# *In Vitro* Studies of Nucleosome Positioning and Stability at the *PHO5* and *PHO8* Promoters in *Saccharomyces cerevisiae*

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A reprint of this paper can be found in the back of this thesis.

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# I. Summary

The *PHO5* and *PHO8* genes in yeast provide typical examples for the role of chromatin in promoter regulation. Both genes are regulated by the same transcriptional activator, Pho4, which initiates nucleosome remodeling and transcriptional activation. In spite of this co-regulation, there are important differences in gene activity and in the way promoter chromatin undergoes chromatin remodeling. First, *PHO5* belongs to one of the most strongly induced genes in yeast being 10-fold more active than the *PHO8* gene (Oshima, 1997; Barbaric et al., 1992). Second, chromatin remodeling at the *PHO5* promoter affects four nucleosomes (Almer et al., 1986), whereas only two nucleosomes are afffected at the *PHO8* promoter (Barbaric et al., 1992). Third, neither the histone acetyl transferase Gcn5 nor chromatin remodeling complex Swi/Snf seem to be critically required for chromatin remodeling at the *PHO5* promoter (Barbaric et al., 2001; Reinke and Hörz, 2003; Dhasarathy and Kladde, 2005; Neef and Kladde, 2003). At the *PHO8* promoter, on the other hand, absence of Swi/Snf results in the complete loss of chromatin remodeling under inducing conditions. Furthermore, Gcn5 is required for full remodeling and transcriptional activation at this promoter (Gregory et al., 1999).

Ever since these differences were recognized there have been speculations about the underlying reasons. This work shows that these discrepancies are not a direct consequence of the position or strength of the UASp elements driving the activation of transcription. Instead, these differences result from different stabilities of the two promoter chromatin structures. The basis for these results was the development of a competitive yeast *in vitro* assembly technique in which differences in nucleosome stability between promoter regions could be directly compared. This technique originated from a yeast *in vitro* chromatin assembly system that generated the characteristic *PHO5* promoter chromatin structre (Korber and Hörz, 2004). As shown here, this system also assembles the native *PHO8* promoter nucleosome pattern. Using the competitive assembly system it was shown that the *PHO8* promoter has greater nucleosome positioning power, and that the properly positioned nucleosomes are more stable than at the *PHO5* promoter. This provided for the first time evidence for the correlation of inherently more stable chromatin with stricter co-factor requirements.

Remarkably, the positioning information for the *in vitro* assembly of the native *PHO5* and *PHO8* promoter chromatin patterns was specific to the yeast extract. Salt gradient dialysis or *Drosophila* embryo extract assemblies did not support the proper nucleosome positioning.

However, nucleosomes in chromatin generated in these systems could be shifted to their *in vivo*-like positions by the addition of yeast extract. This indicates that the nucleosome positioning mechanisms *in vitro* are uncoupled from the nucleosome loading machinery. The nucleosome positioning at the *PHO5* and *PHO8* promoters was energy dependent suggesting a role of chromatin remodeling machines in generation of the repressed promoter chromatin structure. In spite of this, the chromatin remodeling machines Swi/Snf, Isw1, Isw2 and Chd1 were dispensable nucleosome positioning at both promoters.

# II. Zusammenfassung

Die *PHO5*- und *PHO8*-Gene in Hefe stellen typische Beispiele für die Rolle von Chromatin bei der Promotorregulation dar. Beide Gene werden vom selben Transkriptionsaktivator, Pho4, aktiviert, der sowohl Nukleosomenremodulierung als auch Transkriptionsaktivierung initiiert. Trotz dieser Coregulation gibt es bedeutende Unterschiede bei der Genaktivierung und in der Art in der die Chromatinremodulierung am Promoterchromatin stattfindet. Zunächst ist festzustellen, dass *PHO5* zu den am stärksten induzierbaren Hefegenen gehört, es ist 10 mal stärker aktiv als *PHO8*. Darüber hinaus sind von der Chromatinremodulierung am *PHO5*-Promotor vier Nukleosomen betroffen, während es sich am *PHO8*-Promotor nur um zwei Nukleosomen handelt. Zuletzt ist festzustellen, dass weder die Histonacetyltransferase Gcn5 noch der Chromatinremodulierungskomplex Swi/Snf zwingend notwendig für die Chromatinremodulierung am *PHO5*-Promotor zu sein scheinen. Dagegen führt die Abwesenheit von Swi/Snf zum vollständigen Verlust der Chromatinremodulierung am *PHO8*-Promotor unter induzierenden Bedingungen. Außerdem ist Gcn5 für die vollständige Remodulierung und Transkriptionsaktivierung an diesem Promotor notwendig.

Seitdem diese Unterschiede erkannt wurden wird über die zugrundeliegenden Ursachen spekuliert. Die hier vorgestellte Arbeit zeigt, dass diese Unterschiede nicht direkt auf die Position oder Stärke der UASp-Elemente, die die Transkriptionsaktivierung antreiben, zurückzuführen sind. Vielmehr beruhen die Unterschiede auf der unterschiedlichen Stabilität der beiden Promotorchromatinstrukturen. Die Basis für diese Ergebnisse stellte die Entwicklung einer kompetitiven Hefe-*in vitro*-Assemblierungstechnik dar, mithilfe derer Unterschiede in der Nukleosomenstabilität zwischen Promoterregionen direkt verglichen werden konnten. Diese Technik stammte ursprünglich von einem Hefe-*in vitro*-Chromatinassemblierungssystem, das die charakteristische *PHO5*-Promotorchromatinstruktur ausbildet. Wie hier gezeigt wird, assembliert dieses System auch die native *PHO8*-Promotorchromatinstruktur. Unter Verwendung des kompetitiven Assemblierungssystems wurde gezeigt, dass der *PHO8*-Promotor eine größere Nukleosomenpositionierungsstärke besitzt, und dass die korrekt positionierten Nukleosomen stabiler als die des *PHO5*-Promotors sind. Daraus konnten erstmalig Anhaltspunkte dafür bezogen werden, dass eine Korrelation zwischen inhärent stabilerem Chromatin und einem stärkeren Bedarf an Cofaktoren besteht.

Bemerkenswerterweise war die Positionierungsinformation für die *in vitro*-Assemblierung der nativen *PHO5*- und *PHO8*-Promotorchromatinstruktur spezifisch für den Hefeextrakt.

Salzgradientendialyse oder Assemblierung durch Drosophila-Embryoextrakt führte nicht zu korrekter Nukleosomenpositionierung. Dennoch konnten Nukleosomen, die durch dieses System erzeugt worden waren, durch die Zugabe von Hefeextrakt auf deren entsprechende in vivo-Positionen verschoben werden. Das ist ein Anhaltspunkt dafür, dass die Mechanismen zur Nukleosomenpositionierung in vitro nicht an die Maschine, die die Nukleosomen auf die DNA lädt, gekoppelt sind. Die Nukleosomenpositionierung am *PHO5*- und *PHO8*-Promotor war energieabhängig, was darauf schließen lässt, dass Chromatinremodulierungsmaschinen eine Rolle bei der Erzeugung der reprimierten Promotorchromatinstruktur spielen. Trotzdem waren die Chromatinremodulierungsmaschinen Swi/Snf, Isw1, Isw2 und Chd1 an beiden Promotoren nicht für die Nukleosomenpositionierung eforderlich.

# III. Introduction

## *1. Chromatin structure*

The nucleus of eukaryotic cells contains one complement of the genome of the organism. Genome sizes vary among species, e.g., the haploid yeast genome contains $10^7$ and the diploid human genome contains $3x10^9$ base pairs of DNA. If stretched out, the total length of the human DNA is around one meter. As the diameter of an average cell nucleus is only about 10-20 μm the DNA needs to be highly compacted to fit into the nucleus. This task is accomplished by having the DNA in a complex with a set of special proteins, the histones, to form a structure called chromatin which ultimately leads to an up to 100.000 fold compaction of the DNA.

Originally, chromatin was thought of as a DNA packaging device only. However, it soon became clear that chromatin provides also an additional level of regulation for all DNA related processes, such as replication, repair and gene expression, providing a platform where biological signals are integrated and molecular responses take place.

### 1.1. The 10 nm fiber

The association of histone proteins with eukaryotic DNA had long been recognized, however, it was not until 30 years ago that the precise role was understood. At that time, the nucleosome particle was proposed to consist of ~200 base pairs of DNA wrapped around a histone protein core consisting of an octamer of of four histones (H2A, H2B, H3 and H4) forming a structure containing a single dyad axis (Kornberg, 1974; Kornberg and Thomas, 1974; Kelley, 1973). Shortly afterwards it was shown that the nucleosome is the repeating unit of chromatin and that nucleosomes are assembled on DNA like “beads-on-a-string” (Olins and Olins, 1974; Finch et al., 1975; Oudet et al., 1975; Woodcock et al., 1976), generating the so-called 10 nm fiber. This structure allows an approximately six-fold compaction of the DNA and constitutes the first level of chromatin condensation (Griffith, 1976). Digestion of chromatin with nuclease proved that the “linker” DNA between two nucleosomes is readily cleaved by nuclease whereas the so-called nucleosome “core particle” is much more resistant to nuclease digestion. Further characterization of the core particle showed that it consists of 146 bp of DNA wrapped around the histone octamer (Lohr and van Holde, 1975) in approximately 1.7 left-handed superhelical turns (Finch et al., 1977; Klug et al., 1980; Arents et al., 1991).

A high-resolution crystal structure of the nucleosome core particle was solved (Luger et al., 1997; Davey et al., 2002). This structure shows that each core histone contains two separate functional domains: a uniquely structured C-terminal "histone fold domain" sufficient for both histone-histone and histone-DNA contacts within the nucleosome, and the N-terminal "tail" domain that contains sites for posttranslational modifications (Finch et al., 1977; Klug et al., 1980; Arents et al., 1991; Luger et al., 1997). The histone tails promote chromatin fiber formation, possibly by contacting adjacent nucleosomes or by influencing the configuration of the linker DNA (Schwarz et al., 1996). Under physiological conditions the DNA-histone interactions in the nucleosome core particle are stabilized by more than 116 direct and 358 water-bridged interactions rendering the nucleosome a stable particle in the absence of any additional factors (Davey et al., 2002; Luger and Richmond, 1998). The DNA in the nucleosome has a phased helical periodicity (Wang, 1982). Accordingly, the histone octamers interact with the DNA approximately every 10 bp whereby the minor groove of the double helix is faced inwards (Davey et al., 2002; Luger and Richmond, 1998).

### 1.2. Higher order chromatin structures

The first level of compaction, the 10 nm fiber, establishes only a small portion of the condensation necessary to fit the eukaryotic DNA into the nucleus. Additional condensation is accomplished by folding of the chromatin fiber into higher order secondary and tertiary chromatin structures (Woodcock and Dimitrov, 2001) (Figure 1). The secondary level of compaction involves the folding of the beaded 10 nm fiber into a thicker fiber of about 30 nm in diameter (see Hansen, 2002, and references therein). This folding is mediated by histone-histone interactions, especially interactions between the tail domain of H4 and the "charged patch" of H2A are important for the generation of the secondary chromatin structure (Dorigo et al., 2003; Gordon et al., 2005; Dorigo et al., 2004; Fan et al., 2004). In addition, the metazoan linker histone H1 as well as several linker DNA binding proteins are implicated in stabilizing the histone tail-mediated chromatin condensation (reviewed in Adkins et al., 2004b; and Luger and Hansen, 2005). The second level of condensation adds another six- to seven-fold compaction.

The folding of chromatin at the tertiary level to fibers of 100-300 nm in diameter in interphase nuclei remains a contentious issue despite extensive research. However, it is believed that the histone tails are involved in the formation of these tertiary chromatin structures along with several architectural chromatin proteins (see Horn and Peterson, 2002, and references therein).

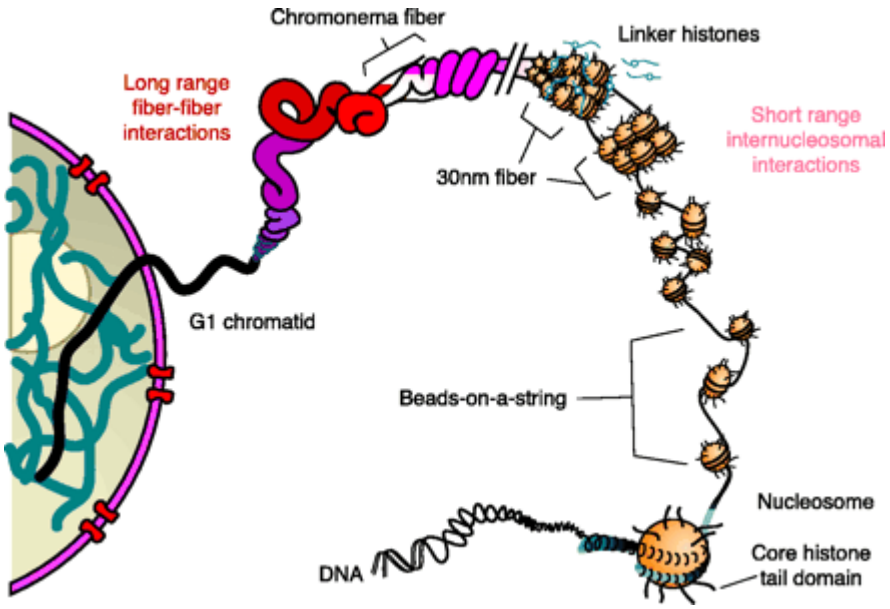


**Figure 1. Multiple levels of chromatin folding.** DNA compaction within the interphase nucleus (depicted on the left) occurs through interactions of the nucleosomes in a hierarchy that can be sub-divided into primary, secondary and tertiary levels of compaction. The 10 nm fiber composes the primary structural unit. The second level of condensation involves the formation of the 30 nm fiber formed by nucleosome-nucleosome interactions. The tertiary level generates the so-called chromonema fiber which is likely to result from histone tail mediated associations within the individual 30 nm chromatin fibers (from Horn and Peterson, 2002).

Although most of the chromatin research was done at the level of the beaded string, this 10 nm fiber may no more physiologically relevant than the mononucleosomes. Recently it was shown that transcriptionally active MMTV promoter chromatin does not decondense into primary chromatin structure but forms secondary chromatin structures *in vivo* (Georgel et al., 2003). As also illustrated in Figure 1, *in vivo* chromatin might only exist as 30-300 nm structures (reviewed by Horn and Peterson, 2002; Luger and Hansen, 2005), and it is still unclear how the different levels of chromatin organization influences DNA related processes. In spite of this, to understand the regulation of chromatin at the secondary and tertiary level one must first understand the basic principles at the level of the nucleosomal array.

## 1.3. Hetero- and euchromatin

Historically, the generic organization of chromatin is divided into two structures; heterochromatin and euchromatin, the identification of which originates from cytogenetic observations. Heterochromatin represents the portion of the genome that remains condensed in interphase. In yeast it corresponds mainly to telomeres and pericentric chromosomal areas and generally localizes to the perinuclear compartment. Heterochromatic areas tend to be rich in repetitive sequences, low in gene content and typically replicate late in the cell cycle (reviewed by Dillon, 2004). Euchromatin on the other hand may be considered as the non-

heterochromatic rest of the genome. It decondenses during interphase, contains most of the genes, is active or proficient for transcription and replicates early. In addition, the organization of euchromatin changes transiently in local areas of the genome as a response to cellular stimuli and/or differentiation programs.

## *2. Chromatin dynamics*

Chromatin is a highly flexible environment, wherein spatially and temporally coordinated changes between transcriptionally active and structurally accessible states as well as transcriptionally repressive and structurally condensed states regulate gene expression.

### 2.1. Chromatin remodeling and nucleosome sliding

Nucleosomes were long perceived as large immovable obstacles in the path of DNA directed processes. However, this view changed dramatically by the finding that ATP dependent chromatin remodeling factors exist, which are capable of mobilizing histone octamers leading ultimately to the exposure or occlusion of the underlying DNA sequences (Eberharter and Becker, 2004; Längst and Becker, 2004). Several cellular mechanisms exists that remodel chromatin in a temporal/spatial manner. Mechanistically especially well understood is the process of nucleosome sliding in which nucleosomes are translocated *in cis* along the DNA. Nucleosome sliding was observed to occur as a spontaneous as well as a catalyzed process. Spontaneous nucleosome sliding can be induced under certain conditions *in vitro* in a temperature- and salt-dependent manner (Pennings et al., 1991; Meersseman et al., 1991). These *in vitro* results suggested that nucleosomes are not static but dynamic. The temperature and the time required for sliding in defined model systems allowed for a comparison of the relative stability of histone-DNA interactions in the nucleosome (Flaus et al., 2004).

*In vivo* chromatin remodeling is catalyzed by large ATP-dependent chromatin remodeling machines. These remodeling machines can be divided into different classes based on their protein composition and functions. This includes the Swi/Snf (Switch/sucrose non-fermenting), Iswi (imitation switch), INO80 (inositol)/Swr1 (Swi/Snf related), and Chd (chromodomain helicase/ATPase DNA binding protein) groups. *In vivo* chromatin remodeling leads to several different outcomes. These include shifting of the positions of nucleosomes, nucleosome sliding (Belikov et al., 2001; Fazzio and Tsukiyama, 2003; Kent et al., 2001), histone eviction (Reinke and Hörz, 2003; Boeger et al., 2003) and exchange of H2A variants (Krogan et al., 2003; Mizuguchi et al., 2004).

## 2.2. Histone modifications

Chromatin structure can also be regulated by post-translational covalent modifications of the amino terminal histone tails, as well as on residues within the globular domain. These modifications include phosphorylation, acetylation, methylation, ubiquitination, and SUMOylation (Figure 2). Such modifications can be binding sites for regulatory proteins like transcription factors, proteins involved in chromatin condensation or DNA repair. There are modifications that co-exist and work in a coorporative manner in the same nucleosome but some are incompatible with others (reviewed by Margueron et al., 2005). The recognition of this dynamic interplay between histone modifications led to the "histone code" hypothesis (Jenuwein and Allis, 2001; Rice and Allis, 2001). This hypothesis proposes that histone modifications on the same or another histone molecule constitute a code. Individual types of histone modifications are read by proteins via for example chromo- and bromodomains that function as binding modules for methylated and acetylated lysines, respectively (reviewed by de la Cruz et al., 2005). Many chromatin remodeling machines contain subunits with one or more bromodomains. The RSC complex, for example, that is closely related to the Swi/Snf complex contains the tandem bromodomain protein Rsc4 that interacts with acetylated H3-K14 (Kasten et al., 2004) directly connecting chromatin remodeling to histone modification marks.

Most post-translational modifications are reversible (except maybe for arginine methylation). The dynamic equilibrium of lysine acetylation *in vivo* is governed by the opposing actions of histone acetyl transferases, HATs, and deacetylases, HDACs. Lysine methylation was long thought to be irreversible. However recently it was shown that a demethylase, LSD1, could reverse this modification on modified peptides (Shi et al., 2004; and reviewed by Wysocka et al., 2005). Interestingly, the activity and specificity of LSD1 can be modulated by association with specific cofactors (Lee et al., 2005; Metzger et al., 2005; Shi et al., 2005), suggesting that lysine methylation is indeed a dynamic mark. Similarly, although the search for arginine demethylases so far were fruitless an alternative pathway for the reversal of arginine methylation was proposeed (Bannister et al., 2002).

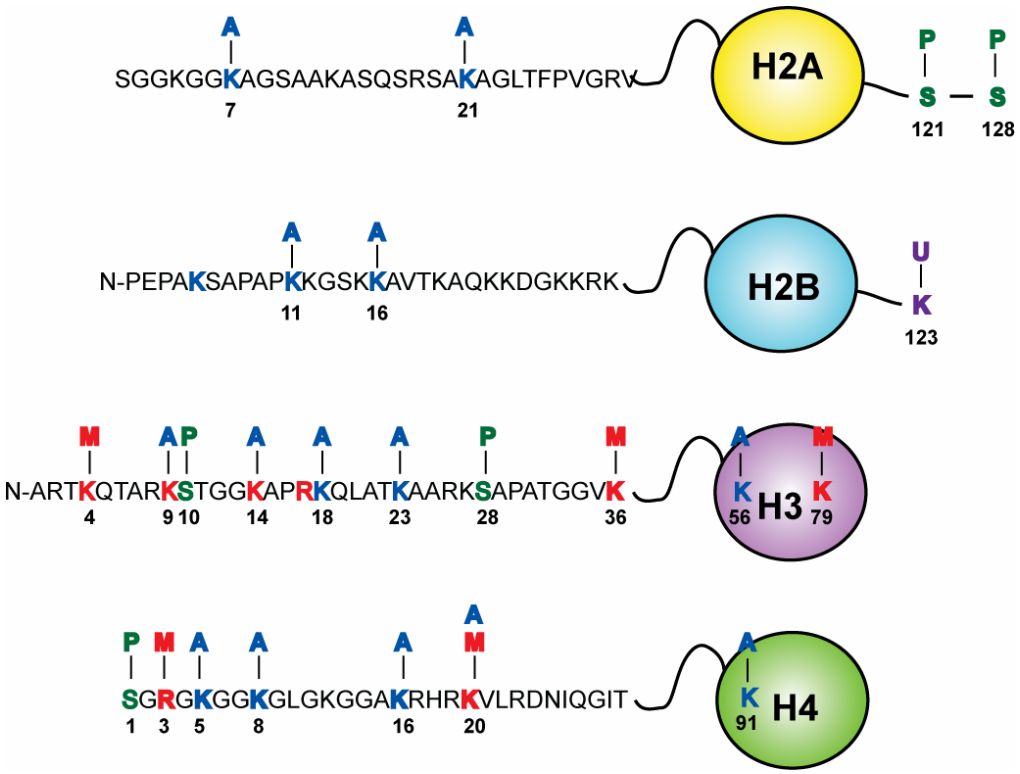


**Figure 2. Histones are subject to a variety of post-translational modifications.** Modifications in *S. cerivisae* include acetylation of lysine residues (A, blue), methylation of lysine and arginine residues (M, red), phosphorylation of serine residues (P, green) and ubiquitylation of lysine residues (U, purple). Arginines can be either mono- or dimethylated, whereas methylated lysines exist in a mono- di- or trimethylated form.

## 2.3. Histone variants

A third mechanism for the regulation of chromatin structure is through substitution of histones by histone variants. Histone variants are variants of the major-type histones with moderate to significant degrees of sequence homology with their corresponding counter-parts. Histone variants can be actively replaced outside of replication. Replication independent nucleosome assembly was demonstrated for the H2A variant Htz1[1] in yeast (similar to the metazoan H2A.Z) by the Swr complex (Krogan et al., 2003; Mizuguchi et al., 2004; Kobor et

1 Here and in the following sections standard nomenclature of proteins, genes and mutated genes for S. cerevisiae will be used (Demerec et al., 1966). This means that "Htz1" refers to the protein, "*HTZ1*" refers to the gene and "*htz1*" refers to the mutated gene.

al., 2004). Furthermore, as nucleosomes are lost from highly active genes in a genome-wide manner (Lee et al., 2004; Bernstein et al., 2004), the deposition of histones upon transcriptional repression might involve the incorporation of histone variants (Ahmad and Henikoff, 2002). The replacement of histone H2A and H3 with their corresponding variants can have several effects on chromatin structure as all histone variants assembled into nucleosomes cause subtle structural variations (Suto et al., 2000; Chakravarthy et al., 2005). This may lead to altered nucleosome stability as was described for some H2A variants (Bao et al., 2004; Park et al., 2004; Gautier et al., 2004; Abbott et al., 2001). The altered stability of a nucleosome might in turn influence subsequent histone eviction during transcriptional activation *in vivo* (Zhang et al., 2005), Nucleosome sliding, chromatin remodeling and histone modifications are also influenced by histone variants (Flaus et al., 2004; Angelov et al., 2003).

## 3. Nucleosome positioning

Nucleosomes were long known to repress transcription. As early as 1979 it was shown that the *in vitro* assembly of SV40 DNA into chromatin leads to the inhibition of initiation and elongation of RNA polymerase I and II (Wasylyk and Chambon, 1979; Wasylyk et al., 1979). A similar inhibitory effect is seen *in vivo*. Turning off histone synthesis by genetic means in yeast results in activation of transcription of a number of genes including the *PHO5* gene (Han et al., 1988). This activation did not require the usual regulatory elements responsible for gene regulation and occurred under otherwise non-inducing conditions (Lorch et al., 1987; Han and Grunstein, 1988; Durrin et al., 1992).

The nucleosome core particle protects against the digestion of nucleases (Elgin, 1981; Reeves, 1984). Consequently, high nuclease susceptibility of DNA regions correlates with the absence of canonical nucleosomes. Digestion of chromatin with both unspecific nucleases such as deoxyribonucleaseI (DNaseI) and miccrococcal nuclease (MNase) and restriction enzymes have proven to be useful tools in the analysis of chromatin structure (Elgin, 1981; Bellard et al., 1980; Almer and Hörz, 1986). Importantly, the absence of canonical nucleosomes as mapped by nuclease digestion does not distinguish between the absence or presence of histones. However, recently it was shown that hypersensitive sites are in fact devoid of histones all together (Boeger et al., 2003; Reinke and Hörz, 2003; Bernstein et al., 2004; Lee et al., 2004)

The majority of genomic DNA in yeast is assembled into distinctly positioned nucleosomes. In yeast, over 69 % of the nucleosomal DNA is found in well positioned nucleosomes (Yuan et al., 2005). The positioning of nucleosomes results from differing nucleosome linker lengths and varies between tissues of the same organism (van Holde, 1989). The exact positioning of a nucleosome can result in important DNA elements being incorporated into a nucleosome thereby being inaccessible for interacting factors. The variability in linker length is therefore, of great significance for the regulation of all chromatin related processes.

