie within a sphere of radius $R$ (which is the radius of confinement). Projection onto the xy plane results in the $r_1 = (\rho_1 \sin \tau_1)$ and $r_2 = (\rho_2 \sin \tau_2)$ defining the projected points by $(r_1, r_2, \varphi_1, \varphi_2)$.

\[
\text{MSD}_{\text{plateau}} = \frac{1}{C^2} \int_0^R \int_0^{2\pi} \int_0^{2\pi} \sqrt{R^2 - r_1^2} \sqrt{R^2 - r_2^2} (r_1^2 - 2r_1r_2 \cos(\varphi_1 - \varphi_2))r_1r_2d\varphi_1d\varphi_2dr_1dr_2
\]

\[
= \frac{4R^2}{5}
\]

This equation could be experimentally confirmed with simulated random walks in a confined volume.

Mathematical description of the MSD curve

Interestingly, the simulated MSD curves can be also described by equation (5.7) (personal communication Lutz Gehlen).
Where (a) determines the height of the plateau and (b) corrects for the slope of the curve.

A comparison between simulated and calculated curve is shown in figure 2.1 underlying the nice fit for a freely diffusing chromatin ring. For all chromosomal loci, the measured MSD curves are flatter, quantifying the additional constraint which is exerted by the context of the fiber (Figures 5.3 A-B and 2.1).

**Figure 5.3.** Experimental and simulated confined random walks and the corresponding standard deviation of the mean. **A)** MSD plots of eight single 7.5 min movies of the *LYS2* locus and **B)** simulations based on the same step size and MSD saturation level (100 nm/s; 0.3 μm²/s) are presented with the corresponding average MSD plot and standard deviation of the mean (error bars).

**Conclusions and perspectives**

Simulations and calculations have led us to a better understanding of chromatin diffusion analyzed by MSD plots. We measured large variability between the single
MSD curves. On one hand they are inherent to the method (see figure 5.3.A), on the other, variability of chromatin diffusion was also observed by other methods (Davis and Bardeen, 2004).

Calculations have allowed us to justify the MSD analysis as a good tool to quantify chromatin mobility. We could establish a tight connection between the radius of constraint and the plateau of the MSD plot and estimate when $<\langle d^2 \rangle(\infty)$ should be reached. Within the time interval of 150 s a plateau was reached only by a subset of loci observed. This suggests that these loci experience different constraints and that eventually they diffuse in a subvolume that for extended periods can diffuse within the entire nucleus (e.g. RPL9A or ATG2, see figure 2.2).

To describe chromatin movements more precisely (i.e. to take into account the fact that saturation levels are reached with different kinetics or the nonlinearity between of the sub-1.5s time scale and our measurements), other models may have to be considered. These may include reptation, non linear random walks, or just take into account the heterogeneous environment of the nucleoplasm or binding events and active processes.

One idea was realized in collaboration with Angelo Rosa (EPFL, Lausanne Switzerland) and Andrzej Stasiak (University of Lausanne, Switzerland). Many chromatin loci are known to be anchored at the nuclear envelope as discussed in Chapter 3 and (Taddei et al., 2004). We therefore wanted to understand how exclusive peripheral localization and diffusion in 2D along the nuclear envelope influences the MSD saturation value $<\langle d^2 \rangle(\infty)$. As for internal movements we assume that movement is restricted to a sphere, of a locus which only moves along the periphery, we assume that its movements are restricted to a circular “cap” region at the nuclear envelope (Figure 5.2.E). Simulating different cap sizes over many different orientations led to a
precise attribution of the surface of confinement. The result of these simulations is shown in figure 5.2.F, where the area of the surface covered is plotted against $<d^2>(\infty)$. This contributed to the understanding that even among loci that are preferentially positioned near the nuclear envelope to a similar extent, locus mobility and the area of constraint can vary significantly. We compared mobility of two telomeres with known differences in mobility, but similar subnuclear localization. Interestingly, telomere 6R (Tel6R) covers the double surface area of telomere 3R (Tel3R) which lies near the HMR locus described in Chapter 3; see figure 5.2.F). A possible cause for this difference could be the particular levels of transcription or silencing, especially when growing on galactose. Whereas genes adjacent to the Tel3R/HMR tag are transcriptionally silent, the gene adjacent to the Tel6R tag ($HXK1$) is transcriptionally active in the conditions observed. Whether these facts are responsible for the differences in the covered surface area has to be investigated elsewhere.