The nucleosome core particle protects against the digestion of nucleases (Weintraub and Groudine, 1976; Elgin, 1981). Consequently, high nuclease susceptibility of DNA regions correlates with the absence of canonical nucleosomes. Digestion of chromatin with both unspecific nucleases such as deoxyribonucleaseI (DNaseI) and miccrococcal nuclease (MNase) and restriction enzymes were used to map chromatin structures at many promoters . Importantly, the absence of canonical nucleosomes as mapped by nuclease digestion does not distinguish between the absence or presence of histones. However, recently it was shown that hypersensitive sites are in fact devoid of histones all together (Boeger et al., 2003; Reinke and Hörz, 2003; Lee et al., 2004)

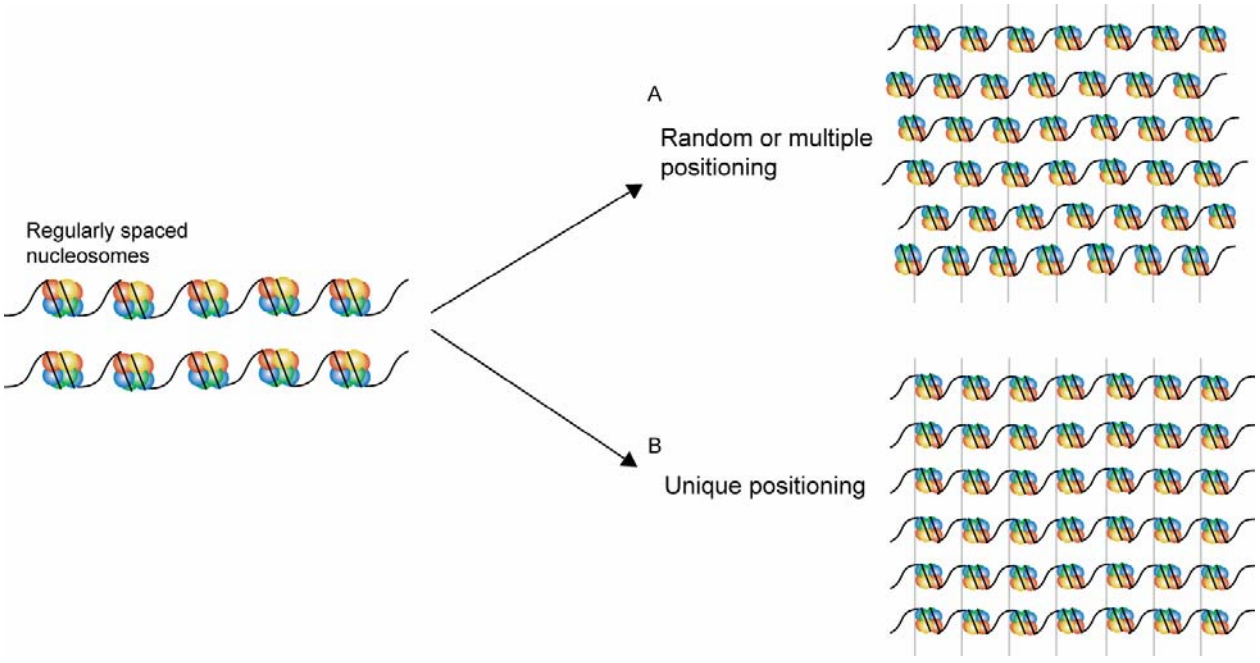


**Figure 3. Concepts of nucleosome positioning.** (A and B) Chromatin can be either irregularly (A) or regularly spaced (B) on a particular DNA region. Additionally, on regularly spaced nucleosomal arrays nucleosomes can adopt either random multiple positioning (C) or unique positioning (D).

The term “positioning” refers to nucleosomes occupying a defined set of positions on a particular DNA region resulting in nucleosomes that are uniquely located throughout a

population of DNA molecules (Figure 3A). In contrast, in a random arrangement of nucleosomes, all DNA sequences will have an equal probability of binding a histone octamer (Figure 3B). A precisely positioned histone octamer might protect regulatory sequences of a gene and thereby have repressing functions (Venter et al., 1994; Anderson and Widom, 2000) or nucleosomes may be positioned such that regulatory regions localize to linker regions (Zhang and Reese, 2004). Nucleosome positioning may also result in location of regulatory sequences on the surface of the nucleosome exposing it to a DNA binding protein (Belikov et al., 2000). Additionally, secondary chromatin structure might bring *cis*-regulatory elements which are located in some distance along the same DNA sequence into closer proximity (Stunkel et al., 1997).

### 3.1. Translational and rotational nucleosome positioning

Two forms of nucleosome positioning are distinguished: translational and rotational positioning. Translational positioning refers to the extent to which a histone octamer selects a particular stretch of 147 bp of DNA in preference to other stretches on the same DNA. Rotational positioning, on the other hand, is a degenerate form of translational positioning in which a set of discrete translational positions, differing by integral multiples of the DNA helical repeat, are all occupied in preference to a set of other possible locations. Rotational positioning is so called because it maintains the rotational orientation of a given face of the DNA with respect to the underlying histone surface. Thus, a site on the DNA that faces outwards in one member of a rotationally related set of nucleosome positions does so for all members of that set of rotational positions (Widom, 2001).

### 3.2. Contribution of DNA structure to nucleosome positioning

Many *in vivo* studies have reported promoters comprising distinctly positioned nucleosomes (Almer and Hörz, 1986; Li and Wrange, 1993; Belikov et al., 2000). However, for most of these promoters it is still not clear which mechanisms direct the generation of these positioned nucleosomal arrays. With classical *in vivo* techniques the mechanistical properties of nucleosome positioning are difficult to assess as all interactions between histone octamers and DNA take place in the context and under the influence of various different cellular factors. Therefore, several *in vitro* approaches were developed to study why some histones prefer some DNA sequences over others. These include chromatin assembly by salt gradient dialysis (Wilhelm et al., 1978) and *in vitro* assembly systems based on cellular extracts such as

*Xenopus* eggs, *Drosophila* embryos and mammalian cells (Laskey et al., 1977; Becker and Wu, 1992; Kamakaka et al., 1993; Gruss et al., 1990).

Chromatin assembly by salt gradient dialysis has the advantage of using purified components (histone octamers and DNA) and has therefore been the prefered *in vitro* system to study nucleosome positioning. Many of the identified rules governing nucleosome positioning are therefore biased by this method (Widom, 2001).

Nucleosome positioning is influenced by the structure of DNA. The persistence length of a DNA molecule refers to the lenght over which a DNA molecule persist bending and twisting. DNA molecules of much shorter lenght than the persistence length are essentially straight in the absence of exogenous forces (Widom, 2001). The persistence length of an arbitrary DNA molecule is suggested to be between 50 and 150 bp (Hagerman, 1988). However, in nucleosomes 147 bp of DNA are wrapped around the histone octamer through 1.67 superhelical turns (Luger et al., 1997; Richmond and Davey, 2003) presenting a severe distortion of the DNA. Additionally, DNA in a nucleosome is twisted less than arbitrary DNA. As estimated by the 2.8 Å crystal structure, the average twist for nucleosomal DNA is around 10.2 bp/turn (Luger et al., 1997; Richmond and Davey, 2003) whereas an average twist for DNA in solution is about 10.5 bp/turn (Travers and Klug, 1987; Olson et al., 1998). Thus, major forces work against the incorporation of DNA into a nucleosome. To overcome this energy barrier, several interactions have to be formed between the histone octamer and the DNA in order to make the nucleosome a stable particle. The structure of the underlying DNA sequence, that is the bendability and/or twistability, contributes significantly to the packaging of DNA into nucleosomes (Drew and Travers, 1985). Accordingly, the energy cost of the incorporation of DNA into a nucleosome is lower if a particular DNA sequence has an increased ability of being distorted. This leads to a higher affinity for the histone octamer, i.e. more or better bonds, e.g. more H-bonds or “salt-bridges”, better H-bond geometry, better steric fit etc. (Widom, 2001).

Therefore, both translational and rotational nucleosome positioning is influenced by the structure of the underlying DNA. Shrader and Crothers designed DNA sequences that were expected to favor incorporation into nucleosomes because of their inherent structure. These sequences proved to bind to histone octamers with enhanced affinity in nucleosome reconstitution experiments, and exhibited rotational positioning according to their curvature (Shrader and Crothers, 1990; Shrader and Crothers, 1989). This incited the mapping of naturally occurring bent DNA structures close to important genomic sequences. However,

although such structures are present in the human beta-globulin locus (Wada-Kiyama and Kiyama, 1996a; Wada-Kiyama et al., 1999) these cannot solely account for nucleosome positioning at this locus.

As rotational positioning is a degenerate form of translational positioning the rules that govern rotational positioning also apply for translational positioning. In agreement with this, the local DNA structure also has profound influence on the translational positioning of a nucleosome. Highly rigid DNA disfavors the incorporation into a nucleosome. An example of such sequences are poly(dT-dA) sequences. These sequences were proposed to repel the histone octamer based on the low yield in *in vitro* reconstitution assays (Simpson and Shindo, 1979; Rhodes, 1979). Later this assumption was supported by X-ray analysis of long poly(dA-dT) stretches showing the rigidity of such a sequence (Nelson et al., 1987). Recently, these sequences were indeed shown to destabilize chromatin by directly destabilizing histone-DNA interactions (Anderson and Widom, 2001). Interestingly, nucleosome free regions present in the majority of all Polymerase II promoters (Sekinger et al., 2005; Yuan et al., 2005) are enriched in Poly(dA-dT) stretches (Yuan et al., 2005).

In contrast, highly flexible DNA readily wraps around a histone octamer. Classical examples are the 5S rRNA positioning sequence which has a high affinity for nucleosomes *in vitro* (Hayes et al., 1990) and natural occurring DNA sequences like the TATAAACGCC repeat which is the strongest known natural occurring nucleosome positioning sequence (Thastrom et al., 1999; Widlund et al., 1999; Widlund et al., 1997). In another study, Lowary and Widom used an adapted SELEX approach to find rules governing histone-DNA interactions. This approach was completely unbiased towards DNA structures that were previously identified as influencing nucleosome positioning. Although, the original strategy was to screen for high nucleosome affinity sequences they identified the today strongest known nucleosome positioning sequence, the so called 601 sequence. This sequence directs nucleosomes into a single predominant translational position on DNA fragments as long as 288 bp in *in vitro* reconstitution experiments (Lowary and Widom, 1998; Thastrom et al., 1999).

*In vivo* several sequences are also organized preferentially in positioned nucleosomes. Some of these sequences contain non-random periodic distributions of certain di-nucleotides related to those discovered in the sequences of isolated nucleosomeal DNA that proved to facilitate packaging of DNA in a nucleosome (Bina, 1994; Staffelbach et al., 1994; Bolshoy, 1995; Ioshikhes et al., 1996). However, this periodic di-nucleotide distribution cannot solely account for nucleosome positioning *in vivo*.

Interestingly, in spite of these findings, many of the sequences that have strong positioning power *in vitro* did not position a nucleosome *in vivo* (Tanaka et al., 1992b; Negri et al., 2001; Buttinelli et al., 1993). In addition, the bulk of the eukaryotic genome is not evolved to aid substantially in its own packaging at the level of individual nucleosomes as assayed by salt gradient dialysis (Lowary and Widom, 1997). Thus, although several DNA sequence rules were identified that govern nucleosome positioning *in vitro* these might be of little functional relevance *in vivo*.

As DNA structure seems to play little role in global nucleosome positioning in the cell, nucleosome positioning was investigated using cellular extract assembly systems (Laskey et al., 1977; Becker and Wu, 1992; Kamakaka et al., 1993; Gruss et al., 1990; see also Lusser and Kadonaga, 2004 and references herein). However, only in a few cases did *in vitro* assembly systems based on cellular extracts result in proper positioning of nucleosomes prior to the addition of a specific DNA binding protein. Assembly with *Xenopus* egg extract for example positioned nucleosomes over the 5 S rDNA sequence (Shimamura et al., 1988; although see Almouzni et al., 1990). Using the *Drosophila* embryo extract assembly system the *Drosophila HSP26* gene was assembled into chromatin with properly positioned nucleosomes (Wall et al., 1995) and *in vivo* like nucleosome positioning over the MMTV promoter region was acchieved (Venditti et al., 1998). However, in the majority of cases a specific DNA binding factor was needed to position the nucleosomes in these systems (Tsukiyama et al., 1994; Pazin et al., 1994; Längst et al., 1997; Pazin et al., 1997; Mcpherson et al., 1993).

In short, although several signals were identified that influence nucleosome positioning, little is still known about the mechanistic determinants *in vivo*. Table 1 summarizes the potential or proposed signals for nucleosome positioning some of which were already introduced in the previous section.

**Table 1. Signals that affect rotational and translational positioning** (adapted from Kiyama and Trifonov, 2002)

| Nucleosome positioning signal | Reference |
|---|---|
| *Specific nucleotide sequences* | |
| SV40 enhanser | (Clarke et al., 1985) |
| Satellite DNA | (Linxweiler and Hörz, 1985) |
| 5S rRNA sequence | (Hayes et al., 1990; but see Blank and Becker, 1996) |
| TATAAACGCC repeat | (Widlund et al., 1999; Widlund et al., 1997) |
| $(A/T)_3NN(G/C)_3NN$ | (Tanaka et al., 1992a; Shrader and Crothers, 1989) |
| 601 sequence | (Lowary and Widom, 1998) |
| AAA/TTT | (Muyldermans and Travers, 1994) |
| Albumin enhancer | (Mcpherson et al., 1996) |
| NGGR | (Travers and Muyldermans, 1996) |
| Non-T(A/T)G (or VWG) | (Stein and Bina, 1999; Baldi et al., 1996) |
| A+T rich sequence in 5S rRNA gene | (Tomaszewski and Jerzmanowski, 1997) |
| *Cis*-elements for TFIIIA | (Pfaff and Taylor, 1998) |
| $(A_5(G/C)_5)_4$ | (Fitzgerald and Anderson, 1998) |
| $(CTG)_n$ | (Wang and Griffith, 1995; Godde and Wolffe, 1996) |
| *Specific DNA structures* | |
| Curving or bending of DNA | (Drew and Travers, 1985; Fitzgerald et al., 1994; Wada-Kiyama et al., 1999; Wada-Kiyama and Kiyama, 1996b) |
| HRE of MMTV promoter | (Pina et al., 1990) |
| *Sequences/structures that disrupts nucleosome formation* | |
| Z-DNA (CA or CG repeats) | (Nickol et al., 1982) |
| Curved DNA at yeast ARS | (Snyder et al., 1986) |
| Triplex DNA (Pu-Py sequences) | (Espinas et al., 1996; Westin et al., 1995) |
| T14A11 of *Alu* element | (Englander and Howard, 1996) |
| TGGA | (Cao et al., 1998) |
| Poly(dA-dT) | (Simpson and Shindo, 1979; Rhodes, 1979; Anderson and Widom, 2001; Yuan et al., 2005) |
| CCG triplet repeat blocks | (Wang et al., 1996) |
| *Binding of proteins/transcription* factors | |
| Sp1 and NFκB | (Widlak et al., 1997) |
| Adf-1 and GAGA factor | (Gao and Benyajati, 1998) |
| GAGA | (Tsukiyama et al., 1994) |
| Gal4-VP16 | (Pazin et al., 1994) |
| CENP-B | (Tanaka et al., 2005) |
| TTF-1 | (Längst et al., 1997) |
| Histone H1(H5) | (Stein and Kunzler, 1983) |
| Histone (H3-H4)2 | (Dong and van Holde, 1991) |
| NF1 of MMTV | (Eisfeld et al., 1997; Archer et al., 1991) |
| Histone H4 | (Roth et al., 1992) |
| *Others* | |
| Histone acetylation | |
| DNA methylation | (Pennings et al., 2005) |
| Superhelicity/higher order chromatin folding | |

## 4. The PHO regulon in S. cerevisiae

The *PHO* regulon in yeast is composed of a number of structural and regulatory genes (Oshima, 1997). The structural genes correspond to phosphatases and phosphate transporters and the regulatory genes provide the cell with the ability to respond to phosphate starvation. Phosphate depletion in the growth media results in an at least 50-fold increased production of secreted acid phosphatase which helps to provide the cells with phosphate by hydrolysing phosphor-monoesters scavenged from the environment. Acidic phosphatase activity results from four isozymes encoded from the *PHO5*, *PHO3*, *PHO10* and *PHO11* genes (Vogel and Hinnen, 1990). The *PHO5* gene product contributes to more than 90% of the acid phosphatase activity generated upon phosphate starvation and is the nost studied gene in the *PHO* regulon. In addition, an alkaline phosphatase encoded by the *PHO8* gene is similarly activated in response to low phosphate levels (Kaneko et al., 1987).

Two DNA binding proteins are responsible for the regulation of the structural genes: an activator of the bHLH family, Pho4, and a pleiotropic homeodomain protein, Pho2 (Vogel and Hinnen, 1990). The *PHO5* gene possesses one of the most strongly inducible promoters in yeast with transcription levels increasing over 100-fold upon phosphate starvation (Oshima, 1997). This induction is brought about by the coordinated action of Pho4 and Pho2 (Barbaric et al., 1998). In contrast, complete promoter opening at the *PHO8* promoter is independent on Pho2 (Barbaric et al., 1992). However at this promoter Pho2 plays a role at the transcriptional level (Münsterkötter et al., 2000).

The activation of Pho4 is regulated at several levels (Figure 4). When cells are grown at high phosphate conditions Pho4 is phosphorylated by the cyclin/cyclin-dependent kinase (CDK) complex Pho80-Pho85. This phosphorylation prevents the binding of Pho4 to DNA (Komeili and O'Shea, 1999) and facilitates recognition by the nuclear export factor Msn5 resulting in its export into the cytoplasm (Kaffman et al., 1998) (Figure 4, top). Conversely, under conditions of phosphate starvation, the CDK inhibitor Pho81 becomes activated. This activation seems to involve the action of the proteins Adk1 and Ado1 (Huang and O'Shea, 2005). Pho81 in turn inhibits the activity of the Pho80-Pho85 CDK complex thereby preventing the phosphorylation of Pho4 (Komeili and O'Shea, 1999). This leads to accumulation of unphoshorylated Pho4 in the nucleus (Kaffman et al., 1998) which in its unphosphorylated state can bind to its target promoters in a cooperative manner with Pho2 (Figure 4, bottom).

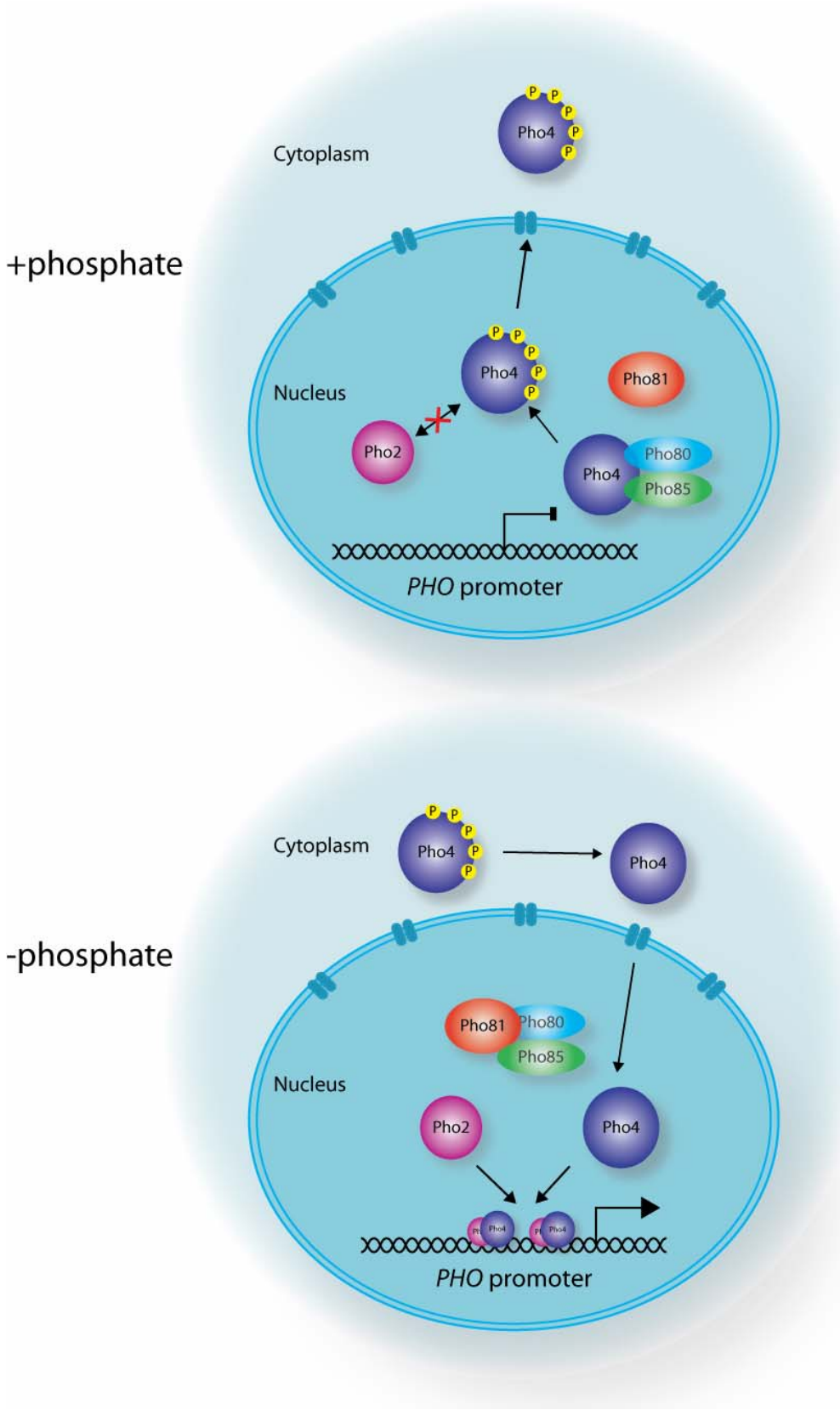


**Figure 4. Phosphate-dependent signal transduction.** When phosphate levels are high, Pho4 is phosphorylated by the Pho80-Pho85 complex which ultimately leads to export of Pho4 to the cytoplasm (top). Phosphate starvation results in inhibition of the Pho80-Pho85 complex leading to accumulation of Pho4 in the nucleus where it binds in corporation with Pho2 to phosphate responsive promoter elements (bottom).

### 4.1. Chromatin structure of the *PHO5* and *PHO8* promoters

Both the *PHO5* and *PHO8* promoters contain regions with positioned nucleosomes under repressing conditions (Svaren and Hörz, 1997; Barbaric et al., 1992; Almer et al., 1986). At the *PHO5* promoter four nucleosomes are positioned, interrupted by a short 80 bp hypersensitive region between nucleosome -2 and -3 (Almer and Hörz, 1986) (Figure 5, top). This hypersensitive site contains one of the two Pho4 binding sites (UASp1) while the second (UASp2) is localized near the center of nucleosome –2. The TATA box is incorporated into nucleosome –1 (Figure 5, top). Thus, under repressing conditions the nucleosomal structure of the *PHO5* promoter prevents both the binding of the specific transcription factor Pho4 to the UASp2 site and the general transcription machinery from accessing the underlying DNA (Venter et al., 1994). The repressed *PHO8* promoter is organized into to two uniquely positioned nucleosomes and one nucleosome (-2), which is less distinctly positioned resulting in two overlapping positions (Barbaric et al., 1992) (Figure 5, bottom). In contrast to the *PHO5* promoter, the two UAS elements at the *PHO8* promoter are both located within hypersensitive sites. The TATA element is nevertheless located within a stable nucleosome, and in analogy to *PHO5* (Han and Grunstein, 1988) loss of nucleosome structure through depletion of histone H4 activates this promoter in the absence of inducing conditions and/or UAS elements (Gregory et al., 1999).

Upon activation of *PHO5* and *PHO8* the positioned nucleosomes are the *in vivo* substrate for remodeling processes that generate extensive nuclease hypersensitive sites (Almer et al., 1986; Almer and Hörz, 1986; Venter et al., 1994) (Figure 5). A series of *in vivo* studies increased our understanding of the mechanism of remodeling leading to the open activated state (Reinke and Hörz, 2004; Svaren and Hörz, 1997). In particular, activation of *PHO5* and *PHO8* upon phosphate starvation leads to the loss of histone DNA contacts, i.e. to histone eviction in the respective promoter regions (Reinke and Hörz, 2003; Adkins et al., 2004a; Boeger et al., 2003; Korber et al., 2006). Remodeling at the *PHO5* promoter is more extensive than at the *PHO8* promoter, resulting in a hypersensitive site of 600 base pairs in length and affecting four nucleosomes (Almer et al., 1986) (Figure 5, top). In the case of the *PHO8* promoter, two nucleosomes are remodeled upon phosphate starvation (Figure 5, bottom) (Barbaric et al., 1992). Furthermore, the histones at the *PHO5* promoter are evicted from the DNA in *trans* (Boeger et al., 2004; Korber et al., 2004). This histone loss is mediated by the histone chaperone Asf1 that is thought to function as a histone acceptor (Adkins et al., 2004a; Korber et al., 2006). Similarly Asf1 also plays a role in the remodeling of *PHO8* (Adkins et

al., 2004a; Korber et al., 2006) and, although not explicitly shown, it is likely that histone eviction at the *PHO8* promoter occurs via a *trans*-mechanism as well.

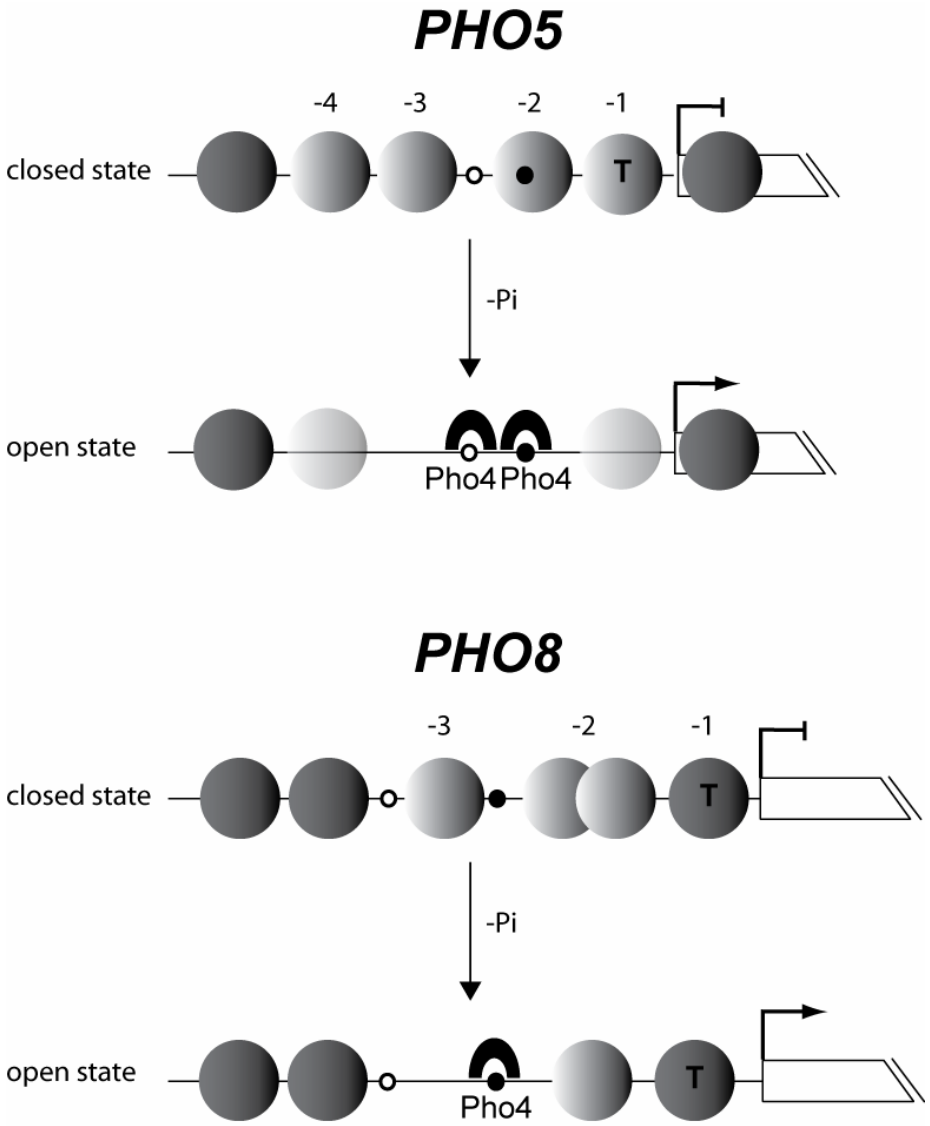


**Figure 5. Promoter chromatin structure of the *PHO5* and *PHO8* promoters.** The repressed *PHO5* and *PHO8* promoters are organized into positioned nucleosomes numbered -1 to -4 according to their position from the transcriptional start site. Upon phosphate starvation Pho4 is bound to the UASp elements leading to nucleosome loss in *trans* rendering the "open state" sensitive to nucleases. On average two nucleosomes are lost from the *PHO5* promoter (Boeger et al., 2003; Korber et al., 2004). The remaining two nucleosomes are drawn transparent. Nucleosome -2 of the *PHO8* promoter represents nucleosome with alternative positions and is drawn as two nucleosomes with overlapping positions. Furthermore, binding of Pho4 to the UASp1 and 2 of the *PHO5* promoter and UASp2 of the *PHO8* promoter is shown.

## 4.2. Co-factor requirements for chromatin opening

Even though both the *PHO5* and the *PHO8* promoter are co-regulated by the same trans-activator Pho4 and both promoter regions loose histones in *trans* upon induction, chromatin remodeling at each promoter has cofactor requirements of differing stringency. At the *PHO8* promoter, no remodeling is detectable in the absence of a functional SWI/SNF complex, and

in a strain without Gcn5 histone acetyl transferase (HAT) activity there is only locally restricted remodeling that does not support induction of *PHO8* activity (Gregory et al., 1999). In contrast, at the *PHO5* promoter the chromatin structure is still completely remodeled without Gcn5 and/or SWI/SNF activity, although with a kinetic delay (Barbaric et al., 2001; Reinke and Hörz, 2003; Dhasarathy and Kladde, 2005; Neef and Kladde, 2003).

## 5. Aims of this work

The hosting lab has a long history in elucidating the role of chromatin structure and remodeling in gene regulation using the *PHO5* and *PHO8* genes as model systems. These studies led to the unraveling of many aspects of the regulation of chromatin opening and closing (see above) that were later proven to be part of universal mechanisms of chromatin regulation in eukaryotes. These studies were mainly been performed using *in vivo* techniques. However, there are clear limitations to this approach and many issues, especially mechanistic questions, are impossible to answer with such techniques. Therefore, a complementary *in vitro* approach using a chromatin assembly system based on whole cell extracts was initiated. This system was able to properly position nucleosomes at the *PHO5* promoter (Korber and Hörz, 2004). Such an *in vitro* system is especially valuable as it has a direct *in vivo* counterpart and therefore the physiological relevance of *in vitro* results can be directly compared and tested *in vivo*.

One aim of this work was to extend the yeast *in vitro* approach to include the *PHO8* promoter. This allows a detailed analysis of requirements for chromatin regulation at the *PHO8* promoter that can supplement the present *in vivo* data. In addition, an *in vitro* system including both the *PHO5* and the *PHO8* promoter provides a powerful tool for studying the differences in chromatin regulation between the two promoters.

It has been a long standing question in the field how the regulation of chromatin opening by one and the same transcriptional activator can differ depending on the promoter context. Previously, it was shown that exchanging the DNA sequence, which assembled into nucleosome -2 at the *PHO5* promoter, for a strong nucleosome positioning sequence, namely an α-satellite DNA fragment, largely abolished chromatin opening (Straka and Hörz, 1991). From these results it was speculated that the inherent stability of a positioned promoter nucleosome directly affects the inducibility of the promoter. In light of this and other studies, it was suggested that the differences in cofactor requirements for chromatin opening at the *PHO5* and *PHO8* promoters reflected differences in the stability of the chromatin substrate

for the two remodeling processes (Münsterkötter et al., 2000). This inherent stability of nucleosomes may be especially relevant for a remodeling mechanism leading to histone eviction in *trans* as it requires the complete disruption of all histone DNA contacts. As nucleosome stability and therefore this hypothesis is not easily testable with classical *in vivo* techniques, one of the aims of this work was therefore to develop an *in vitro* approach to study nucleosome stabilities at the *PHO5* and *PHO8* promoters.

In addition, another aim of the presented work was to modify the *in vitro* chromatin assembly system (Korber and Hörz, 2004) in such a way that mechanistic questions of nucleosome positioning could be addressed successfully. It was recently suggested that the DNA sequence is the determinant of nucleosome positioning at the *PHO5* promoter (Terrell et al., 2002). The authors used an uncatalyzed chromatin assembly system based on the histone chaperone Nap1. However, in this system nucleosome -2 did not affect the binding of Pho4 to its intra-nucleosomal binding site, thereby contradicting *in vivo* data (Venter et al., 1994). Thus, additional factors are likely to influence nucleosome positioning at the *PHO5* promoter. Similarly, the determinants of nucleosome positioning at the *PHO8* promoter are still unknown. The yeast *in vitro* assembly system provided a powerful tool to study nucleosome positioning. However, at the time of initiation of this work this system was rather unstable and proper nucleosome positioning was difficult to obtain. Therefore, this approach was to be improved, making the technique more stable and consistent.

# IV. Results

## 1. An in vitro assembly system capable of proper native-like nucleosome positioning

### 1.1. The yeast extract assembly system generates the native nucleosome positioning at the PHO8 promoter *in vitro*

Previously, an *in vitro* assembly system was developed in the lab using yeast whole cell extract that can generate extensive nucleosomal arrays on naked DNA and establish the correct nucleosome positions at the *PHO5* promoter (Korber and Hörz, 2004). Yeast whole cell extract does not contain large amounts of histones and it was therefore necessary to supplement the extract with exogenous histones. In short, the assembly system comprises yeast whole cell extract, histones purified from *Drosophila* embryo extracts and an energy regenerating system consisting of ATP, creatine phosphate (CP) and creatine kinase (CK). This mixture was added to a DNA template and incubated for up to six hours at 30°C. The generated chromatin was thereafter analyzed by MNase digestion to probe for the generation of regularly spaced nucleosomal arrays or by DNaseI digestion in combination with indirect end labeling to analyze nucleosome positioning.

To test whether this assembly system can also assemble the *PHO8* locus into chromatin with properly[2] positioned nucleosomes, a plasmid was generated similar to the one used as a template for the *in vitro* assembly of the *PHO5* promoter (Korber and Hörz, 2004). Instead of the *PHO5* locus the *PHO8* gene along with 1700 bp of the upstream region was inserted. Using this plasmid as template in an *in vitro* assembly reaction revealed a chromatin pattern virtually identical to that seen at the *PHO8* promoter *in vivo* (Figure 6). Both patterns are clearly different from that of free DNA. Characteristic for the *PHO8* promoter chromatin pattern are two hypersensitive sites at the positions of the UASp elements flanking a

[2] Proper positioning refers to the positions of the native *in vivo* chromatin structure as determined by low resolution mapping techniques.

nucleosome and a third hypersensitive site located close to a *HindIII* site (Figure 6, schematic and lane M). Differences between *in vivo* and *in vitro* generated DNaseI patterns further upstream of the promoter (in the upper part of the lanes) reflect differences in the underlying DNA sequences, i.e., vector versus chromosomal sequences. Thus, the established *in vitro* assembly system completely recapitulates the native nucleosome positions not only at the *PHO5* promoter but also at the *PHO8* promoter.

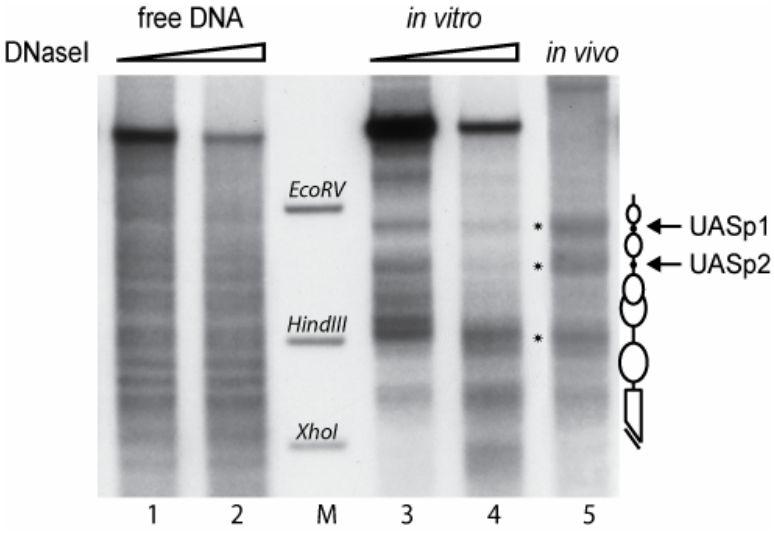


**Figure 6. *In vitro* chromatin assembly with yeast whole cell extract generates the native chromatin structure at the *PHO8* promoter.** Limited DNaseI digestion and secondary cleavage with BglII for indirect end labeling was performed with free DNA (lanes 1 and 2), chromatin assembled with yeast extract in vitro for 6 hours (lanes 3 and 4) and yeast nuclei from a wild type strain (lane 5). The marker bands correspond to the EcoRV-BglII, HindIII-BglII and XhoI-BglII fragments of the PHO8 promoter (lane M). Schematics of the chromatin structure at the PHO8 promoter are shown on the right side of the gel. Nucleosomes are depicted as ovals, UASp elements as black dots and the open reading frame as broken bar. Asterisks in the gel refer to the most distinguishing bands of the PHO8 promoter chromatin pattern. All samples were digested with a range of DNaseI concentrations (ramps on top of the lanes). However, due to space limitations only representative lanes are shown in this and the following figures.

## 1.2. The kinetics of nucleosome positioning at the *PHO5* and *PHO8* promoter in a *de novo in vitro* assembly reaction are different

The generation of extensive nucleosomal arrays as well as proper nucleosome positioning at the *PHO5* promoter *in vitro* is a slow process which takes approximately 6 hours (Korber and Hörz, 2004). The kinetics of nucleosome assembly and positioning at the *PHO8* promoter was therefore analyzed and compared to that of the *PHO5* promoter. The plasmids carrying the *PHO5* and *PHO8* loci are identical except for the respective loci. Furthermore, the size of the *PHO5* and *PHO8* loci are approximately similar. Accordingly, when both templates were added at equimolar ratios in one and the same assembly reaction, the kinetics of nucleosome assembly and positioning could be directly compared.

The extent of chromatin assembly was monitored by MNase digestion (Figure 7A). The generation of extensive nucleosomal arrays on the *PHO8* template, detected by a probe recognizing part of the *PHO8* promoter region without preceding secondary cleavage, took 3 to 4 hours (Figure 7A). Similar results were obtained by re-hybridizing with a probe recognizing the *BamHI-ClaI* fragment of the *PHO5* promoter (data not shown). These results are in agreement with previously published data (Korber and Hörz, 2004).

The generation of nucleosomal arrays and positioning of the nucleosomes need not follow the same kinetics. Therefore, in addition to kinetics of overall chromatin assembly, kinetics of nucleosome positioning were also investigated by DNase I digestion followed by indirect end labeling (Figure 7B and C). In keeping with published data (Korber and Hörz, 2004), the proper nucleosome positioning pattern over the *PHO5* promoter was not discernible prior to 3 hours after the beginning of the assembly reaction but was clearly established after 6 hours (Figure 7B). Surprisingly, however, the generation of the native chromatin structure at the *PHO8* promoter was much more rapid. The nucleosomes were properly positioned already after 30 min to 1 hour (Figure 7C) even before extensive nucleosomal ladders were generated (Figure 7A). It was also noted repeatedly, that the generation of proper nucleosome positioning over the *PHO5* promoter required certain buffer conditions and careful titration of DNA, histones and extract. In contrast, the assembly of properly positioned nucleosomes at the *PHO8* promoter proved to be much more lenient to changes in experimental conditions. In many cases, proper positioning could be observed at the *PHO8* promoter however, at the *PHO5* promoter either no pattern was present or the pattern was only hardly discernible even though both plasmids were present in the same assembly reaction (data not shown).

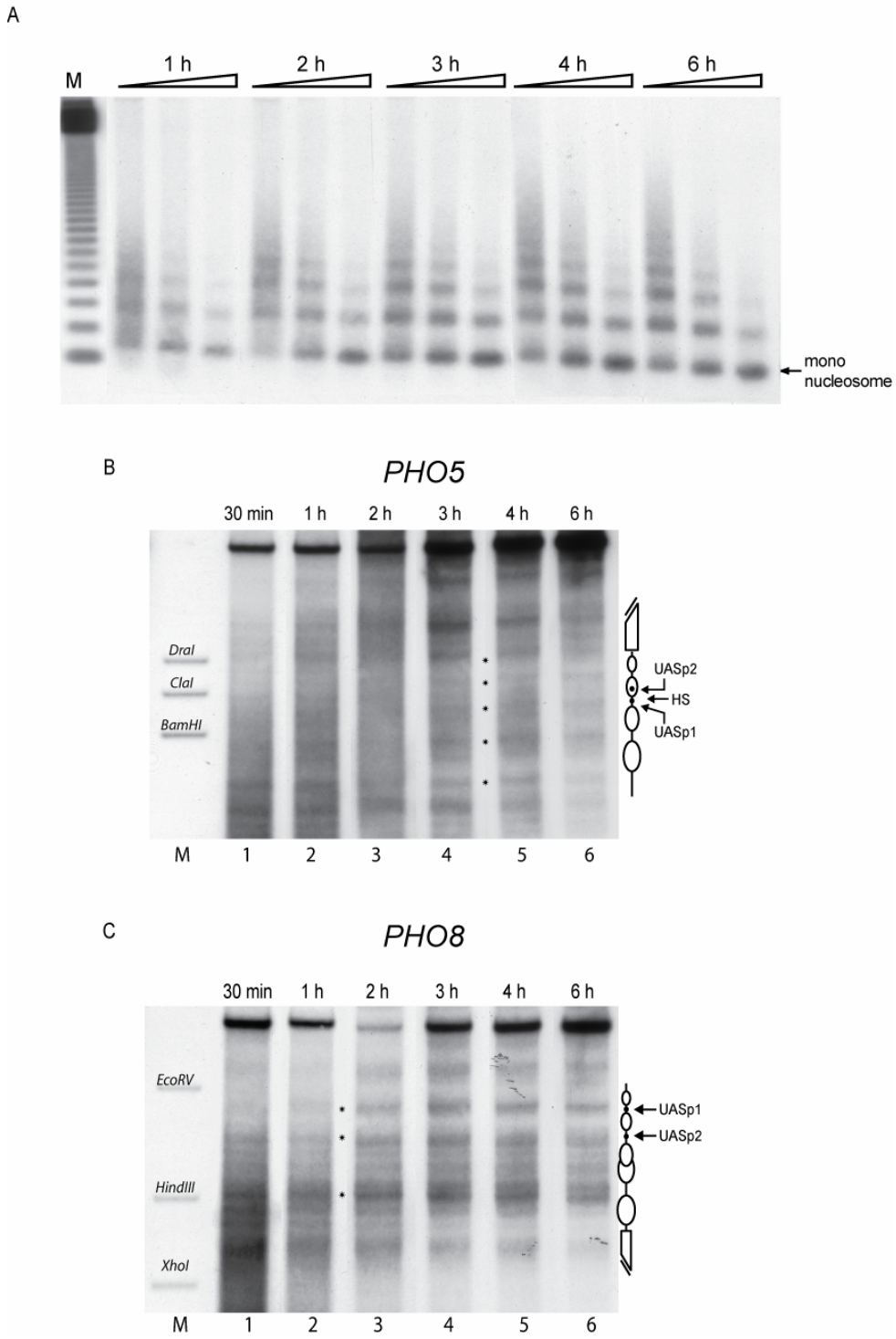


**Figure 7. In a *de novo in vitro* assembly reaction nucleosomes become positioned more rapidly at the *PHO8* than at the *PHO5* promoter.** Assembly kinetics of a yeast extract *in vitro* assembly reaction with plasmids containing the *PHO5* and *PHO8* locus in the same reaction were monitored at the indicated time points by MNase digestion followed by specific probing for the *PHO8* promoter region (A) and DNaseI mapping followed by probing for the *PHO5* (B) or the *PHO8* (C) promoter. Equivalent results as in panel (A) were also obtained by using a *PHO5* promoter probe (not shown). Ramps on top of the lanes represent increasing MNase digestion times. Lane M shows a 123 bp ladder (Gibco). The marker in (B) corresponds to the *ApaI-BamHI*, *ApaI-ClaI* and *ApaI-DraI* fragments of the *PHO5* promoter and in (C) the *EcoRV-BglII*, *HindIII-BglII* and *XhoI-BglII* fragments of the *PHO8* promoter. Schematics of the chromatin structure at the *PHO5* and *PHO8* promoters are on the right side of the gels. Nucleosomes are depicted as ovals, UASp elements as black dots and open reading frames as broken bars. HS in panel (B) denotes the linker region between nucleosomes –2 and –3 at the *PHO5* promoter.

## 2. The nature of the nucleosome positioning information

### 2.1. The UASp elements have no influence on the kinetics of nucleosome positioning *in vitro*

The UASp elements of the *PHO8* promoter are both located in linker regions (Barbaric et al., 1992). On the contrary, at the *PHO5* promoter one UASp element, the UASp2, is located in a nucleosome. This means that binding of a protein to the UASp elements during an assembly reaction would result in different outcomes at the *PHO5* and the *PHO8* promoters, respectively. Binding of a protein to the UASp2 site of the *PHO5* promoter might compete with the assembly of a nucleosome, and thus negatively influence the assembly kinetics. Conversely, such a factor could help to position the nucleosomes at the *PHO8* promoter as the binding could function as a "signpost" for the nucleosomes. Therefore the influence of the UASp sites were further analyzed.

Both UASp2 elements of the *PHO5* and the *PHO8* promoter are consensus E box motifs. Apart from binding the transcriptional activator Pho4 these E-box motifs are also a potential target site for another protein, Cpf1 (Baker and Masison, 1990; Cai and Davis, 1990; Mellor et al., 1990). At the *PHO8* promoter this protein binds to the UASp2 under repressing conditions (Moreau et al., 2003). Furthermore, the transcriptional co-activator Pho2 had also been shown to bind to several binding sites within the *PHO5* promoter (Barbaric et al., 1996; Magbanua et al., 1997).

In order to address the question if any of these proteins have an effect on the positioning of nucleosomes *in vitro* at either promoter, chromatin was assembled using an extract from a strain triply deleted in Pho4, Pho2 and Cpf1. The simultaneous deletion of these three proteins had no effect on the nucleosome positioning at either promoter (Figure 9B and C; lanes 1 and 2).

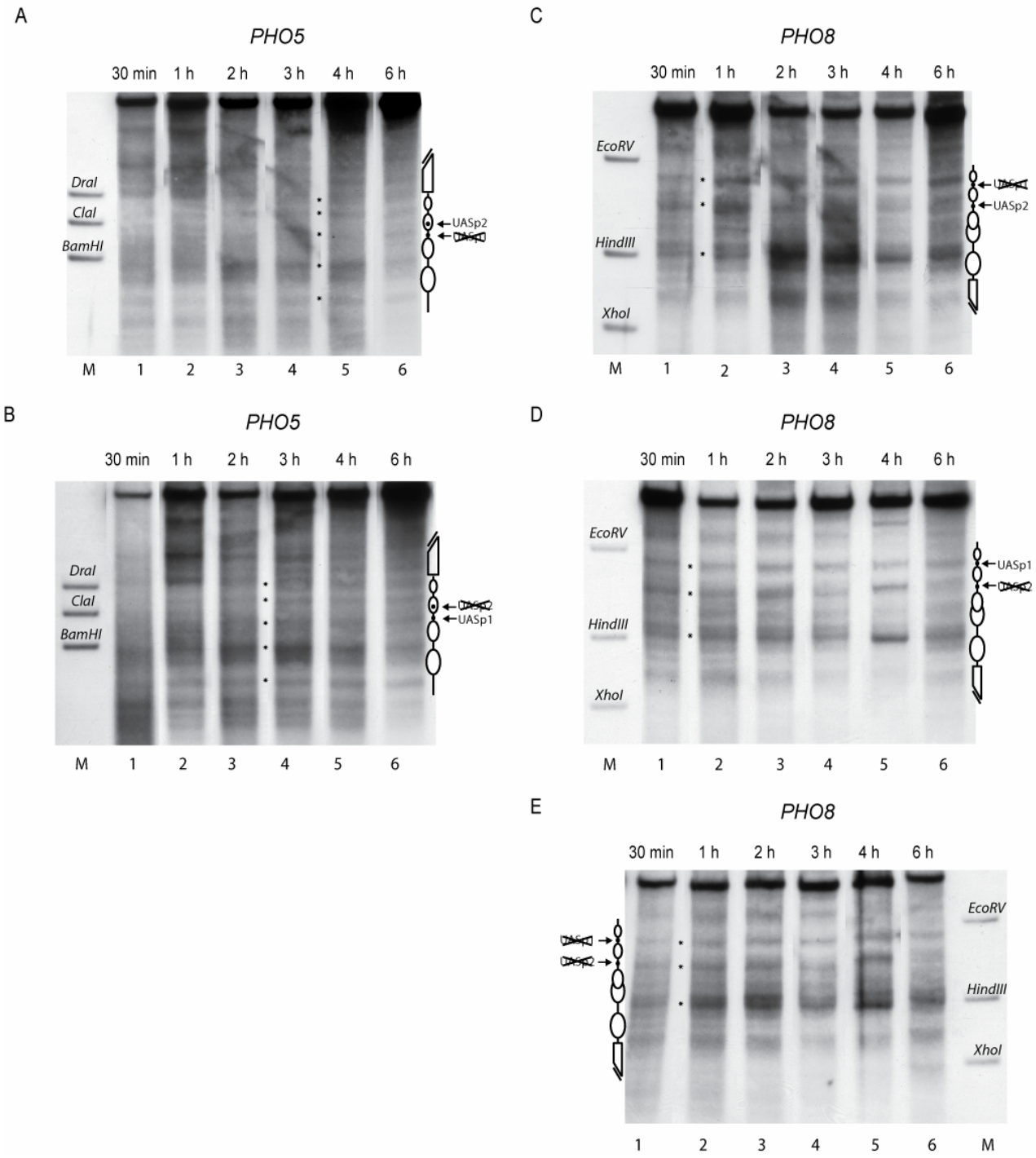


**Figure 8. Also in the absence of functional UASp elements the *in vitro* assembly kinetics of nucleosome positioning at the *PHO8* promoter are more rapid than at the *PHO5* promoter.** *In vitro* chromatin assembly was performed as in Figure 7 but using plasmids with deletions in either UASp1 (A) or UASp2 (B) at each promoter or UASp1 and UASp2 together at the *PHO8* promoter (C). Chromatin was mapped by DNaseI digestion followed by indirect end labeling at the indicated times and probed for the *PHO5* (left side of each panel) or the *PHO8* (right side) promoter. Schematics, asterisks and marker lanes are the same as in Figure 7.

E boxes are binding sites for some bHLH proteins and the whole cell extract is rather abundant in such proteins (Robinson and Lopes, 2000). The possibility was therefore considered that a fortuitous E-box binding protein from the whole cell extract could bind to the UASp sites of both promoters. To address this theory experimentally, DNA templates were generated in which the E-box consensus sequence 5'-CANNTG-3' was mutated.

Importantly, mutation of the intra-nucleosomal UASp site of the *PHO5* promoter did not alter the kinetics of nucleosome positioning neither did mutation of the inter-nucleosomal binding site (Figure 8A and B). Similar results were obtained at the *PHO8* promoter; mutation of either UASp site or mutation of both sites did not show any influence on the kinetics of nucleosome positioning (Figure 8C-E). Thus, the difference in the kinetics of nucleosome positioning at the *PHO5* and *PHO8* promoters can not be explained by a fortuitous E box binding activity in the yeast extract.