5.6 Outlook and future developments

A key to better understanding of chromosome mobility by fluorescence microscopy is technical innovations. It is instrumental to increase resolution in space and time. Several techniques on the level of the microscope, camera or image analysis are being developed and will help to improve these issues. Next, a short selection of these improvements is described. Especially combining these techniques will ameliorate sensitive and rapid life fluorescence microscopy a lot (Garini et al., 2005).
5.6.1 The fluorophores

Despite the large variety of GFP variants or small chemical compounds used to label proteins in vivo, major efforts are focused on their improvement. One main focus of the work is to find GFP variants which are more stable, more resistant to low pH or which have new emission spectra. These developments will inevitably improve the sensitivity of the fluorophores and lead to increased temporal and spatial resolution. The current developments very carefully review by (Zhang et al., 2002).

5.6.2 The microscope

The improvements of spatial resolution are mainly due to revolutions in microscopy technology. Generally, resolution is determined by the optics of the microscope as first described by Abbe (Abbe, 1873). He described that diffraction of light at an object (which can be as small as a few nm) depends on the wavelength and the finite size of the objective lens. The intensity distribution of every small object is called the point-spread-function (PSF). The PSF usually has radial symmetry in the focal plane and a larger broadening in the optical axis. The Rayleigh criterion then describes the minimal distance of two points that can still be resolved. It is approximately equal to the width of the PSF and is defined (equation 5.8a and b), where λ is the wavelength of the light and NA is the numerical aperture of the lens, which is half of the observation angle of the lens multiplied by the refractive index n of the material that is between the lens and the sample.

Focal plane resolution: \[ d_{x,y} = \frac{0.61\lambda}{N_A} \] (5.8a)

Optical axis resolution: \[ d_z = \frac{2\lambda}{N_A^2} \] (5.8b)
The aim of any approach improving resolution thus is to make the PSF smaller. An image of a round fluorescent bead therefore results in an ellipsoid which is elongated in the z-axis.

The basic types of microscopy are discussed in section 5.1.1. In spinning disk microscopy recent developments allow to better focusing of the light onto the individual pinholes and therefore decrease the acquisition time.

As discussed above, for optical reasons the resolution along the optical axis (z dimension) is about three times poorer than in the focal plane (x and y dimensions). A revolutionary idea circumvent this stretch is the use of two opposing objectives with the thin sample plane in-between. Interference patterns produced by the overlapping spectra of both objectives lead This type of microscopy is known as 4 Pi microscopy and is now commercially available (Leica, Mannheim, Germany). With 4 Pi microscopy z-resolution can be improved to ~100 nm. Importantly in all approaches above resolution remains limited by the laws developed by Abbe.

Recent approaches aimed to reduce resolution below the classical resolution limit which is described in equation (5.8a and b). They are based on the physical concept of reversible saturable optical fluorophore transitions (RESOLFT) (Hell, 2003) and reviewed in (Garini et al., 2005). The best established technique based on this phenomenon is the stimulated emission depletion (STED) microscopy which uses two kinds of pulsed lasers. With the first laser excites the fluorophore is excited, with the second laser which has a doughnut-like shape, excited fluorophores are depleted back to the ground state. This technique has proven to resolve to points at 30 nm distance in the focal plane (Klar et al., 2000; Westphal and Hell, 2005). Theoretically in
RESOLFT microscopy, the resolution limits are not yet reached which opens possibilities for even better optical resolution.

### 5.6.3 The camera

Different suppliers have developed more sensitive but equally fast charge-coupled device cameras (Andor, USA and Hamamatsu, Japan). They are based on an electron multiplying step, similar to that observed in a photomultiplier tube in the confocal scan head (PMT), in front of every CCD for a standard CCD camera. This results in an amplification of the signal whereas the background remains unchanged. Generally, more sensitive cameras lead to shorter exposure times which in turn allow increasing the sampling frequency, number of slices in z or the absolute length of image acquisition.

### 5.6.4 Post acquisition techniques

In past years powerful deconvolution algorithms have ameliorated and have been implemented in standard imaging software such as Metamorph (Universal Imaging, USA) or Imaris (Bitplane, Switzerland) (Schaefer et al., 2001). Based on the measured or calculated PSF which is specific for every microscope setup, the out-of-focus haze is removed from the image after processing. Deconvolution seems to become a standard technique for treating wide-field fluorescent images captured on a CCD camera since in some cases these images are equally good as confocal images.
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