## 2.2. The DNA sequence information alone is not sufficient to position the nucleosomes at the *PHO5* and *PHO8* promoters by salt gradient dialysis chromatin assembly

As the templates only differ in the *PHO5* and *PHO8* loci, respectively, the observed difference in the kinetics of nucleosome positioning must lie within this DNA region. Strong nucleosome positioning information present in the DNA sequence of the *PHO8* promoter could explain why nucleosomes become positioned more rapidly at the *PHO8* promoter compared to those at the *PHO5* promoter. In this scenario the *PHO8* promoter would have higher nucleosome positioning power than the *PHO5* promoter. Such a difference in nucleosome positioning power might already become apparent in an un-catalyzed assembly system like salt gradient dialysis. In this system histones and DNA are mixed at high salt concentration (2 M NaCl) that prevents histone-DNA interactions. The salt concentration is then slowly reduced allowing first histone H3 and H4 to bind DNA between 1.2 M NaCl and 0.85 M NaCl imposing a nucleosome like structure to which the histone H2A and H2B are bound at lower salt concentrations (Wilhelm et al., 1978). Further reduction of the salt concentration to physiological levels (100-150 mM NaCl) or below (50 mM) "freezes in" the resulting chromatin state. Generation of chromatin by this protocol has the advantage of working with purified components, i.e., only DNA and histones, and was previously been widely used in order to study the influence of the DNA sequence on the formation of a nucleosome (Widom, 1998 and references herein).

If strong positioning information were present in the *PHO8* promoter sequence it was possible that the proper nucleosome positioning at the *PHO8* promoter could be generated in such an un-catalyzed system. At the *PHO5* promoter nucleosome positioning might be less defined through the DNA sequence alone and would therefore have to rely on factors from the yeast extract to properly position the nucleosomes. To test this hypothesis the *PHO5* and *PHO8* DNA constructs were assembled into chromatin by salt gradient dialysis whereby several ratios of histones to DNA were tested. Nucleosomal arrays as assayed by MNase digestion were generated at various different ratios of histones to DNA in the range between 0.9 to 1.1 (Figure 9A). At the highest ratio tested (1.1) the lowest amount of sub-nucleosomal DNA, an indicator of incomplete chromatin assembly, was visible on the gel (Figure 9A), arguing for a more complete assembly under these conditions. At histone to DNA ratios below 0.9 extensive nucleosomal arrays were not generated and at ratios higher than 1.1 the generated chromatin began to aggregate during the assembly reaction, probably due to the high amount of histones (data not shown).

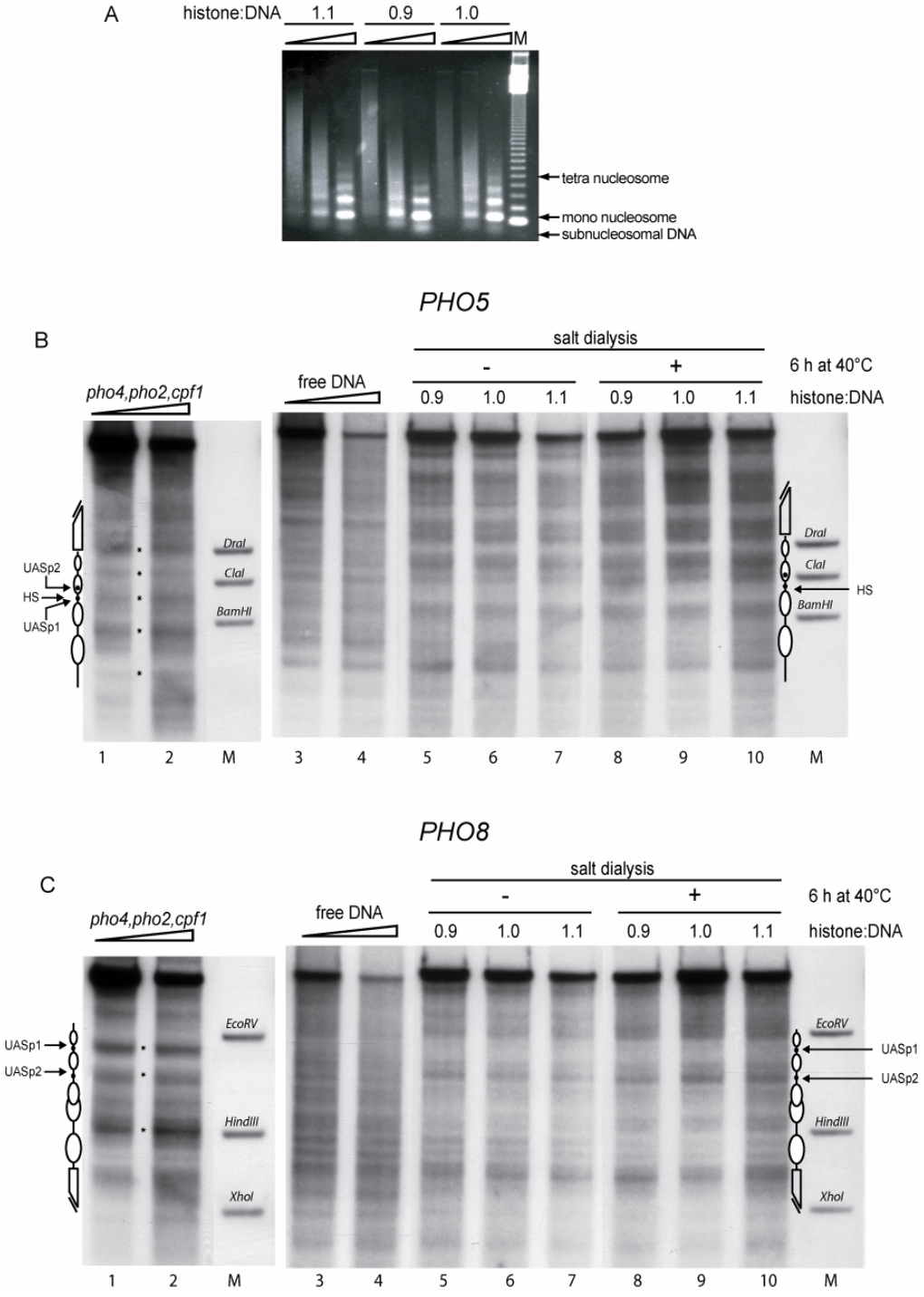


**Figure 9. Chromatin assembly by salt gradient dialysis does not generate the proper chromatin structure at the *PHO5* or the *PHO8* promoter.** (A) Chromatin assembled by salt gradient dialysis at histone to DNA mass ratios of 0.9, 1.0 and 1.1 (as indicated) was subjected to MNase digestion (ramps on top of lanes represent increasing MNase digestion times) and visualized by ethidium bromide staining. Lane M shows a 123 bp ladder (Gibco). The same chromatin preparations as in (A) were subjected to DNaseI mapping and probed for the *PHO5* (B) or the *PHO8* (C) promoter before and after a 6 hour incubation at 40°C (as indicated). DNaseI mapping of free DNA (lanes 3 and 4) as well as chromatin assembled with yeast extract made from a *pho4*, *pho2*, *cpf1* triple mutant strain (lanes 1 and 2) is shown for comparison. Ramps on top of the lanes 1 to 4 in panel (B) and (C) denote increasing DNaseI concentrations. Marker bands (lanes M), schematics and asterisks are the same as in Figure 7.

The same chromatin shown in Figure 9A was subjected to DNaseI mapping and probed for both the *PHO5* and the *PHO8* promoter regions subsequently on the same blot membrane (Figure 9B and C). The resulting nucleosomal patterns were distinct patterns and not similar to the patterns of free DNA but they were also clearly different from the patterns obtained with the yeast extract assembly system. In particular, the hypersensitive site in the *PHO5* promoter between nucleosomes -2 and -3 was strongly protected in the chromatin assembled by salt dialysis, and the region of nucleosome -3 contained a strong hypersensitive site. Furthermore, a band at the position of the *ClaI* marker band was visible both in the patterns of free DNA and in the salt dialysis chromatin. This region is protected by nucleosome -2 in the native yeast pattern (Figure 9B; compare lanes 1 and 2 with lanes 3 to 10). Notably, however, the promoter nucleosome -1 seemed to have been formed properly in the chromatin assembled by salt dialysis suggesting that a strong nucleosome-positioning DNA sequence may be involved in determining the position of this nucleosome. At the *PHO8* locus the hypersensitive site at UASp1 that is clearly visible in the yeast pattern was strongly protected in the chromatin assembled by salt dialysis, while the hypersensitive sites at the position of UASp2, at the position of the *HindIII* marker band, and at the beginning of the open reading frame were present in both patterns of free DNA and in chromatin generated by salt dialysis (Figure 9; compare lanes 1 and 2 with 5 to 10).

It was shown repeatedly that the assembly of chromatin by salt dialysis need not result in nucleosomes occupying the energetically most favorable positions right away. Many protocols therefore apply an additional heat shifting step at 37 to 55°C in order to allow the nucleosomes to adopt their preferred positions (Flaus and Richmond, 1998; Luger et al., 1999; Meersseman et al., 1992; Pennings et al., 1991). To test whether this might change the patterns of the *PHO5* and *PHO8* promoter after assembly by salt dialysis, chromatin was generated by salt dialysis and then incubated up to 6 hours at 40°C. This did, however, not lead to a significant change in the chromatin structure of either promoter (Figure 9B and C, compare lanes 5 to 7 with 8 to 10). Thus, the DNA sequence alone, as read by the formation of histone-DNA interactions after salt gradient dialysis assembly is not sufficient to properly position the nucleosomes at either promoter.

## 2.3. An *in vitro* assembly system based on *Drosophila* embryo extract does not generate the proper chromatin structure at the *PHO5* and *PHO8* promoters

The fact that the proper chromatin pattern at either the *PHO5* or the *PHO8* promoter was not generated by salt dialysis alone does not exclude that the positioning information is in fact present in the DNA sequence at physiological conditions, but that it takes an ATP-dependent chromatin remodeling machine to move the nucleosomes into their proper positions. In accordance with this theory, the yeast whole-cell extract, which contains such machines, is been shown to be sufficient for proper nucleosome positioning at the *PHO5* promoter (Figure 6) (Korber and Hörz, 2004). Maybe one or several other remodeling machines (different from the ones present in the yeast extract) might also be able to position the nucleosomes at the *PHO5* and *PHO8* promoters. To test this hypothesis the well-established cell-free chromatin reconstitution system based on *Drosophila* embryo extracts was employed. These extracts are rich in histones and chromatin-remodeling activities (Becker et al., 1994; Becker and Wu, 1992; Kamakaka et al., 1993) (Figure 10). Using this system, *Drosophila* embryo extracts were incubated with the yeast DNA templates and an energy regenerating system for up to six hours. In agreement with previously published data (Becker et al., 1994; Becker and Wu, 1992), arrays of regularly spaced nucleosomes were assembled on both DNA templates (Figure 10B and data not shown). However, mapping the positions of the nucleosomes on both promoters showed that this *in vitro* system could not generate the native yeast chromatin patterns. Instead, the resulting nucleosome pattern was very similar to the pattern of free DNA (Figure 10C and D, compare lanes 1 and 2 with 3 and 4). Again, the interference of a fortuitous E box binding protein from the *Drosophila* extract was controlled for. Mutating the UASp sites at either promoter did not alleviate the inability of the *Drosophila* extract to generate the characteristic yeast pattern (data not shown). Notably, in all three chromatin assembly systems histones from *Drosophila* embryos were used, and therefore differences in the generated nucleosomal patterns cannot be due to the source of histones.

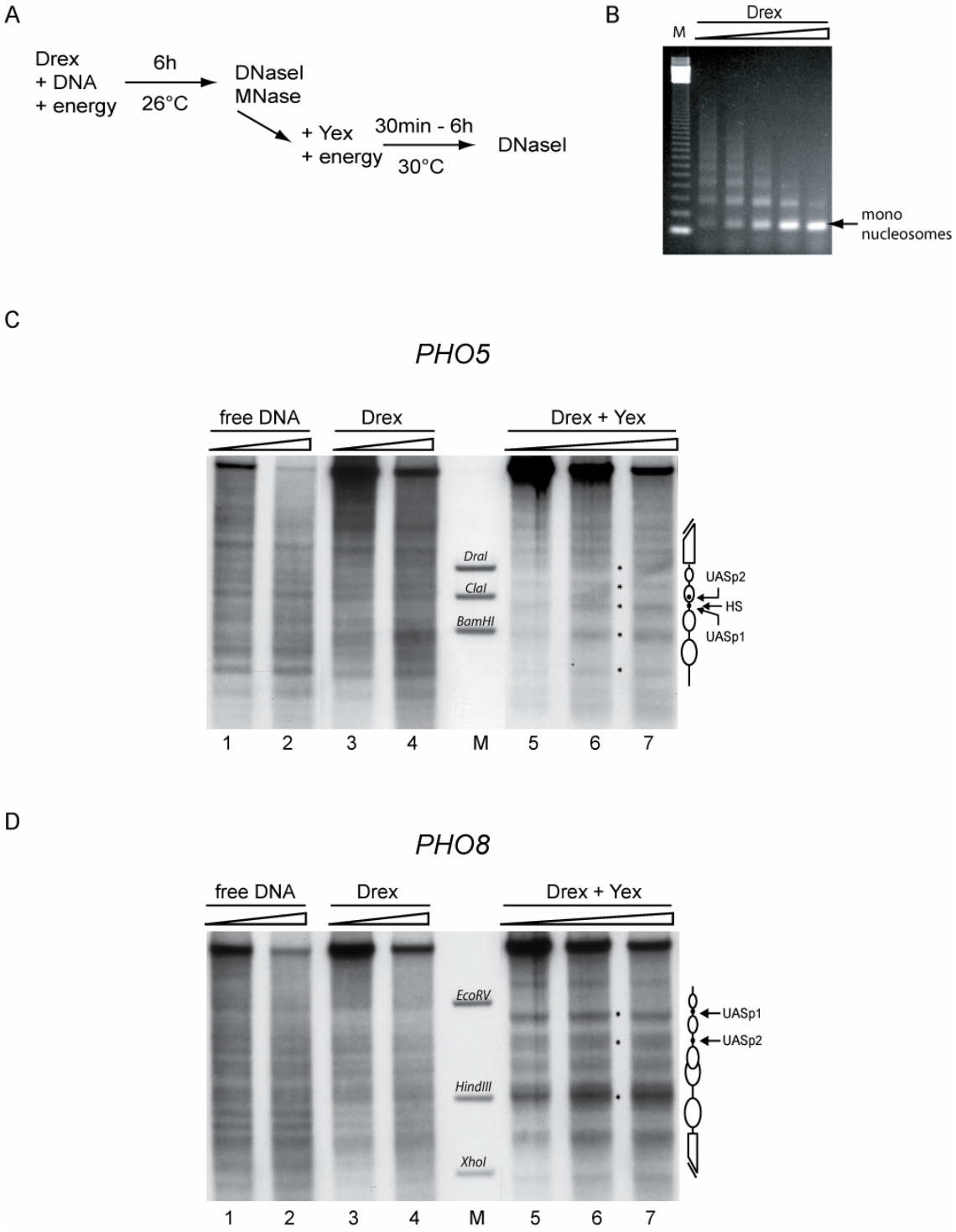


**Figure 10. A *Drosophila* embryo extract assembly system cannot position the nucleosomes at the *PHO5* and *PHO8* promoter.** (A) Scheme of the assembly reaction. Chromatin was generated by incubating *Drosophila* embryo extract (Drex) with DNA and an energy regenerating system for 6 hours at 26°C. The chromatin was analyzed by MNase digestion and DNaseI mapping (DNaseI) either directly or after addition of yeast extract (Yex), a fresh energy mix and incubation for up to 6 more hours at 30°C. (B) The chromatin generated with the *Drosophila* embryo extract system was subjected to MNase digestion and visualized by ethidium bromide staining. Ramps on top of lanes represent increasing amounts of digestion time and lane M shows a 123 bp ladder (Gibco). (C and D) DNaseI mapping of free DNA (lanes 1 and 2) or chromatin prior (lanes 3 and 4) or after (lanes 5 to 7) addition of yeast extract and further incubation for 3 hours, probed for the *PHO5* (B) or the *PHO8* (C) promoter. Schematics, asterisks and marker lanes (M) are as in Figure 7. Ramps on top of the lanes in panel (B) and (C) denote increasing DNaseI concentrations.

These results can be explained in two ways: (i) the underlying DNA sequence contains sufficient positioning information, but as *Drosophila* embryo extract is rich in chromatin remodeling activities these machines override these specific determinants responsible for nucleosome positioning and thereby randomize the pattern, or (ii) neither the underlying DNA sequence nor the *Drosophila* embryo extract contain sufficient positioning information to position the *PHO5* and *PHO8* promoter nucleosomes.

## 2.4. The addition of yeast extract to chromatin preassembled by *Drosophila* extract can shift the nucleosomes to the proper positions at both the *PHO5* and *PHO8* promoter

To address the question if the lack of proper nucleosome positioning in *Drosophila* extract-assembled chromatin could be compensated by the yeast extract, the *PHO5* and *PHO8* plasmids were first assembled into chromatin using the *Drosophila* extract system and then yeast extract was added followed by an incubation for up to six more hours. Strikingly, this experimental setup indeed led to the generation of proper nucleosome patterns at both promoters (Figure 10C and D, lanes 5 to 7). Interestingly, nucleosome repositioning at both promoters by addition of the yeast extract was completed after just 30 min, and the chromatin retained the same pattern for up to six more hours of incubation (data not shown). These results show that the *Drosophila* embryo extract can assemble chromatin as assayed by the formation of regularly spaced nucleosomes but it cannot position the nucleosomes correctly on a yeast DNA template because it lacks the necessary nucleosome positioning information.

## 2.5. The addition of yeast extract to chromatin preassembled by salt gradient dialysis also repositions nucleosomes to the native chromatin patterns

Apparently, yeast extract can shift nucleosomes on a chromatin template pre-assembled by *Drosophila* extract into the native nucleosome pattern much more rapidly compared to a *de novo* assembly. This could be due to a specific property of the *Drosophila* extract that generates an ideal template for the yeast extract. Alternatively, the fact that the nucleosomes were already loaded on the DNA could greatly enhance the speed of nucleosome positioning in the yeast extract. In order to differentiate between these two possibilities, re-positioning of the promoter nucleosomes to the native *PHO5* and *PHO8* patterns by addition of yeast extract to chromatin pre-assembled by salt gradient dialysis was tested next. In this system only purified components are used and therefore an initial effect on nucleosome loading and

subsequent nucleosome positioning by cellular factors besides the yeast extract could be excluded.

Indeed, a clear shift from the pattern characteristic of the chromatin generated by salt dialysis to the proper *PHO5* and *PHO8* promoter chromatin structure was seen for both promoters (Figure 11B and C, lanes 1 to 4). This shift in positioning was energy dependent as no change was seen in the absence of ATP (Figure 11B and C, lanes 6). Again, as opposed to *de novo* chromatin assemblies where proper nucleosome positioning at the *PHO5* promoter was not observed until after three to four hours, the kinetics of the pattern switch were very rapid, and were complete within 30 min at both promoters. No major changes in the pattern occurred after incubation for up to 6 hours (data not shown). Thus, chromatin assembly and nucleosome positioning in this *in vitro* system can be mechanistically separated into two processes: chromatin assembly being a very slow process taking three to four hours, whereas nucleosome positioning is very rapid, accomplished within 30 min. Furthermore, shifting of the nucleosomes to the proper chromatin structure at the *PHO5* promoter using pre-assembled chromatin templates proved to be much more lenient to both buffer conditions and extract concentrations, as opposed to *de novo* assemblies of the *PHO5* promoter which required a careful titration of histones and yeast extract and was very sensitive to buffer conditions. Interestingly, titrating in the amount of yeast extract needed to switch the pattern showed that only 1:80 of the amount that is normally needed for a *de novo* yeast extract assembly was able to switch the pattern at the *PHO8* promoter on a pre-assembled chromatin template whereas for *PHO5* promoter dilutions lower than two-fold failed to generate the pattern (data not shown).

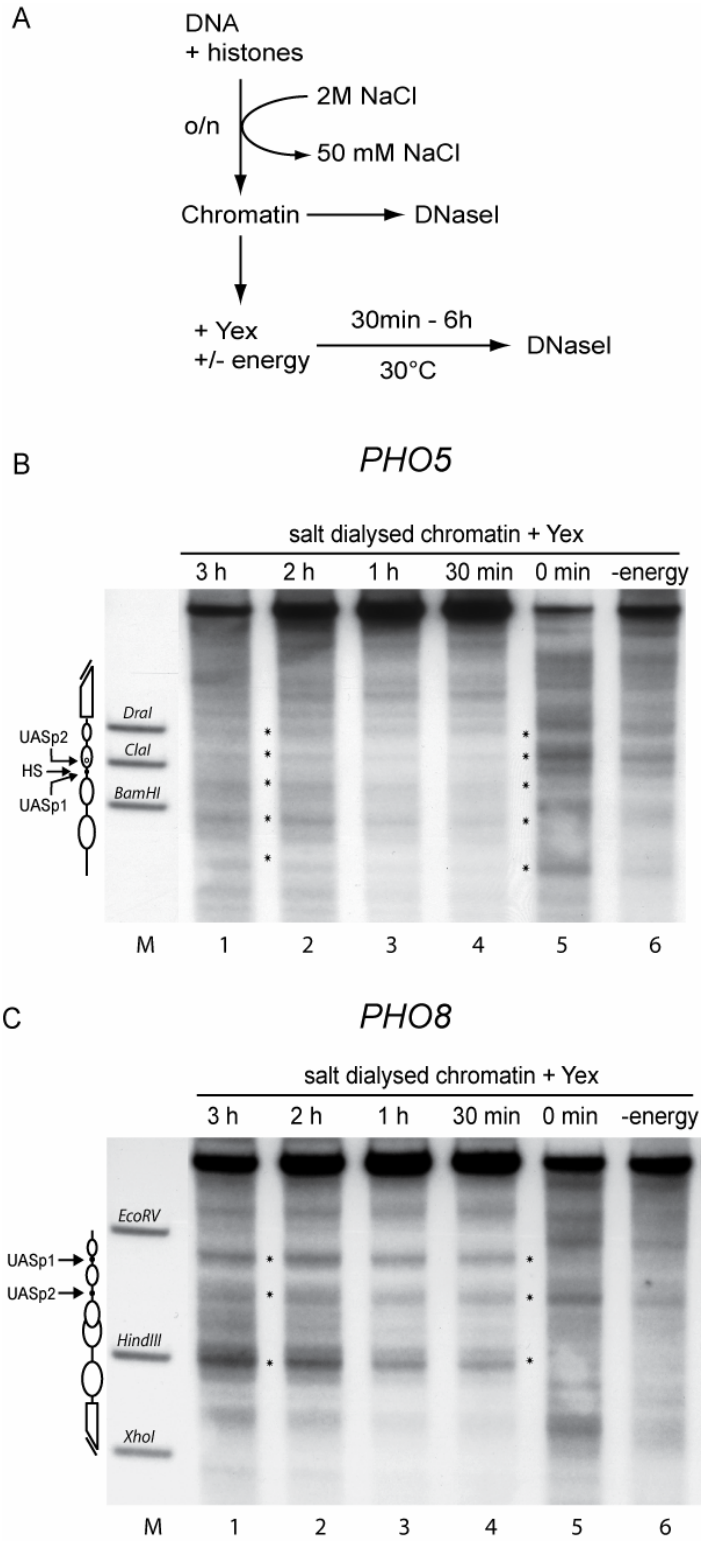


**Figure 11. Nucleosomes preassembled by salt gradient dialysis become rapidly and properly repositioned after the addition of yeast extract.** (A) Reaction scheme. Chromatin was generated by mixing DNA and histone octamers at a mass ratio of 1.1 and over night (o/n) salt gradient dialysis from 2M to 50mM NaCl. The resulting chromatin was analyzed by DNaseI mapping (DNaseI) either immediately afterwards or following an additional incubation step with yeast extract (Yex) with or without an energy regenerating system (energy) for up to 6 hours at 30°C. (B) and (C) DNaseI mapping of chromatin after addition of yeast extract for the indicated times in the presence (lanes 1 to 5) or for 3 hours in the absence of energy (lane 6) as probed for the *PHO5* (B) or the *PHO8* (C) promoter. Marker lanes (M), schematics and asterisks are the same as in Figure 7.

## 2.6. Swi/Snf, Isw1, Isw2 or Chd1 are not required for nucleosome positioning at the *PHO5* and *PHO8* promoters

Taking advantage of the fact that chromatin assembly and nucleosome positioning can be functionally dissected, the latter could now be investigated more carefully. First, the requirement for ATP-dependent chromatin remodeling machines for correct nucleosome positioning was addressed. Extracts from yeast strains deleted in ATPase subunits of the chromatin remodeling complexes Swi/Snf, Isw1, Isw2 and Chd1 were prepared. In *de novo* chromatin assemblies the *isw1, isw2* double mutant and the *isw1, isw2, chd1* triple knockout extracts could not assist the generation of extensive nucleosomal arrays and these extracts was therefore not able to generate the proper *PHO5* chromatin structure in *de novo* assemblies (data not shown and unpublished data from the lab). However, the *snf2* extract, the *isw1, isw2* double mutant extract and the *isw1, isw2*, *chd1* tripple mutant extract were able to properly position the nucleosomes at the *PHO5* and *PHO8* promoters in nucleosome shifting reactions (Figure 12A and B, lanes 1 to 4). This suggests that the observed deficiency in generation of extensive nucleosomal arrays in the double and triple mutant occurs at the level of chromatin assembly and not at nucleosome positioning. Thus, at least Isw1 and Isw2 are essential for *in vitro* chromatin assembly, however neither Swi/Snf, Isw1, Isw2 nor Chd1 are strictly required for nucleosome positioning at the *PHO5* and *PHO8* promoters.

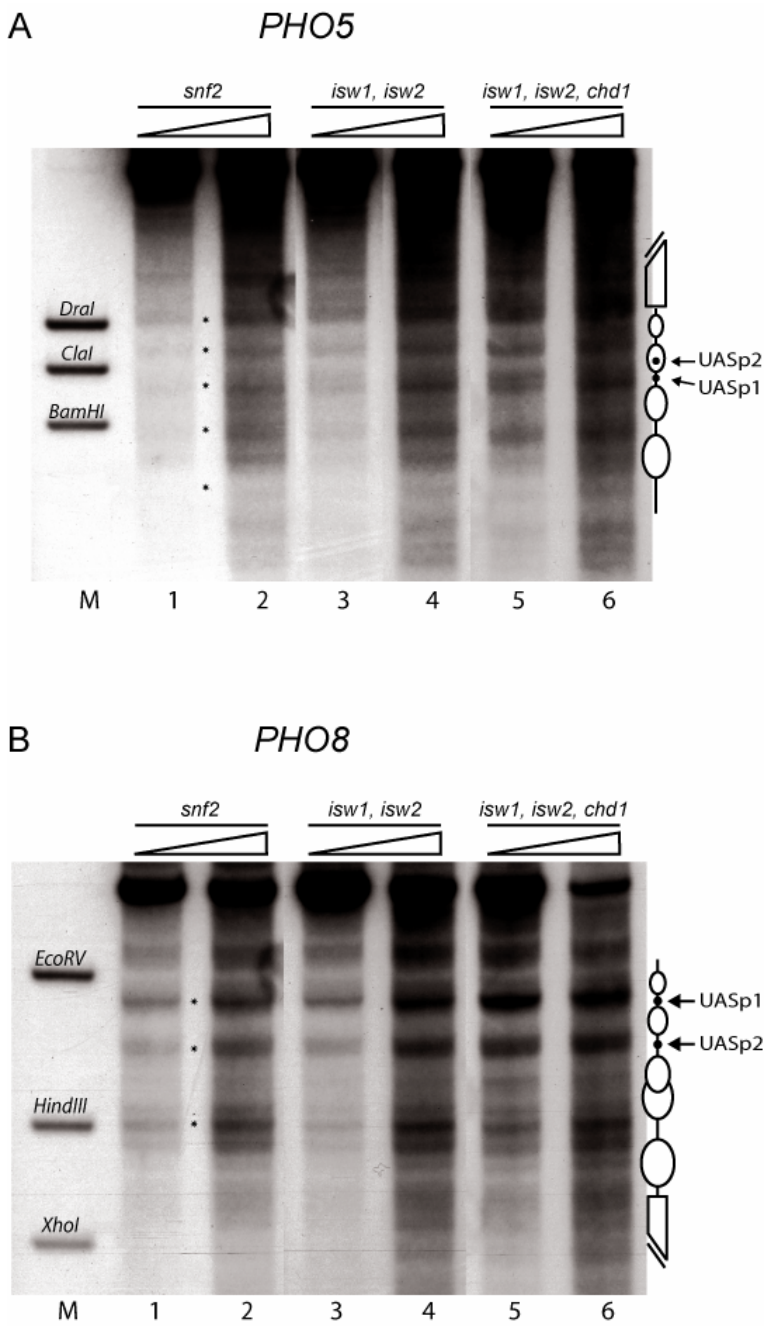


**Figure 12. The absence of various Snf2-type ATPases has no effect on the proper repositioning of chromatin preassembled by salt gradient analysis.** The analogous experiment to the one in Figure 10 was done by adding yeast extracts from strains deleted for the indicated gene(s) to salt dialysis assembled chromatin followed by further incubation for 2 hours in the presence of energy and subsequent DNaseI mapping and probing for the *PHO5* (A) or the *PHO8* (B) promoter. Schematics, asterisks and marker lanes are the same as in Figure 7. Ramps on top of the lanes indicate increasing DNaseI concentrations.

## 2.7. Treatment with high concentrations of RNaseA abolishes the nucleosome positioning activity of a whole cell yeast extract

RNaseA treatment of a *Drosophila* embryo extract prior to a *de novo* chromatin assembly completely abrogates its ability to generate nucleosomal arrays (data not shown and Gernot Längst, personal communication). The function of RNA in *in vitro* chromatin assembly may be a histone chaperone function similar to that of Nap1, which binds to the free histones thereby preventing protein aggregation (Lusser and Kadonaga, 2004 and references herein). Therefore, it is not possible to test whether RNA could play a role in nucleosome positioning at the *PHO5* and *PHO8* promoter in *de novo* chromatin assemblies. However, using pre-assembled chromatin as a substrate in a nucleosome shifting reaction allowed testing of a possible involvement of RNA in nucleosome positioning. Prior to the nucleosome shifting reaction yeast extract was digested with different concentrations of RNaseA for one hour. Treatment of the extract with low amounts of RNaseA did not have any effect on nucleosome positioning at either promoter (Figure 13A and B, lanes 3 to 14), but nucleosome positioning was lost at both promoters after treatment of the extract with high concentrations of RNaseA (48 mM[3]) (Figure 13A and B, lanes 1 and 2). The resulting DNaseI patterns looked very similar to that of the salt dialyzed chromatin (compare Figure 9B and C, lanes 5-10 with Figure 13A and B, lanes 1 and 2). Extract treated with high amounts of RNaseA (48 and 4.8 mM) were also tested in *de novo* assemblies. In these extracts no RNA was visible by Ethidium Bromide staining and these extracts could not generate extensive nucleosomal arrays (data not shown)

[3] 48 μM Rnasee corresponds to approximately 50 μg of protein. In comparison, in a nuclesome shifting reaction approximately 300 μg of protein are added with the extract.

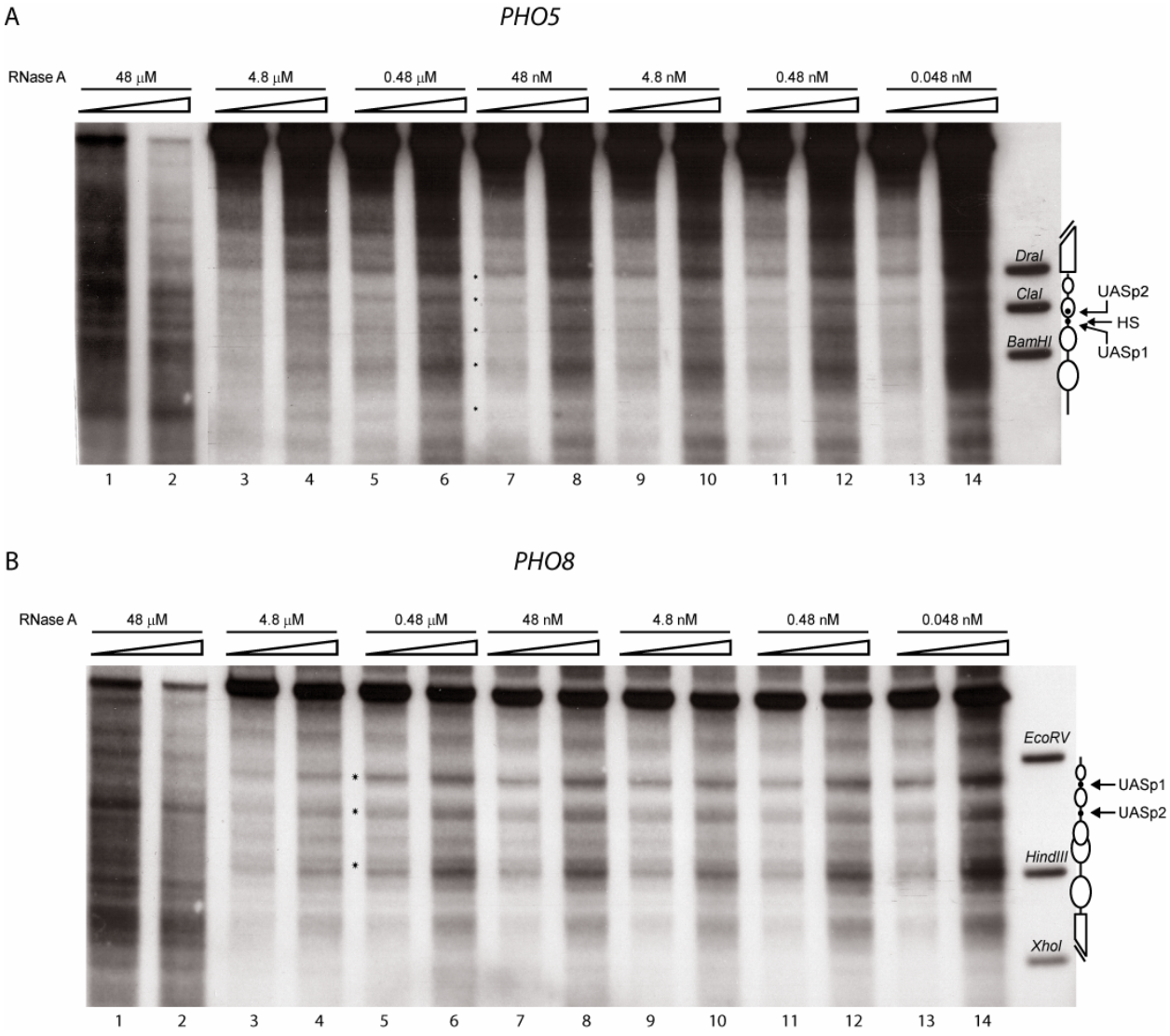


**Figure 13. Treatment with high concentrations of RNaseA abolishes the nucleosome positioning activity in a whole cell yeast extract.** Whole cell extract was treated with the indicated concentrations of RNaseA for one hour and then added to pre-assembled chromatin. After 2 hour incubation in the presence of energy DNaseI mapping was performed and blots were probed for the *PHO5* (A) or the *PHO8* (B) promoter. Schematics, asterisks and marker lanes are the same as in Figure 7. Ramps on top of the lanes indicate increasing DNaseI concentrations.

## 2.8. RNA Polymerase II transcription is not required for nucleosome positioning

The *PHO5* promoter has baseline activity even under repressing conditions in high phosphate medium so that low levels of mRNA and acid phosphatase activity can still be measured (Gregory et al., 1998). Furthermore, under repressing conditions the accessibility of the *ClaI* site of the *PHO5* promoter is around 10-20 % in a wild type strain (Almer et al., 1986).

It was therefore considered that RNA-Polymerase II (Pol II) transcription may be a prerequisite for nucleosome positioning. To test this possibility, nucleosome positioning at the *PHO5* and *PHO8* promoters was analyzed in a strain with a temperature-sensitive mutation of

the largest subunit of the RNA Polymerase II, Rpb1-1 (Nonet et al., 1987). When the temperature is increased to 36ºC for two to four hours in this strain, a complete block in Pol II transcription occurs whereby the catalytic activity of the enzyme is impaired but the integrity of the PolII complex is not compromised (Nonet et al., 1987). Furthermore, yeast whole cell extracts harvested from this strain grown at the permissive temperature (24ºC) have no detectable *in vitro* transcription activity (Nonet et al., 1987, and Jesper Svejstrup, personal communication).

Whole cell extract from a temperature sensitive *rpb1-1* strain grown at the permissive temperature were prepared and tested in a nucleosome shifting reaction. This extract could indeed properly position the nucleosomes at the *PHO5* and *PHO8* promoters (Figure 14A and B, lanes 3 and 4) in an energy-dependent way (compare Figure 9B and C, lanes 5-10 with Figure 14A and B, lanes 5 and 6).

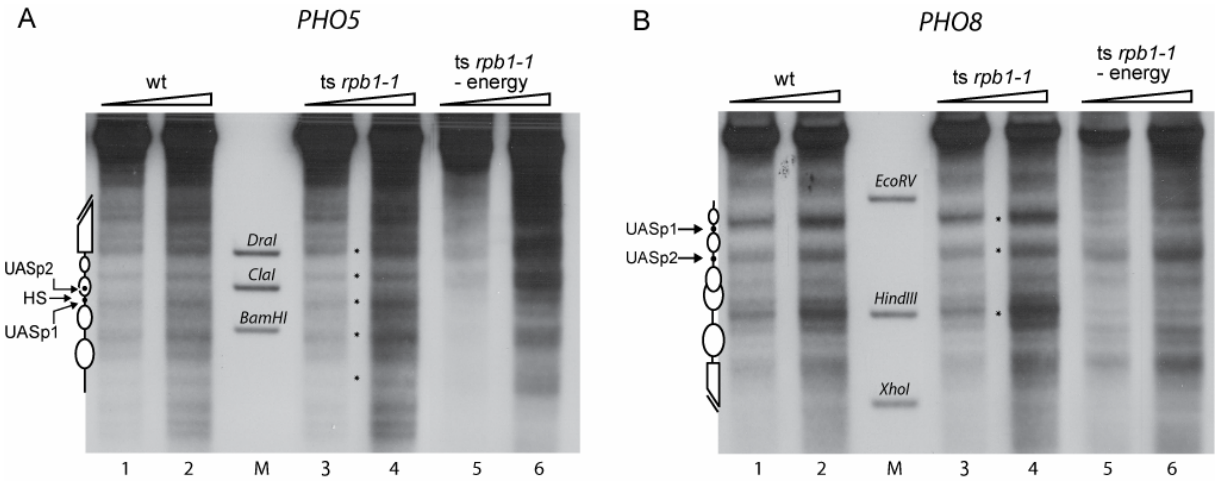


**Figure 14. Whole cell extract from a temperature sensitive *rpb1-1* strain can position the nucleosomes at the *PHO5* and *PHO8* promoters.** Whole cell extract, prepared from a temperature sensitive *rpb1-1* strain grown at the permissive temperature was added to chromatin assembled by salt gradient dialysis with and without energy for two hours and subsequently DNaseI mapping was performed and visualized by probing for the *PHO5* (A) and the *PHO8* (B) promoter. Schematics, asterisks and marker lanes are the same as in Figure 7. Ramps on top of the lanes indicate increasing DNaseI concentrations.

In parallel to the *in vitro* approach, nucleosome positioning was also analyzed *in vivo* in the *rpb1-1* temperature-sensitive strain. Nuclei were prepared from the temperature sensitive *rpb1-1* strain grown at the permissive (24°C) or after a shift to the restrictive temperature (36°C) for 12-16 hours. To rule out any effects on chromatin structure caused solely by the elevated temperature, nuclei from the isogenic wild type strain were prepared in parallel. In the wild type strain the chromatin structure of both promoters appeared less distinct upon incubation at 36°C. This was apparent both from the DNaseI indirect end labeling experiment

(Figure 15A and C, compare lanes 1 and 2 with 3 and 4) and the restriction enzyme accessibility assay (Figure 15B and D). In particular the *ClaI* site of the *PHO5* promoter, which is normally protected by nucleosome -2, became more accessible at elevated temperatures (Figure 15B), but also both the *StuI* and *NdeI* site of the *PHO8* promoter (Figure 15D) were more accessible to restriction enzymes under these conditions arguing that high temperature has a negative effect on the chromatin structure *in vivo*. In the temperature sensitive *rpb1-1* mutant the DNaseI patterns at both promoters also became less distinct when the cultures were grown at the restrictive temperature (Figure 15A and C, compare lanes 5 and 6 with 7 and 8). However, as judged both by DNaseI indirect end labeling (Figure 15A and C) and restriction enzyme accessibility (Figure 15B and D) this effect was not as severe as in the wild type strain. Interestingly, the *ClaI* site of the *PHO5* promoter became less accessible when the cells were grown at the restrictive temperature (Figure 15B) However, this could also be explained by the lack of basal level transcription. At the *PHO8* promoter the accessibility of both the *StuI* and *NdeI* site did not change significantly (Figure 15D). These results clearly show that PolII transcription *per se* is not a determinant of nucleosome positioning at the *PHO5* and *PHO8* promoters.

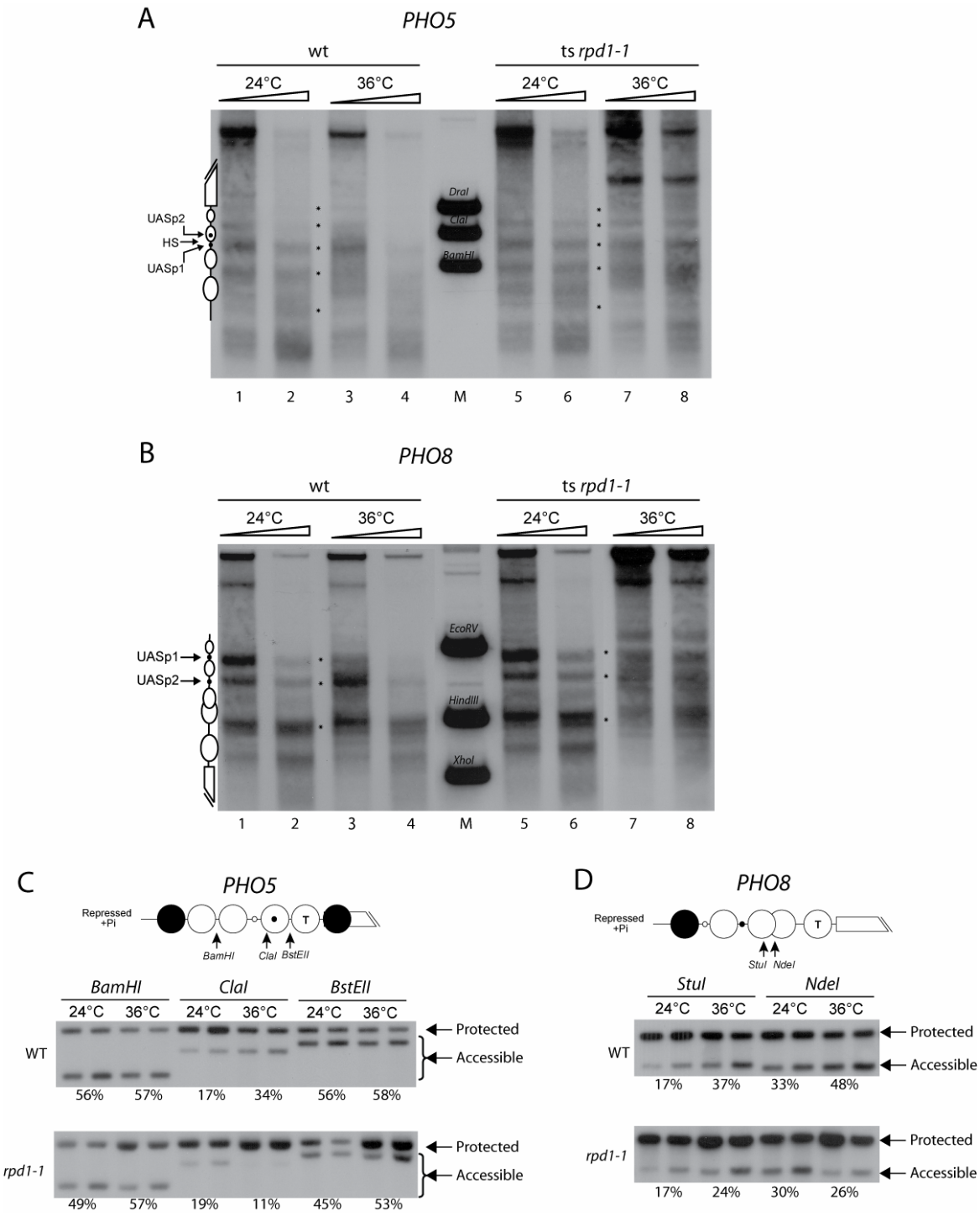


**Figure 15. Inhibition of RNA PolII transcription *in vivo* has no effect on nucleosome positioning at the *PHO5* and *PHO8* promoters.** Yeast nuclei prepared from a temperature sensitive *rpb1-1* strain and from its wild type strain grown at the permissive (24°C) and restrictive (36°C) temperature were subjected to DNaseI mapping and restriction enzyme accessibility. (A) and (B) Schematics, asterisks and marker lanes are the same as in Figure 2 and ramps on top of the lanes indicate increasing DNaseI concentrations. (C) Nuclei were digested with two concentrations of four fold difference of *BamHI*, *ClaI* and *BstEII*, subsequently the DNA was prepared and digested with *HaeIII* and probed for the *PHO5* promoter. The accessibility of each restriction enzyme was normalized to the accessibility of the *ClaI* site of the *PHO3* gene (data not shown) and is shown as percentage of the total signal. (D) Same as C except that nuclei were digested with *StuI* and *NdeI*, subsequently the DNA was prepared and digested with *BglI/EcoRV* and probed for the *PHO8* promoter.

# 3. Stability of positioned nucleosomes

## 3.1. Proper nucleosome positioning at the *PHO5* promoter is dependent on higher degrees of chromatin assembly than at the *PHO8* promoter

In a *de novo* assembly reaction nucleosomes at the *PHO8* promoter were positioned much more rapidly than at the *PHO5* promoter (Figure 7), however, in a nucleosome shifting reaction no difference in nucleosome positioning kinetics was observed after 30 minutes (Figure 11). This suggests that there is no difference between the two promoters when the template is already fully assembled. If this was true it would suggest that nucleosome positioning at the *PHO5* promoter can only occur when an extensive nucleosomal array is present whereas positioning of the nucleosomes at *PHO8* promoter is largely independent on the nucleosome occupancy of neighboring sequences.

In accordance with this hypothesis, proper nucleosome positioning at the *PHO5* promoter was sometimes impaired when using chromatin pre-assembled by *Drosophila* embryo extract in a nucleosome shifting reaction. Such an assembly can lead to more or less extensive nucleosome arrays depending on the quality of the extract and the buffer conditions. The repositioning of the pattern at the *PHO8* promoter to yield the proper chromatin structure by addition of yeast extract was largely independent of the extent of nucleosomal arrays as preassembled by the *Drosophila* embryo extract. However, repositioning at the *PHO5* promoter worked properly only if extensive nucleosomal arrays were pre-assembled by the *Drosophila* embryo extract (data not shown).

This could however be also be a result of differences in nucleosome affinities between the *PHO5* and *PHO8* promoters. If the *PHO8* promoter had a higher affinity for nucleosomes than the *PHO5* promoter the *PHO8* promoter region may become more fully assembled under conditions of limiting histones (low histone to-DNA ratios) whereas the *PHO5* promoter region would not. To test this directly, chromatin templates with different degrees of nucleosomes assembly were generated by varying the histone-to-DNA mass ratio in the salt gradient dialysis assembly (0.6 to 1.4). Using this strategy different assembly states were purposely generated. The plasmids harbouring the *PHO5* and *PHO8* loci were present at equimolar ratios in all reactions. Following assembly by salt gradient dialysis the different reactions were pooled and the differently chromatinized DNA molecules were separated according to their sedimentation velocity, i.e. assembly state on a sucrose gradient by ultracentrifugation.

First, the assembly state of the chromatin in each fraction for each promoter plasmids were determined by limited MNase digestion followed by quantification of the signals remaining after digestion (Figure 16A). DNA that is not assembled into chromatin is readily digested by MNase whereas DNA assembled into chromatin is resistant to MNase digestion. The ratios between nucleosomal and non-nucleosomal DNA can therefore serve as a measure for the assembly state of the DNA. The signals remaining after the MNase digest were determined with specific probes of the *PHO5* (data not shown) and *PHO8* promoter regions (Figure 16A), respectively, and is therefore a direct measure of the assembly states of the promoter regions. The ratios between nucleosomal and non-nucleosomal DNA for the *PHO5* and the *PHO8* promoter region were similar in each fraction (data not shown) indicating that both promoter regions are assembled to the same degree.

Next, the relative amount of chromatinized *PHO5* and *PHO8* plasmids in each fraction (determined by the undigested DNA) were quantified using a probe recognizing the *PHO5* and the *PHO8* promoter region of the plasmid, respectively, and compared to the total amount of chromatinized DNA loaded on the gradient. As the chromatin sediments according to its nucleosome density this is a measurement of the assembly state of each plasmid. These relative amounts were similar for the *PHO5* and the *PHO8* plasmids (Figure 16B) indicating that both plasmid are assembled into chromatin equally efficient. Taken together, it can therefore be excluded that the *PHO8* plasmid locally at the promoter region has a higher nucleosome density than the *PHO5* promoter region under limiting histone conditions.

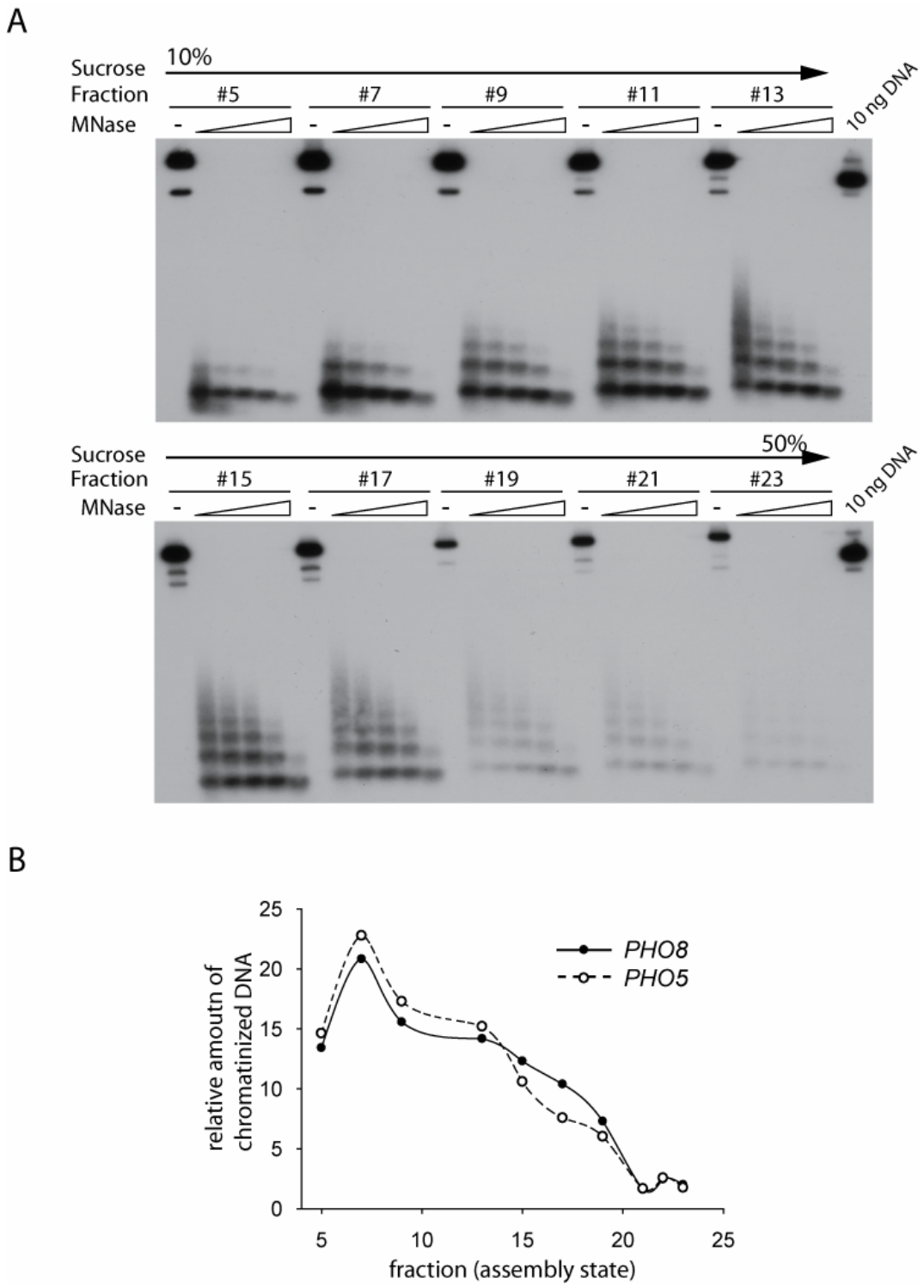


**Figure 16. The *PHO8* and *PHO5* promoter regions have the same affinity for nucleosomes.** 50 µg of chromatin assembled by salt gradient dialysis with different histone-to-DNA mass ratios (0.6 to 1.4) were loaded onto a 10-50% sucrose gradient. The chromatin in each fraction was analyzed by limited MNase digestion and probed for the *PHO8* promoter (A). Equivalent results as in panel (A) were obtained by using a *PHO5* promoter probe (not shown). One sample from each fraction was left un-digested (marked -). As loading reference 10 ng of each construct was added on each gel. Ramps on top of the lanes indicate increasing digestion times. (B) The amount of DNA in each fraction was estimated comparing the signal of the undigested sample (-) with the signal corresponding to 10 ng DNA for both the *PHO5* (data not shown) and the *PHO8* promoter (A).The percentage of total DNA loaded on the gradient (50 µg) was calculated and plotted against fraction number.

As chromatin assembly under limited amounts of histones leads to less assembled chromatin templates and as both promoter regions are assembled to the same degree under such conditions it was possible to directly test if nucleosome at the *PHO8* promoter could be positioned at lower nucleosome densities that the *PHO5* promoter.

Underassembled chromatin was generated by salt gradient dialysis with limiting amounts of histones (0.4 to 0.8) and both plasmids at equimolar ratios. Again the different chromatin preparations were tested by limited MNase digestion to determine the degree of assembly. In accordance with the results in Figure 16, the ratios between nucleosomal and total DNA were the same for both the *PHO5* and *PHO8* promoters (Figure 17A and data not shown). Next, this chromatin was shifted by addition of whole cell yeast extract. In all under-assembled chromatin preparations, nucleosomes at the *PHO5* promoter did not become properly positioned (Figure 17B, lanes 1 to 8), whereas at the *PHO8* promoter the correct positioning of the nucleosomes was already discernible at low histone-to-DNA mass ratios (Figure 17C, lanes 1 to 4) and became more distinctive with increasing mass ratios (Figure 17C, lanes 5 to 8). Thus, the shift to proper nucleosome positioning at the *PHO5* promoter requires higher degrees of chromatin assembly than at the *PHO8* promoter. These results, together with the results in Figure 7 (nucleosome positioning kinetics in *de novo* assemblies), suggest that the *PHO8* promoter has stronger nucleosome positioning power than the *PHO5* promoter, i.e. the properly positioned nucleosomes at the *PHO8* promoter have higher relative stability compared to those of the *PHO5* promoter.

A

| Histone:DNA ratio | 0.4 | 0.5 | 0.6 | 0.8 |
|---|---|---|---|---|
| Relative nucleosome density *PHO8*:*PHO5* | 1 | 1.2 | 1.1 | 1.2 |

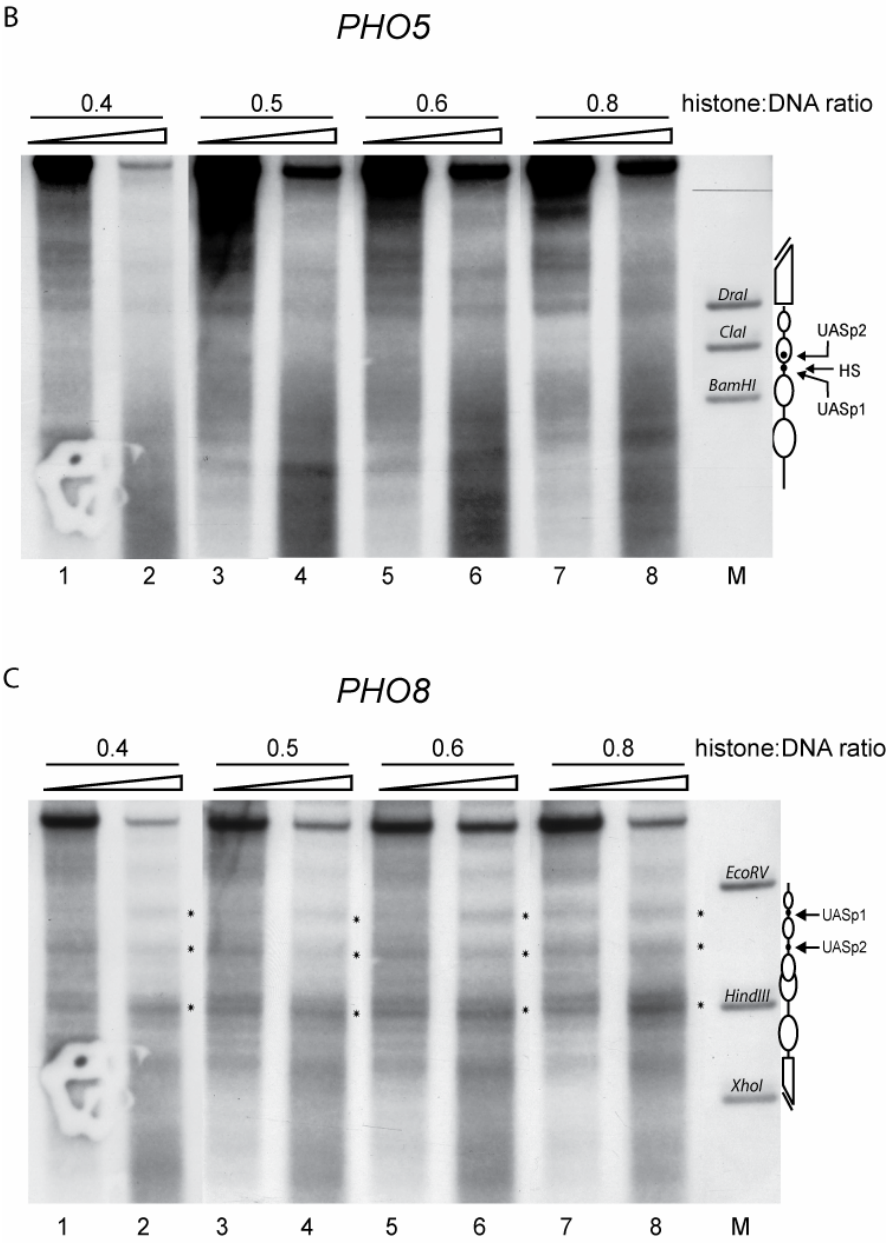


**Figure 17. The *PHO8* promoter has higher nucleosome positioning power than the *PHO5* promoter.** (A) Chromatin was generated by salt gradient dialysis with increasing histone to DNA mass ratios. The ratio of DNA protected from limited MNase digestion versus total DNA, reflecting the nucleosome density after chromatin assembly, was determined by specific probing for both the *PHO5* and the *PHO8* promoters (data not shown). The quotient of this ratio for the *PHO8* to that for the *PHO5* promoter is given for the histone octamer to DNA mass ratios used in the respective salt gradient dialysis assembly reaction. (B and C) The same chromatin preparations as in (A) were mixed with yeast extract as in Fig. 5, incubated for 90 min at 30°C and then subjected to DNaseI mapping and probed for the *PHO5* (B) or the *PHO8* (C) promoter. Schematics, asterisks and marker lanes are the same as in Figure 7. Ramps on top of the lanes indicate increasing DNaseI concentrations.

## 3.2. Nucleosomes at the *PHO8* promoter are more resistant to thermally induced loss of positioning than nucleosomes at the *PHO5* promoter

The studies on relative stability of nucleosomes at the *PHO5* and *PHO8* promoter were extended to chromatin assembled *in vivo* . Yeast nuclei were prepared and used as an *in vitro* substrate for the reverse process of nucleosome assembly, i.e., the loss of positioned nucleosome structure.

Nucleosomes exhibit temperature-dependent mobility on DNA (Flaus and Richmond, 1998; Luger and Richmond, 1998; Meersseman et al., 1992; Pennings et al., 1991). By incubating nuclei at elevated temperatures loss of nucleosome positioning can be accomplished. Changes in chromatin structure were assayed by DNase I mapping at three time points during incubation at 55°C (Figure 18A and B). At the *PHO5* promoter, the nucleosome pattern was largely lost after 20 min (Figure 18A, lanes 5 and 6) whereas the nucleosomes at the *PHO8* promoter were still properly positioned at this time point (Figure 18B, lanes 5 and 6). After 1 hour the pattern was lost at both promoters (Figure 18A and B, lanes 7 and 8). These results show that the nucleosomes at the *PHO8* promoter have a higher kinetic stability towards thermally induced loss of nucleosome positioning.

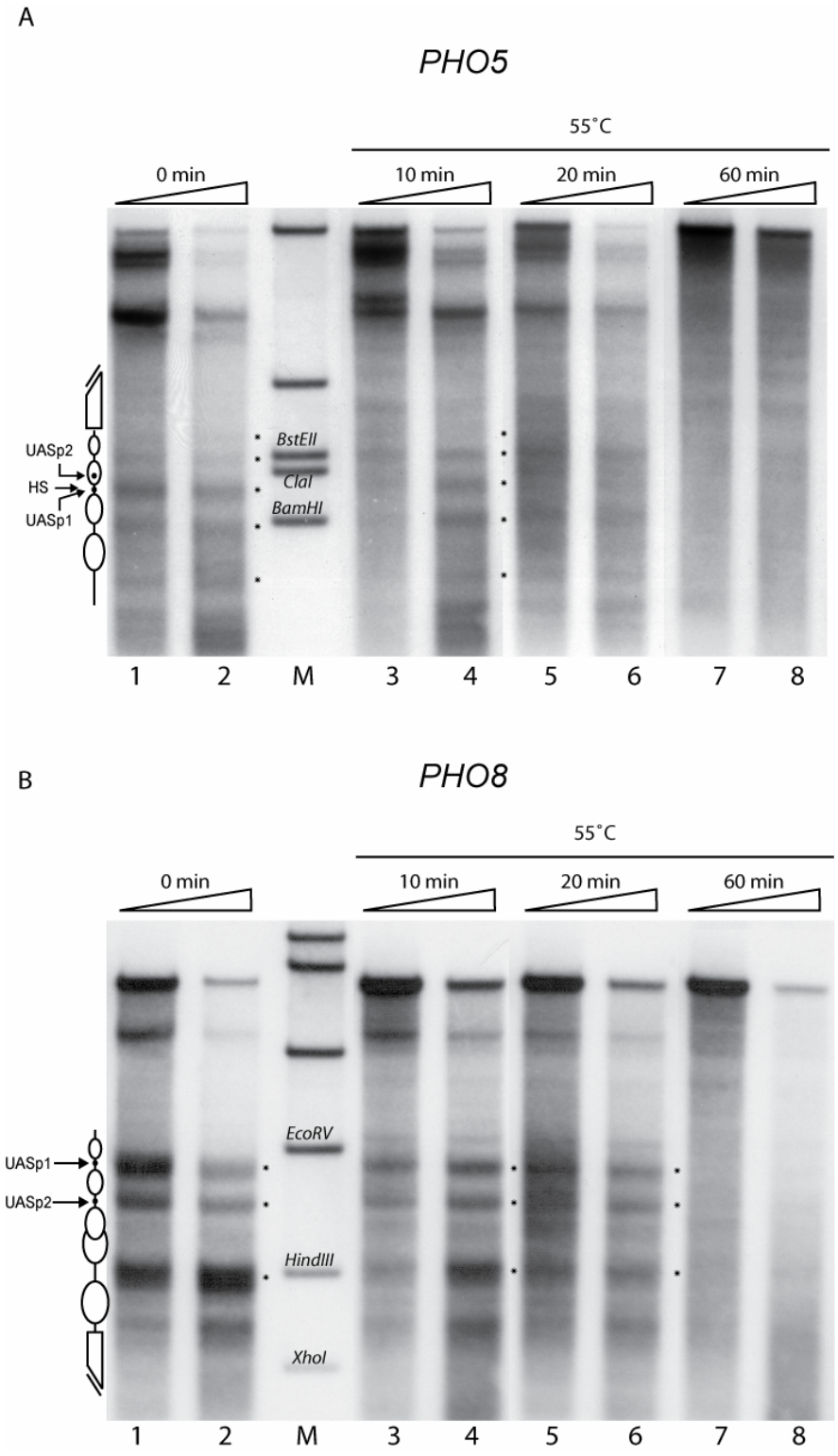


**Figure 18. Using *in vivo* assembled chromatin, nucleosomes at the *PHO8* promoter are more resistant to temperature induced loss of nucleosome positioning than those at the *PHO5* promoter.** (A) and (B) Yeast nuclei were incubated at 55°C for the indicated times and subjected to DNaseI mapping and probed for the *PHO5* (A) or the *PHO8* (B) promoter. Schematics and asterisks are the same as in Figure 7, ramps on top of the lanes indicate increasing DNaseI concentrations. The bands in the marker lanes (M) are generated by restriction digests of genomic DNA and correspond to the restriction sites indicated on the gel. Additional bands outside the region of interest stem from the digestion of the *PHO5* locus with BglII used as secondary digest for *PHO8* (A) and from the digestion of the *PHO8* locus with ApaI used as secondary digest for *PHO5* (B).

# 4. The influence of UASp elements on co-factor requirements for chromatin opening

## 4.1. Co-factor requirements are not determined by position or strength of UASp sites

Chromatin remodeling at the *PHO8* promoter proceeds through a dedicated pathway, stringently requiring the cofactors Snf2 and Gcn5 (Gregory et al., 1999). These cofactors are also involved in chromatin remodeling at the *PHO5* promoter (Barbaric et al., 2001; Reinke and Hörz, 2003). However, the absence of either factor leads only to a kinetic delay in opening and after prolonged induction chromatin remodeling goes to completion. This argues for redundant pathways of chromatin remodeling at the *PHO5* promoter. Here, the UASp2 site is located within a nucleosome. This is a strong binding site for the transcriptional activator Pho4. The UASp1 site, which is a weak binding site for Pho4, is located in a linker region (Svaren and Hörz, 1997; Barbaric et al., 1998). In contrast, at the *PHO8* promoter both UASp sites are present in linker regions and again only UASp2 is a strong binding site for Pho4.

The possibility existed that the presence of an intra-nucleosomal binding site in the *PHO5* promoter makes this promoter more amenable to chromatin remodeling because the binding of the transcriptional activator Pho4 would compete with the binding of a nucleosome. If that was the case, mutation of the intra-nucleosomal binding site should make the promoter more dependent on co-factors such as Snf2 and Gcn5. To test this hypothesis, the activity of a *PHO5* promoter construct without the intra-nucleosomal UASp2 site was measured in wild type, s*nf2* and g*cn5* strain backgrounds. However, this construct was never induced to normal wild type levels even when Pho4 was over-expressed (data not shown) as this construct only contains the weak UASp1 site. To circumvent this problem the weak UASp1 site in the linker region of the *PHO5* promoter was exchanged with the strong UASp2 site of the *PHO5* promoter. In order to have a more direct comparison with the *PHO8* promoter the similarly strong UASp site of the *PHO8* promoter was also introduced. In this way two constructs were created without an intra-nucleosomal binding site and with a strong UASp2 element of the *PHO5* or *PHO8* promoter located in the linker region (Figure 19A). The activity of these constructs was significantly induced in a wild type background however, not as highly as a wild type construct. Similarly, in a *gcn5* background phosphate starvation resulted in low but significant levels induction of the *PHO5* gene. Interestingly induction of both constructs in a *snf2* background was similar to that of a wild type construct (Figure 19B).

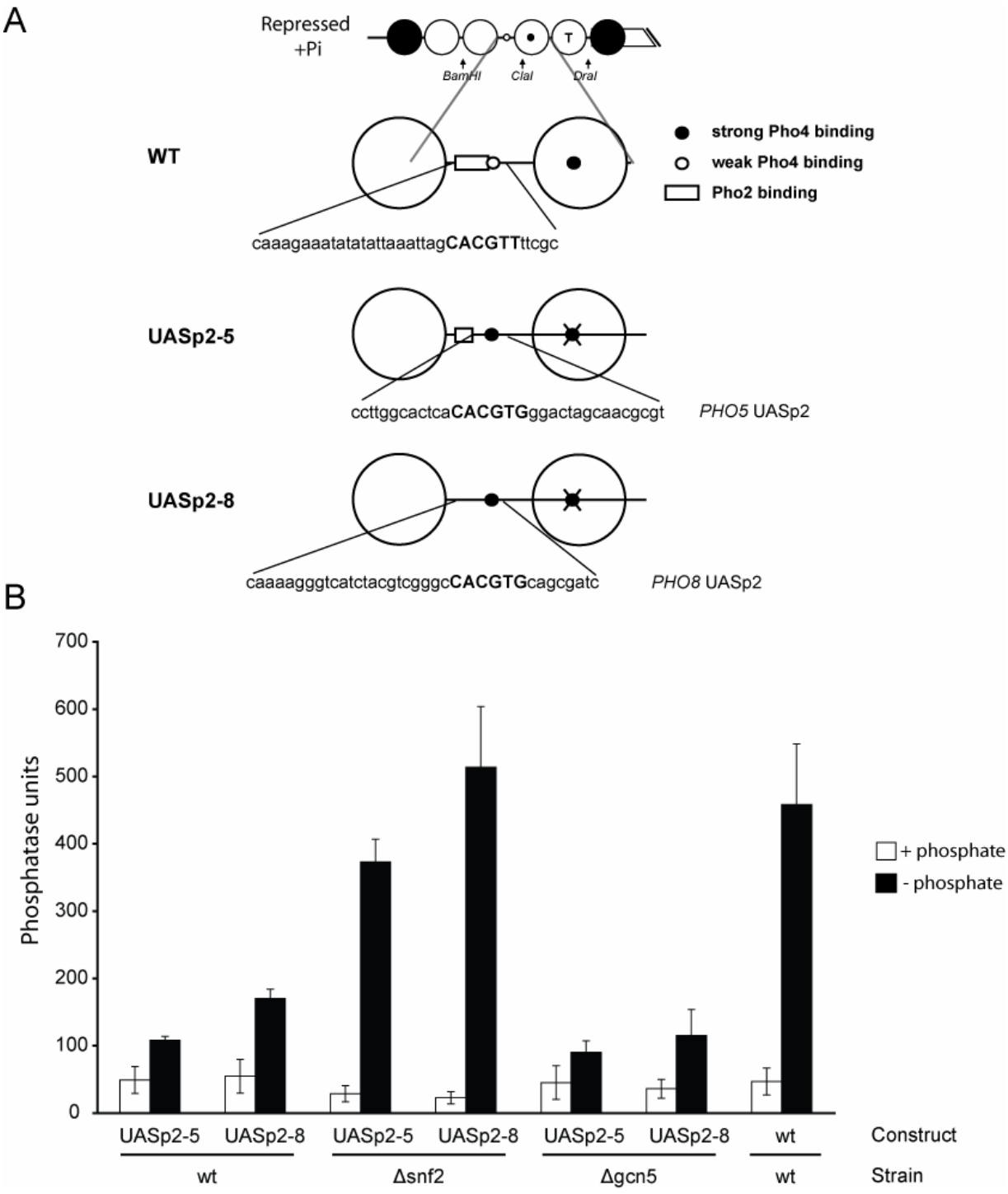


**Figure 19. Position and strength of the UASp sites does not influence cofactor requirements.** (A) schematics of constructs transformed into *pho5*, *pho5*,*snf2* and *pho5*,*gcn5* strain backgrounds. (B) Acid phosphatase activity of the transformed constructs was measured in the aforementioned strains in logarithmic growth in phosphate containing and after over night induction in phosphate-free medium. Error bars indicate standard deviations of four independent experiments.

The surprisingly strong activation of both constructs in a *snf2* strain background could be a consequence of an atypically closed promoter structure making it easier to remodel. It was therefore important to establish whether both constructs were assembled into the characteristic *PHO5* promoter chromatin structure under repressing conditions *in vivo*. Therefore nuclei

were prepared from this strain grown in phosphate containing medium, and the chromatin structure was mapped with DNaseI indirect end labeling and restriction enzyme accessibility. Due to cross-hybridization of the plasmid locus with the partially deleted chromosomal locus it was not possible to use the probe normally used to map the chromatin structure at the *PHO5* promoter. Instead, a probe corresponding to the *BamHI-ClaI* fragment of the promoter was used. This probe has the disadvantage that only nucleosome -1 and -2 is visible on the DNaseI blot, however, using *BamHI* in accessibility assays the position of nucleosome -3 can also be determined. Both constructs assembled into the characteristic chromatin structure under repressive conditions (Figure 20A and B). Thus, the full induction of *PHO5* in a *snf2* strain background is not due to a difference in chromatin structure of the closed state.

Taken together, these results indicate for one that although the full induction is not achieved in a wild type and *gcn5* strain background the presence of an intra-nucleosomal binding site does not render the *PHO5* promoter more dependent on co-factors for chromatin remodeling. Second, the difference in strength of Pho4 binding between the *PHO5* and *PHO8* promoter does not account for the difference in co-factor requirements.

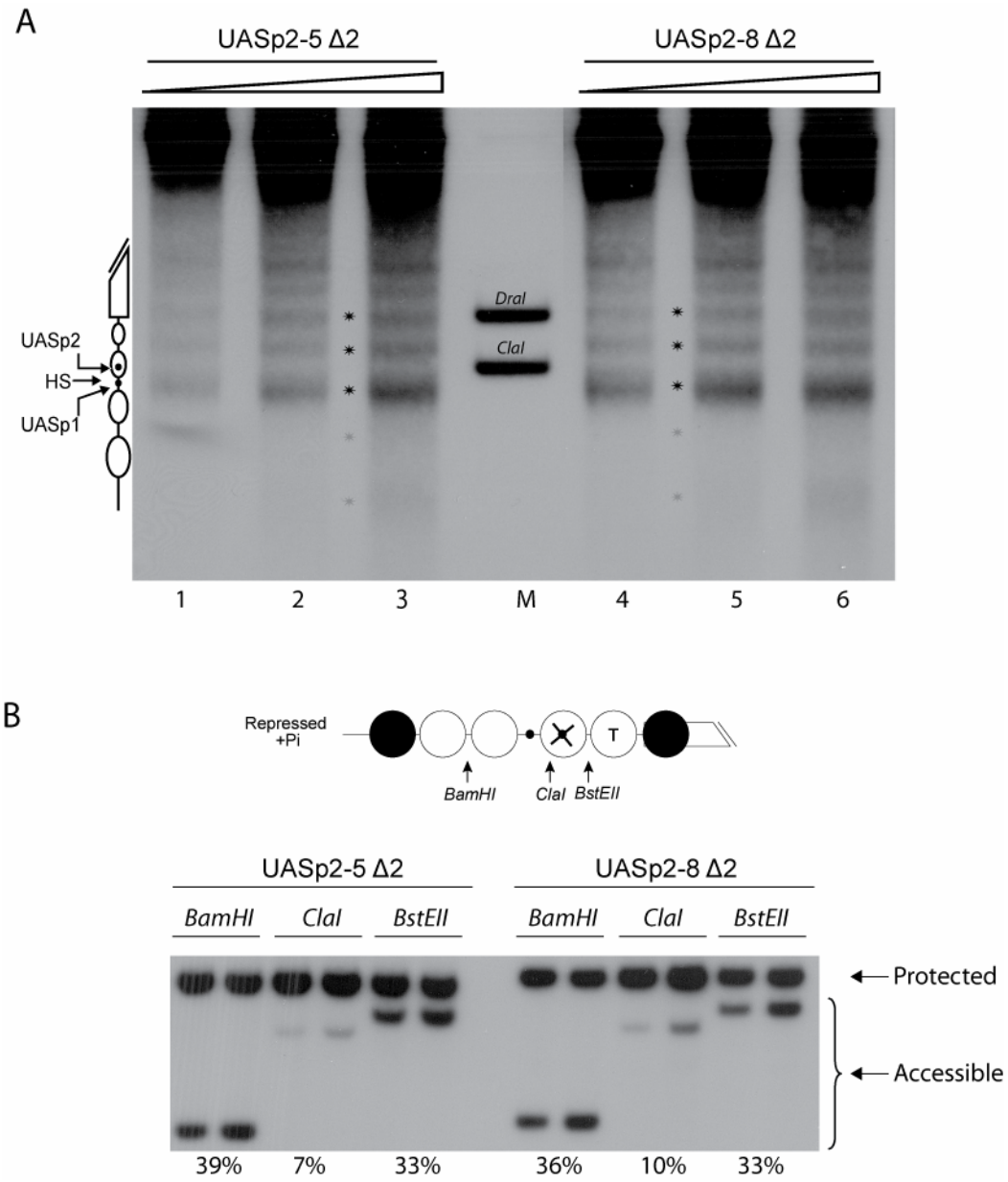


**Figure 20. The *PHO5* promoter constructs from fFigure 19 are assembled into the characteristic chromatin structure of the *PHO5* promoter under repressing condition in a *snf2* strain background.** Nuclei were prepared from a *pho5, snf2* strain transformed with the promoter constructs from Figure 19A. (A) Chromatin was analyzed with DNaseI mapping (A) and restriction enzyme accessibility (B) and probed with a probe recognizing the *BamHI-ClaI* fragment of the *PHO5* promoter. (A) Schematics, asterisks and marker lanes are the same as in Figure 2. Ramps on top of the lanes indicate increasing DNaseI concentrations. (B) Nuclei were digested with two concentrations with a four fold difference of *BamHI*, *ClaI* and *BstEII*, DNA was prepared and digested with *HaeIII*. The accessibility of each restriction enzyme is indicated as percentage of the total signal.

# V. Discussion

## 1. Differences between the PHO5 and PHO8 promoters

Several differences in gene activity and chromatin regulation between the *PHO5* and *PHO8* promoters are recognized, although both are regulated by the same transcriptional activator. For one, the difference in promoter strength resulting in 10-fold higher activity of the *PHO5* gene compared to the *PHO8* gene (Oshima, 1997; Barbaric et al., 1992). Second, chromatin remodeling at the *PHO5* promoter affects 4 nucleosomes (Almer et al., 1986), whereas only two nuclesomes are affected at the *PHO8* promoter (Barbaric et al., 1992). Third, the *PHO8* promoter is dependent on SWI/SNF and Gcn5 for chromatin remodeling whereas the *PHO5* promoter is not (Barbaric et al., 2001; Gaudreau et al., 1997; Gregory et al., 1999).

There have been several speculations about the underlying reasons for these differences. One attractive possibility is that differences inherent in the chromatin structures render one promoter more amenable for chromatin remodeling and chromatin opening than the other. According to this hypothesis, nucleosomes that are more stably associated with the underlying DNA will be more difficult to remodel and subsequently lost in *trans* than nucleosomes that are less stably associated with the underlying DNA. In the case of the *PHO5* and *PHO8* promoters, nucleosomes at the *PHO8* promoter are speculated to be more stable than nucleosomes at the *PHO5* promoter. Another possibility is the position and/or strength of the UASp elements. In a promoter with an intra-nucleosomal UASp site the activator binding competes with the formation of a nucleosome. This may make one promoter easier to remodel than the other. In line with this, one strong UASp site at the *PHO5* promoter is located within a nucleosome whereas the strong UASp site of the *PHO8* promoter is located in a linker region.

## 2. The PHO8 promoter has greater nucleosome positioning power, and properly positioned PHO8 promoter nucleosomes are more stable than their PHO5 counterparts.

The hypothesis that the stability of nucleosomes is linked to their remodeling was inspired by results from studies of the *PHO5* promoter. Insertion of a fragment from the African green monkey α-satellite DNA at the position of nucleosome -2 of the *PHO5* promoter completely abolished inducibility of this promoter. In contrast, insertion of a pBR322 DNA segment at the same position of the *PHO5* promoter had the opposite effect (Straka and Hörz, 1991). The

α-satellite DNA fragment was known to be a strong nucleosome positioning sequence (see introduction section 3 and Linxweiler and Hörz, 1985). It associates with histones in a highly specific manner to give a uniquely positioned nucleosome determined solely on the histone-DNA interactions (Neubauer et al., 1986). Therefore, the quality of the histone-DNA interactions contributes to the regulation of the gene. These results were later backed up by studies of the *PHO8* promoter (Münsterkötter et al., 2000). By replacing the UASp elements of the *PHO8* promoter with those of the *PHO5* promoter it was shown that the nature of the UASp elements does not account for the differences in inducibility between the *PHO5* and *PHO8* promoters. Rather, the difference in extent of remodeling and promoter strength between *PHO5* and *PHO8* relies on the presence of persistent nucleosomes at the *PHO8* promoter that are more resistant to chromatin remodeling (Münsterkötter et al., 2000). Thus, several results from studies of the *PHO* system point toward differences in inherent chromatin structures between the *PHO5* and *PHO8* promoters. However, as these results all rely on classical *in vivo* techniques it is difficult to directly address this hypothesis.

As an alternative approach to the classical *in vivo* chromatin techniques an *in vitro* chromatin assembly system was recently established. This system was initially shown to properly positioning nucleosomes over the *PHO5* promoter (Korber and Hörz, 2004). The advantages of such an approach are numerous as several parameters in the assembly reaction can be specifically controlled. Here, the *in vitro* assembly approach was successfully extended to the *PHO8* promoter. As with the *PHO5* promoter, this yeast extract system was capable of properly positioning the nucleosomes at the *PHO8* promoter. Including both the *PHO5* and the *PHO8* promoter sequences in one and the same assembly reaction allowed to directly compare the assembly and positioning of nucleosomes at the two promoter regions under the same experimental conditions. Therefore, this method for the first time provided a tool to directly study differences in nucleosome stability between the *PHO5* and the *PHO8* promoter.

The results presented in this work show that nucleosomes at the *PHO8* promoter nucleosomes become positioned much more rapidly in a *de novo in vitro* assembly reaction than at the *PHO5* promoter. Such a difference could result from a difference in nucleosome affinities. If the *PHO8* promoter had a higher overall affinity for nucleosomes compared to the *PHO5* promoter, nucleosomal arrays could be present over the *PHO8* promoter already at very early time points of assembly kinetics, whereas it would take longer to generate nucleosomal arrays over the *PHO5* promoter. However, the kinetics of nucleosome assembly as assayed by MNase digestion where similar at the two promoter regions. Therefore both promoter regions

have the same overall assembly states during assembly kinetics. Hence, nucleosome positioning at the *PHO8* promoter is truly generated at lower overall nucleosome densities than at the *PHO5* promoter. This suggests that the properly positioned nucleosomes of the *PHO8* have a higher stability than those of the *PHO5* promoter.

This concept relies on the following. As each significantly long stretch of DNA has a certain propensity to be assembled into a nucleosome, principally every base pair can serve as a starting point for a nucleosome position. Overlapping positions can not be occupied at the same time, resulting in a competition of positions along the DNA. As some nucleosome positions may be energetically more favorable than others, such positions will be occupied more often. If the energetic difference is high, this leads to more or less exclusively occupied major positions at so called strong nucleosome positioning sequences (see also Introduction section 3). According to this, sequences having lower or greater relative affinities for the histone octamer, that is lower or greater relative free energy for nucleosome formation, must create nucleosomes having correspondingly lower or greater relative equilibrium stabilities (Widom, 2001). As a consequence, the proper nucleosome positions at the *PHO8* promoter have a higher stability relative to alternative positions in the same region than the proper positions at the *PHO5* promoter have relative to alternative positions in that region. This relative stability of nucleosome positions is equivalent to the "nucleosome positioning power" of the corresponding DNA regions (Lowary and Widom, 1998). Importantly, this stability of nucleosomes is defined relative to other positions on the same sequencer. This type of stability should not be confused with the stability of positioned nucleosomes at the *PHO8* promoter relative to the stability of positioned nucleosomes at the *PHO5* promoter. This stability comparison between nucleosomes at two different promoter regions is made on an absolute scale and referred to as "absolute stability". If, at a DNA region of sufficient length, one nucleosome position exists that has a high absolute stability for a nucleosome this DNA region would accordingly have high nucleosome positioning power. However, importantly, absolute stability and positioning power need not correlate with each other. A DNA sequence of sufficient length may have a very high overall affinity for nucleosomes, i.e. this sequence confers high absolute stability for nucleosomes. However, several possible nucleosome positions on the same template may have very similar high stabilities. In such a case the nucleosome positioning power would be low. Nonetheless, it is reported for short *in vitro* selected DNA fragments that the overall affinity of a DNA sequence for a nucleosome correlates with the ability to position nucleosomes either rotationally or translationally

(Lowary and Widom, 1998). In the same sense, higher positioning power of the *PHO8* promoter also goes together with higher absolute stability as compared to the *PHO5* promoter.

These nucleosome stability arguments are supported by two results presented in this work. For one, nucleosomes at the *PHO8* promoter became properly positioned even under conditions of limiting histone octamers whereas the proper positioning at the *PHO5* promoter was not generated. Under such conditions, where low nucleosome densities are deliberately generated, there is competition of all possible nucleosome positions with each other for the formation of a nucleosome, and only those positions will be occupied that are significantly more stable than others. Therefore, the proper positions at the *PHO8* promoter are more stable than all alternative positions in this region. Secondly, under the same conditions of limiting histone octamers the overall assembly states[4] of the *PHO5* and *PHO8* promoter regions in the same assembly reaction were compared. As both plasmids were present at equimolar ratios, this directly assays the overall, or average, nucleosome affinity of both regions regardless of the particular positions of the nucleosomes. Sucrose gradient fractionation of a pool of chromatin assembled by salt gradient dialysis under differing histone to DNA ratios showed that both the chromatinized plasmids were distributed equally over the gradient. This indicates that chromatin assembly under limiting histone conditions results in similar assembly states at both promoters. Similarly, quantification of the amount of each plasmid resistant to MNase digestion present in one particular chromatin assembly reaction by salt gradient dialysis under a range of limiting histone conditions also showed that both plasmids were assembled to the same degree. Therefore, the average histone affinities for each promoter region is very similar and provide a common reference point for comparison of nucleosome stabilities: As the proper nucleosome positions at the *PHO8* promoter are more stable than the average positions under the limiting conditions, whereas the proper positions at the *PHO5* promoter are not, it can be concluded that the proper positions at the *PHO8* promoter are more stable than the proper positions at the *PHO5* promoter.

[4] Assembly states refers to the extent of nucleosomal arrays as monitored by MNase digestion..

Importantly, higher stabilities of nucleosomes at the *PHO8* promoter compared to those of the *PHO5* promoter are not just a property of *in vitro* assembled chromatin. In *in vivo* assembled chromatin the nucleosomes at the *PHO8* promoter resist thermally induced nucleosome sliding longer than the nucleosomes at the *PHO5* promoter. As finally both chromatin patterns are lost, this assay measures the kinetic stability of the nucleosomes at the promoter regions. Thus, nucleosomes at the *PHO8* promoter have a higher kinetic stability than nucleosomes at the *PHO5* promoter.

These conclusions are summarized in Figure 21. Positioned nucleosomes at the *PHO8* promoter have high intrinsic stability as they adopt their proper positions in spite of competition for the formation of a nucleosome with neighboring sequences on the same DNA template under conditions of limiting histones. During a *de novo in vitro* assembly when the nucleosome density increases and more and more of the neighboring sequences become occupied by nucleosomes, the proper positions persist through all assembly states. Thus, at the *PHO8* promoter the nucleosomes are positioned under any condition and are therefore concluded to have a high intrinsic stability. Conversely, positioned nucleosomes at the *PHO5* promoter have low intrinsic stability since these positions are only achieved when competition from neighboring sequences is diminished by the increasing incorporation of more stable nucleosomes. Under conditions of limiting histones nucleosomes are first formed on preferred positions. With the exception of nucleosome -1 which may be properly positioned already during salt gradient dialysis, these are not the position of the native *PHO5* promoter. Nucleosome -1 is therefore depicted as having a high intrinsic stability. As the alternative positions at the *PHO5* promoter that are occupied first under limiting histone conditions are overridden by the positions of the native *PHO5* promoter structure at high nucleosome densities they are assumed to be of medium intrinsic stability.

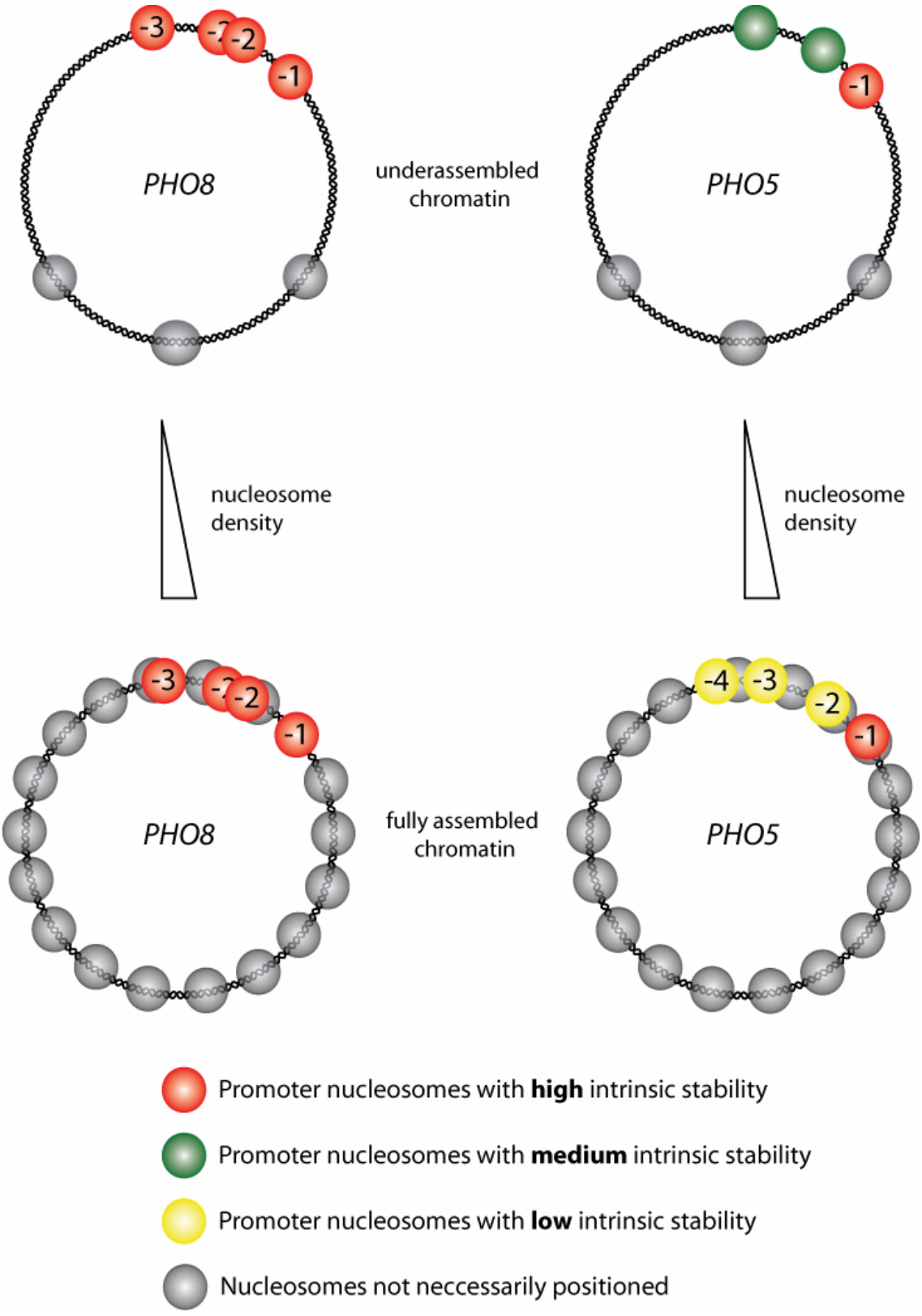


**Figure 21. Nucleosomes at the *PHO8* promoter have higher intrinsic stability than nucleosomes at the *PHO5* promoter.** Nucleosomes at the *PHO8* promoter are positioned as soon as they are deposited onto the DNA in spite of competition from neighboring sequences. In contrast, nucleosomes at the *PHO5* promoter are only positioned at high nucleosome densities.

Notably, affinities can only be directly compared under equilibrium conditions. It was previously shown, that the yeast *in vitro* chromatin assembly system generates an equilibrium of positions of the nucleosomes as prolonged incubations under conditions of sustained nucleosome mobility did not alter the final chromatin structure (Korber and Hörz, 2004). The same independence of the generated chromatin patterns on prolonged incubation times was confirmed for all assembly reactions presented in this work.

## 3. The differences in nucleosome stability correlate with differential cofactor requirements for chromatin opening

Chromatin remodeling at the *PHO8* promoter proceeds through a dedicated pathway. In the absence of Swi/Snf, the chromatin structure at the *PHO8* promoter is retained in its repressed state even under inducing conditions and the absence of Gcn5 permits only partial and localized perturbation of the chromatin immediately adjacent to the upstream transcription factor binding site (Gregory et al., 1999). Similarly, the chromatin transition upon induction of the *PHO5* gene also seems to involve these cofactors as it is delayed in their absence (Barbaric et al., 2001 ; Neef and Kladde, 2003; Dhasarathy and Kladde, 2005; Reinke and Hörz, 2003). However, after prolonged induction, chromatin remodeling still goes to completion arguing for redundant pathways that can support promoter opening at the *PHO5* promoter even without Snf2 and Gcn5. The same is true in the absence of Ino80, Asf1, Swr1, Isw1, Isw2, Chd1, Rad54, Mot1, Esa1, and other cofactors (Kent et al., 2001; Korber et al., 2006; Gaudreau et al., 1997; Boeger et al., 2004, and unpublished data from the lab). Further, while chromatin opening affects four nucleosomes at the *PHO5* promoter, only one nucleosome is fully affected at the *PHO8* promoter. This results in much weaker promoter strength of the *PHO8* promoter compared to the *PHO5* promoter with the *PHO5* being 10 fold more active than the *PHO8* (Almer et al., 1986; Barbaric et al., 1992).

The differences in the extent of chromatin remodeling and promoter strength between the *PHO5* and *PHO8* promoter are unlikely due to differences in the recruitment of cofactors as both promoters are regulated by the same trans-activator Pho4. However, the differences could be a direct consequence of differences in binding strength and position of the UASp elements. First, simply the number of functional UASp elements could influence the extent of chromatin remodeling. The *PHO8* promoter contains only one functional UASp element (Münsterkötter et al., 2000), whereas both UASp elements at the *PHO5* promoter bind Pho4, although with different affinity (Venter et al., 1994; Barbaric et al., 1998). Nevertheless,

introduction of the *PHO5* UASp elements at the *PHO8* promoter did not yield the completely open chromatin structure typical for the *PHO5* promoter (Münsterkötter et al., 2000).

Second, differences in promoter strength could be a direct consequence of the strength of the UASp elements at each promoter. Several experiments addressed this speculation (Figure 22M-Q). Insertion of the strong UASp2 element of the *PHO5* promoter at the position of the UASp1 element of the *PHO8* promoter, thereby generating a promoter driven by two strong UASp elements, did result in somewhat higher gene activity, however only two-fold (Figure 22M). As mentioned previously, exchanging the UASp elements of the *PHO8* promoter with those of the *PHO5* did not result in full gene activity (Figure 22N) nor in more extensive chromatin opening (Münsterkötter et al., 2000).

A third possibility for the observed differences between *PHO5* and *PHO8* could be the influence of the transcriptional co-activator Pho2. The *PHO5* promoter is strictly Pho2-dependent. Pho2 plays a dual role in the activation process, as it is critical both for binding of Pho4 to the weak UASp1 site and is required for the ability of Pho4 to trans-activate from the UASp2 site (Barbaric et al., 1998). Pho2 is not required for Pho4 binding to the *PHO8* promoter and for chromatin remodeling (Barbaric et al., 1992), however, Pho2 significantly increases the expression of *PHO8* (Münsterkötter et al., 2000). Even though Pho2 has a role in chromatin opening at the *PHO5* promoter, this role can be compensated for by the overexpression of Pho4 (Fascher et al., 1990). In this case the *PHO5* promoter is still remodeled whereas the *PHO8* promoter is not. Based on these results it seems unlikely that the action of Pho2 can account for the differences between the two promoters. Thus, neither Pho2 nor the number of binding sites for the transcriptional activator Pho4 nor their quality can adequately explain the apparent “weakness” of the *PHO8* promoter.

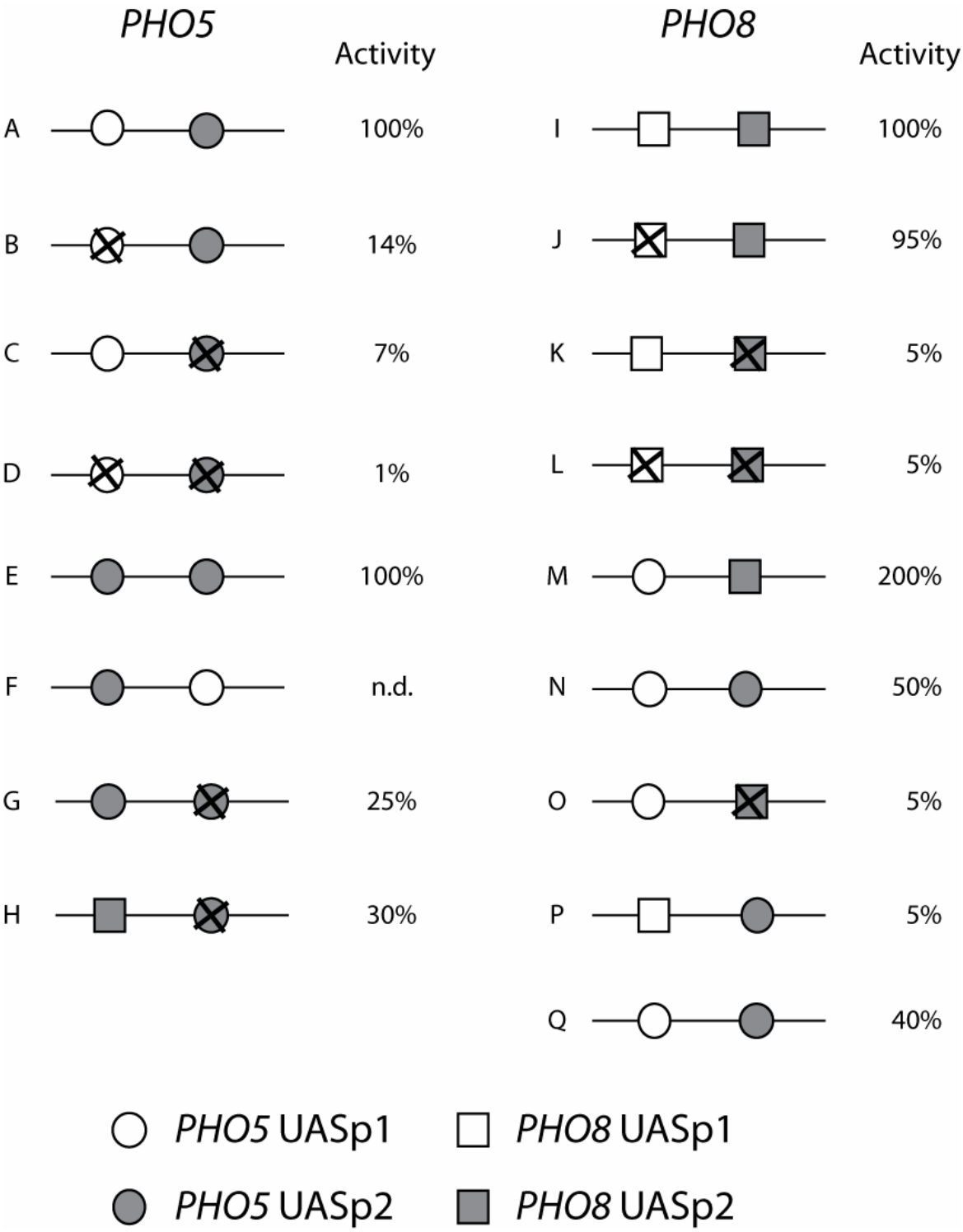


**Figure 22. The effect of various UASp mutations on gene activity of the *PHO5* and the *PHO8* promoter in a wild type background.** Filled circles and squares denote strong UASp sites and open circles and squares denote weak UASp sites (Data summarized from Venter et al., 1994; Svaren and Hörz, 1997; Barbaric et al., 1998; Münsterkötter et al., 2000, and results in this work (G and H)).

Recent data showed that the Pho4 concentration and its occupancy at the *PHO5* promoter play crucial roles in defining the extent to which chromatin opening requires Swi/Snf and Gcn5 (Dhasarathy and Kladde, 2005). Similarly, data from the lab showed that under certain sub-maximal induction conditions deletion of the histone chaperone Asf1 prevents *PHO5*

induction, whereas it only causes a kinetic delay under maximal induction conditions (Korber et al., 2006). Thus, under some sub-maximal induction conditions the co-factor requirements for *PHO5* promoter opening are more stringent. In line with this, it was possible that a partial crippling of the *PHO5* promoter (e.g., by mutation of the UASp elements) would render this promoter variant strictly dependent on co-factors for chromatin opening. Although the nature of the UAS elements proved not to be responsible for the difference in promoter strength and the extent of chromatin remodeling at the *PHO5* and *PHO8* promoters in a wild type background, possibly some promoter variants would behave significantly different in co-factor mutant strain.

Especially the presence of an intra-nucleosomal Pho4 binding site could potentially render the *PHO5* promoter less stable and therefore easier to remodel because the binding of Pho4 would compete with nucleosome -2. This could lead to a redundancy in co-factor requirements. The deletion of the intra-nucleosomal Pho4 binding site of the *PHO5* promoter, leaving only the weak UASp1 site intact, completely abolished promoter inducibility probably because only a weak UASp site was present in the promoter. Therefore, a *PHO5* promoter variant with one strong UASp site present in a linker region and no intra-nucleosomal site was constructed. Induction of such a construct proved to be very weak, although significant, in a wild type background. However, in a *snf2* strain levels of acid phosphatase activity upon induction was similar to the wild type promoter construct. Thus, the absence of an intra-nucleosomal UASp element does not make the *PHO5* promoter more dependent on at least the co-factor Swi/Snf. Taken together, the position and strength of the UASp elements are unlikely to be responsible for the difference in co-factor requirements between the *PHO5* and *PHO8* promoter.

An alternative explanation for the differences in co-factor requirements for chromatin remodeling between the *PHO5* and the *PHO8* promoter is the properties of the substrate for the chromatin remodeling reaction, i.e. the positioned nucleosome structure. Especially for a mechanism leading to histone eviction in *trans*, the absolute stability of nucleosomes would be an important feature as a chromatin remodeling machine needs to completely disassemble the nucleosome. In this context, it cannot be excluded that a remodeling mechanism leading to histone eviction in *trans* may not involve an initial phase of nucleosome sliding (Korber et al., 2004). For such a phase the intra-molecular relative stability of positioned nucleosomes compared to alternative positions along the same DNA molecule would be relevant. Whether or not histone loss involves an initial sliding phase, not only the thermodynamic stabilities of

proper and alternative positions have to be considered. Also the kinetic energy barrier for dislocating nucleosomes from their starting positions, i.e. their kinetic stability will influence the process of histone loss.

The data presented in this work show that the absolute nucleosome stability, the relative intra-molecular nucleosome stability and the kinetic stability of the properly positioned nucleosomes at the *PHO5* promoter are lower compared to those at the *PHO8* promoter. These results suggest that the inherent nucleosome stability determines to what extent the promoter chromatin will open up when it is provided with the right trigger, i.e. Pho4 and factors recruited by Pho4. In the case of the *PHO5* promoter the process of chromatin opening seems to be energetically so favorable that it is supported by a redundant set of co-factors.

## 4. The nature of the nucleosome positioning information at the PHO5 and PHO8 promoters

Even though positioned nucleosomes were described *in vivo* for a long time and these were shown to play important roles in gene regulation (Lu et al., 1995; Schild et al., 1993; Kornberg and Lorch, 1995; Straka and Hörz, 1991; Venter et al., 1994; Yuan et al., 2005) (see also Introduction), the molecular nature of the positioning information remains largely unresolved. The DNA sequence certainly plays an important role but there is no algorithm available to reliably predict nucleosome positions from the DNA sequence alone (Widom, 2001). Most studies on the role of DNA sequence in nucleosome positioning are done *in vitro* using salt gradient dialysis. During this procedure the histone H3/H4 tetramers are the first to bind DNA in the range of 0.75 – 1 M salt imposing a nucleosome-like structure to which histone H2A and H2B then bind at lower salt concentrations (Wilhelm et al., 1978). This means that the positioning of nucleosomes during this procedure is mainly determined by the H3/H4 tetramer (for review of this argument see Widom, 2001). This could explain why some sequences, which are strong nucleosome positioning sequences *in vitro*, are not preferably assembled into a nucleosome *in vivo* (Tanaka et al., 1992b; Negri et al., 2001; Buttinelli et al., 1993). Indeed it has been shown that > 95% of a eukaryotic genome did not sufficiently constrain nucleosome positioning in salt gradient dialysis reconstitution (Lowary and Widom, 1997). In spite of this, over 69% of all nucleosomes in yeast are positioned (Yuan et al., 2005).

The results presented in this work show that in the case of the *PHO5* and *PHO8* promoters salt gradient dialysis does not reflect the same preferences for nucleosome positions as seen *in*

*vivo*. Nevertheless, salt gradient dialysis may properly assemble sub-regions of both promoters. A region of the *PHO8* promoter (between UASp2 and *HindIII*), which has been characterized as being especially repressive for transcription (Münsterkötter et al., 2000), was assembled in salt gradient dialysis with the same nucleosome positioning as in the native pattern. Similarly, although slightly shifted compared to the native yeast pattern, nucleosome -1 of the *PHO5* promoter also seems to be positioned by salt gradient dialysis. Interestingly, nucleosomes in both of these regions are not completely remodeled *in vivo* during chromatin opening (Almer et al., 1986; Barbaric et al., 1992). This may be a direct consequence of high nucleosomes stabilities and it might also point to strong nucleosome positioning sequences being present in these regions. However, aside from these regions, strong nucleosome positioning sequences are not sufficient for setting up the repressive chromatin structure at the *PHO5* and *PHO8* promoters.

The here modified yeast *in vitro* assembly system provides a strong tool to identify the nucleosome positioning information. The positioning information at the *PHO5* and *PHO8* promoter is specific to the yeast extract as a *Drosophila* embryo extract does not support proper positioning at either promoter, although this system can support proper nucleosome positioning on other DNA sequences (Blank and Becker, 1996; Wall et al., 1995; Varga-Weisz et al., 1995). Rather, nucleosome positions are completely randomized in an *in vitro* assembly reaction using *Drosophila* embryo extracts. Even the potential positioning information present in the above-mentioned DNA regions of the *PHO5* and *PHO8* promoters are overridden. Nevertheless, chromatin pre-assembled by *Drosophila* embryo extract can be properly positioned by addition of yeast extract. This suggests that the *Drosophila* embryo extract is devoid of the information needed to properly position both the *PHO5* and the *PHO8* promoter nucleosomes.

Interestingly, this shifting of nucleosomes to the positions of the native chromatin structure is much faster than the *de novo* assembly starting from free DNA. Therefore, positioning of nucleosomes seems to be uncoupled from their loading onto the DNA and the generation of chromatin with physiological spacing is the slow step in *de novo* chromatin assemblies (Korber and Hörz, 2004, and results presented in this work). Additionally, nucleosome positioning is energy dependent. On one hand this energy dependence may indicate that a chromatin remodeling complex is part of the positioning information, on the other hand it could also mean that ATP dependent nucleosome sliding is necessary to overcome the kinetic barrier of nucleosome repositioning.

At this point one can only speculate about the molecular nature of the nucleosome positioning information beyond the DNA sequence information. It may be a specific or unspecific DNA binding protein, a chromatin remodeling complex or a combination of several types of factors. It is remarkable that this information appears to be species-specific as it is not contained in a *Drosophila* embryo extract. As an assembly reaction contains many copies of the *PHO5* and *PHO8* promoters it is unlikely that the amount of yeast extracts used can contains enough *PHO5* and *PHO8* promoter-specific factors to induce the proper positioning in all these copies. If the nucleosome positioning information were a sequence specific DNA binding factor it would have to be rather abundant. One such factor could be the protein Reb1p. Reb1p is an essential, abundant DNA-binding protein (Ju et al., 1990), and recent studies showed that its binding site is the single most conserved motif found in yeast, even more conserved across species than the TATA box (Elemento and Tavazoie, 2005). At the *GAL1-10* promoter the presence of Reb1p binding sites correlated with a nucleosome free region and it was therefore claimed that Reb1p was involved in nucleosome positioning by excluding the binding of a nucleosome (Fedor et al., 1988). However, this speculation was however disputed later (Axelrod et al., 1993; Reagan and Majors, 1998). Furthermore, a Reb1p binding site of the *ILV1* promoter was shown to be dispensable for nucleosome positioning at this promoter (Moreira et al., 2002). In contrast, another study showed that the Rep1p biding site was indispensable for nucleosome positioning at the *PFY1* gene (Angermayr et al., 2003). Thus, the general importance of Reb1p in nucleosome positoining is still unclear. Interestingly, it was recently published that a binding site of Reb1p together with adjacent dA:dT tracts was sufficient to direct the formation of a nucleosome free region flanked by H2A.Z containing nucleosomes (Raisner et al., 2005). This might suggest that Reb1p could be involved in setting up nucleosome positioning on a global level either directly or indirectly through recruitment of H2A.Z.

Similarly, most yeast genes contain a nucleosome-free region around 200 bp upstream of the transcriptional start site that includes conserved dA:dT stretches (Yuan et al., 2005). dA:dT stretches were shown to disfavor the incorporation into a nucleosome (Anderson and Widom, 2001). This is in agreement with the reported difference in nucleosome occupancy between coding and non-coding DNA sequences. Coding regions that are low in dA:dT were found to be more stably associated with nucleosomes than non-coding regions (Sekinger et al., 2005; Lee et al., 2004; Pokholok et al., 2005). Additionally, Struhl and colleagues also showed that the lower histone density can be explained by an intrinsically poor nucleosome stability of such regions as assayed in salt gradient dialysis (Sekinger et al., 2005). Taken together, these

results suggest that nucleosome positioning at many promoter regions may be determined by the presence of a nucleosome-free region due to DNA sequences of intrinsically low affinity to nucleosomes.

However, this seems not to be the case for the *PHO5* promoter. For one, the results presented in this work clearly show that the hypersensitive site containing the UASp1 site is not generated by salt gradient dialysis. On the contrary, in chromatin generated by salt gradient dialysis this region was protected from DNaseI digestion, indicating that a nucleosome actually forms on this particular stretch of DNA and ruling out intrinsically poor nucleosome stability in this region. Second, deleting most of the hypersensitive region, including UASp1, leaves positioning of the adjacent nucleosomes in the repressed *PHO5* promoter intact (Fascher et al., 1993). Thus, in the case of the *PHO5* promoter, it seems unlikely that the hypersensitive site or a factor binding to this DNA region is involved in positioning of the promoter nucleosomes. At the *PHO8* promoter salt gradient dialysis does recapitulate part of the native pattern. The hypersensitive site at the position of UASp2 is generated in salt gradient dialysis. However, the hypersensitive sites at the positions of UASp1 and the *HindIII* site are not generated. Thus, although the hypersensitive site at the position of UASp2 might be characterized by poor intrinsic nucleosome stability, this is not sufficient to position the adjacent nucleosomes at the *PHO8* promoter.

A more likely hypothesis is that an abundant protein like, e.g. HMG proteins or remodeling factors rather than sequence specific DNA binding proteins are involved in setting up the repressive chromatin structure at the *PHO5* and *PHO8* promoters. From this group of proteins the members Nhp6A and Nhp6B can be excluded as being solely responsible for nucleosome positioning at the *PHO5* and the *PHO8* promoters. Double mutant strains showed no defects in setting up the repressive chromatin structure *in vivo* (Moreira and Holmberg, 2000). Similarly, it can be excluded that the Swi/Snf, Isw1, Isw2 and Chd remodeling complexes are involved, as results in this work show that mutation in their respective catalytic subunits does not destroy nucleosome positioning *in vitro*. Another candidate for a chromatin remodeling complex involved in nucleosome positioning is the Rsc complex. The Rsc complex is closely related to the Swi/Snf complex. However, in contrast to the Swi/Snf complex it is required for yeast viability (Angus-Hill et al., 2001). Furthermore, the Rsc complex is very abundant (Cairns et al., 1996) making it a conceivable candidate for being involved in nucleosome positioning on a genome-wide level. Although, this complex was suggested to be involved in setting up the repressive chromatin structure at the *CHA1* gene (Moreira and Holmberg,

1999), the same publication also showed that depletion of Swh3p or Sth1p (two essential components of the Rsc complex) did not have any effect on nucleosome positioning at the repressed *PHO5* promoter (Moreira and Holmberg, 1999). Further experiments will clarify whether the Rsc complex is responsible for nucleosome positioning at the *PHO8* promoter.

Another attractive hypothesis is that yeast-specific RNA might play a role in nucleosome positioning. Recently it was shown that many intergenic regions including the *PHO5* promoter are transcribed in an *rrp6* mutant in yeast (Davis and Ares, Jr., 2006). Rrp6 is a component of the nuclear exosome and contributes to the quality control system that retains and degrades aberrant mRNA in the nucleus and mutations in *RRP6* lead to the accumulation of several species of RNA (Wyers et al., 2005; Kuai et al., 2004; van Hoof et al., 2000). In agreement with this, treatment of high amounts RNaseA abolished nucleosome positioning at both the *PHO5* and *PHO8* promoters in a nucleosome shifting reaction. However, blocking RNA PolII transcription *in vivo* did not result in loss of nucleosome positioning at neither promoter.

## 5. Outlook

The results presented in this work answered one of the long-standing questions regarding the differential co-factor requirements of the co-regulated *PHO5* and *PHO8* promoters. The basis for this was the development of a reliable *in vitro* assay that could directly compare nucleosome stability. It showed a direct correlation between intrinsic stability of promoter nucleosomes and co-factor requirements for chromatin remodeling at the *PHO5* and *PHO8* promoters. This prompted the question whether intrinsic stability of nucleosomes directly dictates the co-factor requirements. Experiments are under way using the methods developed in this work to study the stability of nucleosomes at promoters of genes known to be dependent on co-factors for chromatin remodeling. The result from these experiments will answer whether the correlation between nucleosome stability and co-factor requirements for chromatin opening is a universal feature.

In addition, the methods established here provide an feasible way to generate fully positioned chromatin templates. This opens up wide possibilities for *in vitro* studies of chromatin structure and remodeling in yeast systems. Especially, studies on the mechanisms involved in nucleosome positioning might benefit from these techniques. Ongoing work in the lab is trying to further characterize the molecular nature of nucleosome positioning at the *PHO5* and *PHO8* promoters.

# VI. Materials and Methods

## 1. Standard methods

All standard methods were done according to standard protocols (Sambrook et al., 1989). This includes agarose gel electrophorese, cloning, SDS PAGE and subsequent staining, western blotting etc. In addition, diverse DNA purifications were performed with kits from Qiagen.

## 2. Media for growing S. cerevisiae and E. Coli

### 2.1. YPDA medium

1% (w/v) yeast extract (Difco), 2% (w/v) peptone (Difco), 2 % (w/v) glucose, 100 mg/l adenine.

### 2.2. YNB minimal media

6.7 g/L Yeast Nitrogen Base w/o amino acids, 2% (w/v) glucose, 1.6 mg/L amino acid drop-out mix (2 g adenine, 2 g alanine, 2 g arginine, 2 asparagine, 2 g aspartate, 2 g cysteine, 2 g glutamine, 2 g glutamate, 2 g glycine, 2 g meso-inositol 2 isoleucine, 2 g lysine, 2 g. methionine 0,2 g p-aminobenzoic acid 2 g phenylalanine, 2 g proline, 2g serine, 2 g threonine, 2 g Tryptophane, 2 g tyrosine, 2 g valine, 2 g histidine, 2 g uracile, 2 g leucine)

### 2.3. Phosphate-free minimal media

2 g/l L-asparagine; 500 mg/l $MgSO_4$ x $H_2O$; 100 mg/l NaCl; 100 mg/l $CaCl_2$ x 2 $H_2O$; 2 mg/l Inositol; 500 µg/l $H_3BO_3$; 40 µg/l $CuSo_4$ x $H_2O$; 100 mg/l KJ; 200 µg/l $Fe(III)Cl_3$ x 6 $H_2O$; 400 mg/l $MnSO_4$ x $H_2O$; 200 µg/l $(NH_4)_6Mo_7O_{27}$ x 4 $H_2O$; 200 mg/l $ZnSO_4$ x 7 $H_2O$; 200 µg/l Riboflavin; 200 µg/l p-aminobezoesäure; 2 µg/l biotin; 2 µg/l folic acid; 400 µg/l nicotin acid; 400 µg/l pyridoxin-HCl; 400 µg/l thyaminchlorid; 13,4 mM KCl; 50 mM natriumcitrate pH 5,0, 2% (w/v) glucose; 1,6 g/l aminoacid drop out mix.

## 3. Extract and protein preparations

### 3.1. Whole cell yeast extract

Yeast extracts were prepared as described (Korber and Hörz, 2004). The protocol is based on the protocol from (Schultz et al., 1997; Schultz, 1999) with modifications by S.E. Kong and J. Q. Svejstrup. Briefly, cells were grown to an $OD_{600}$ of 2-4, harvested at 3000 x g for 5 min at

room temperature. The cell pellet was washed subsequently with water and extraction buffer (0.2 M Tris/HCl pH 7.5, 10 mM $MgSO_4$, 20% glycerol, 1 mM EDTA, 390 mM $(NH_4)_2SO_4$, 1 mM DTT and Complete™ protease inhibitor without EDTA (Roche Applied Science)). The pellets were shock frozen and lysed by grinding in liquid nitrogen in a mortar with an additional 1.5 ml of extraction buffer. After slow thawing the lysed cells were cleared by centrifugation (2 hours at 100,000 x g at 4°C). The middle part of the supernatant containing the soluble proteins was withdrawn with a syringe, leaving behind the cloudy layer on top of the pellet and the lipid-rich layer at the meniscus. Proteins were precipitated by adding 337 mg/ml $(NH_4)_2SO_4$ while stirring until complete dissolution. The solution was then centrifuged for 20 min at 41,000 x g at 4°C and the pellet was resuspended in 500-700 µl dialysis buffer (20 mM Hepes/KOH pH7.5, 20% glycerol, 50 mM NaCl, 1 mM EGTA, 5 mM DTT and Complete™ protease inhibitor without EDTA) and dialyzed three times for 30 minutes against the same buffer. Aliquots of the extract were frozen in liquid nitrogen and stored at -80°C.

### 3.2. *Drosophila* embryo extract

*Drosophila* embryo extract was essentially prepared as described (Becker et al., 1994; Bonte and Becker, 1999). 0-2 hours preblastoderm *Drosophila* embryos were harvested several successive collections and washed in wash buffer (0.7 % NaCl, 0.05 % Triton-X-100). Embryos were resuspended in 200 ml wash buffer. For dechorionation 60 ml of 13 % hypochloric acid was added per 200 ml embryo suspension and stirred vigorously for 3 min. Following dechorionation the embryos were washed extensively in 1) tap water, 2) embryo wash, 3) embryo wash without triton and 4) twice in cold EX buffer (10 mM Hepes-KOH, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 % glycerol). After washing the embryos were resuspended in EX buffer supplemented with $MgCl_2$ to 5 mM, 0.2 mM PMSF and 0.1 mM DTT and homogenized by 6 complete strokes at 2000-3000 rpm with a Teflon pestle connected to a motor driven drill press. The extract was then centrifuged for 10 min at 17,000 x g and the supernatant was withdrawn. This fraction was then cleared by ultra-centrifugation for 2 hours at 150,000 x g at 4°C. The supernatant containing the soluble proteins was collected with a syringe and aliquots of the extract were frozen in liquid nitrogen and stored at -80°C.

### 3.3. Yeast nuclei

Yeast nuclei were performed as described (Almer et al., 1986; Gregory and Hörz, 1999). Cells were grown to an $OD_{600}$ of 2-4, harvested at 3000 x g for 5 min at room temperature and washed once with water. After centrifugation the pellet was resuspended in 2 x volumes of pre-incubation buffer (0.7 M β-mercaptoethanol, 2 mM EDTA) and incubated for 30 min at 30°C with shaking. After incubation the cells were centrifuged and the pellet was washed with 1 M sorbitol and resuspended in 5 ml 1 M sorbitol, 5 mM β-mercaptoethanol per gram wet weight. The absorbance ($OD_{600}$) was measured to determine cell density and spheroblast were generated by adding zymolyase 100 T (ICN) was added to a final concentration of 2% and incubated for 30 min at 30°C with shaking. After incubation the efficiency of the enzymatic removal of the cell wall was determined by absorbance ($OD_{600}$) (normally in the range of 60- 95%). Spheroblasts were washed once in 1 M sorbitol and lysis was performed by resuspending the spheroblasts in a hypotonic buffer (18% ficoll, 20 mM $KH_2PO4$, 1 mM $MgCl_2$, 0.25 mM EGTA, 0.25 mM EDTA, pH 6.8 adjusted with KOH). Following lysis the nuclei were pelleted by centrifugation 30 min at 24,000 x g at 4°C and aliquots were frozen and stored at -80°C.

### 3.4. *Drosophila* embryo histone-octamer purification

Purification of *Drosophila* embryo histone octamers was performed as described (Simon and Felsenfeld, 1979). *Drosophila* embryos were collected, washed in tap water, resuspended in lysis buffer (15 mM Hepes-KOH, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 0.05 mM EDTA, 0.25 mM EGTA, 10 % glycerol, 1 mM DTT, 0.2 mM PMSF) and homogenized by 6 complete strokes at 1000-2000 rpm. The homogenized embryos were then centrifuged for 10 min at 10,000 x g at 4°C resulting in three fractions; a solid pellet, soft layer on top of pellet containing the nuclei, and supernatant layer. The supernatant layer was carefully withdrawn and the nuclei layer was resuspended in sucrose containing buffer (15 mM Hepes-KOH, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 0.05 mM EDTA, 0.25 mM EGTA, 1.2 % sucrose, 1 mM DTT, 0.2 mM PMSF) transferred to new tubes and centrifuged again for 10 min at 10,000 x g at 4°C. The supernatant was resuspended in sucrose buffer, $CaCl_2$ was adjusted to 3 mM and protease inhibitor (aprotinin, pepstatin, leupeptin) in addition to the PMSF was added. The nuclei was digested with approximately 200 u/µl MNase (Roche) for 10 min at 26°C, stopped with 10 mM EDTA and centrifuged for 10 min at 10,000 x g at 4°C. The resulting nuclei pellet was resuspended in TE, pH 7.6 with 1 mM DTT and 0.2 mM PMSF and lysed by rotation for 30-45 min at 4°C. After lysis the nuclei were centrifuged for 30 min at 23,000 x g

at 4°C and the supernatant containing the soluble nuclear proteins was withdrawn. Salt concentration was adjusted to 0.63 M KCl and added to 30 ml pre-washed hydroxylapatite (Biorex) and mononucleosomes were allowed to bind the hydroxylapatite by rotation for 60 min at 4°C. The bound mononucleosomes were washed with 0.63 M KCl buffer and loaded into a FPLC column. The histone octamers were eluted with a salt gradient between 0.63 M and 2 M KCl (octamers usually eluted at approximately 1 M KCl). Glycerol concentration was adjusted to 40-50 % and supplemented with Complete$^{TM}$ protease inhibitor without EDTA (Roche) and kept at -20°C. Concentration was estimated by SDS-PAGE in comparison to other histone preparations and Bradford assays.

## *4. In vitro chromatin assembly*

### 4.1. DNA templates

The DNA templates for all chromatin assembly reactions were circular, supercoiled, 10 kb plasmids that are derivatives of plasmid pCB/wt (*LEU2*) (Fascher et al., 1993), where the *TRP1* marker in pCB/wt is replaced by the *LEU2* marker and with either the *PHO5* ORF plus 1311 bp upstream region or the *PHO8* ORF plus 1661 bp upstream region inserted analogously to the *PHO5* insertion in pCB/wt. Mutation of the UASp sites in these plasmids were as described (Münsterkötter et al., 2000; Venter et al., 1994). All cloning and ligation reactions were performed according to Maniatis. Both plasmids were combined at equimolar ratio in all *in vitro* assembly reactions.

### 4.2. *Drosophila* embryo extract assembly

Chromatin assembly with *Drosophila* embryo extracts was performed according to published procedures (Becker et al., 1994; Tsukiyama et al., 1994). A standard chromatin assembly reaction contained 0.9 µg DNA, 40-80 µl *Drosophila* extract in a total of 150 µl assembly buffer (80 mM KCl, 10 mM Tris/HCl pH 7.6, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10% Glycerol, 1 mM DTT) supplemented with a regenerative energy system of 3 mM ATP/$MgCl_2$, 30 mM creatine phosphate (Sigma) and 5 ng/µl creatine kinase (Roche) and incubated for 6 hours at 26°C.

### 4.3. *De novo* yeast extract assembly

Chromatin assembly with yeast extracts was performed as described (Korber and Hörz, 2004). For each preparation of DNA, extract and histones, the ratio of DNA:extract:histones had to

be carefully optimized. A normal assembly reaction contained approximately 1.8 µg DNA, 300 µg of extract protein and 6 µg of *Drosophila* histone octamers in 150 µl assembly buffer (20 mM Hepes pH 7.5, 80 mM KCl, 25 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 12% Glycerol, 2.5 mM DTT), supplemented with a regenerative energy system of 3 mM ATP/$MgCl_2$, 30 mM creatine phosphate (Sigma) and 5 ng/µl creatine kinase (Roche) and incubated for up to 6 hours at 30°C. The assembly reaction was performed in siliconized tubes which were blocked with a solution of 2 mg/ml bovine serum albumin, 0.1% Nonidet-40. Prior to any manipulation or analysis, the assembly reaction was always centrifuged for 2 min at room temperature and maximum speed in a table-top centrifuge.

### 4.4. Salt gradient dialysis assembly

Salt gradient dialysis was performed as described (Längst et al., 1999). A typical assembly reaction contained 4-20 µg DNA, 4 µg BSA, 3.6-4.4 µg *Drosophila* embryo histone octamers (see Materials and Methods section 3.4) in 50 µl high salt buffer (10 mM Tris/HCl pH 7.6, 2 M NaCl, 1 mM EDTA, 1 mM ß-Mercaptoethanol, 0.05% Nonidet P40). This was dialyzed for 12-16 hours against low salt buffer (10 mM Tris/HCl pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM ß-mercaptoethanol, 0.05% Nonidet P40). Chromatin was stored at 4°C for up to 6 months.

### 4.5. Adding yeast extract to pre-assembled chromatin (nucleosome shifting reaction)

Yeast extract (100-900 µg protein for *Drosophila* embryo extract chromatin or 3-500 µg protein for salt gradient dialysis chromatin) was added to 2 µg DNA preassembled chromatin along with a fresh complement of the regenerative energy system (3 mM ATP/$MgCl_2$, 30 mM creatine phosphate (CP) (Sigma) and 5 ng/µl creatine kinase (CK) (Roche) and further incubated at 30°C for up to 6 hours.

## *5. Chromatin analysis*

### 5.1. DNaseI digestion of in vitro assembled chromatin

DNase indirect endlabeling was performed as described in (Almer and Hörz, 1986). *In vitro* assembled chromatin was digested with a range of bovine deoxyribonucleaseI (DNaseI, Roche) concentrations for 5 min at room temperature and the reaction was stopped by adding SDS to a final concentration of 0.5 % and EDTA to a final concentration of 2 mM. 80 mg of Proteinase K (Roche) was added to the digested chromatin incubated for 12-15 hours at 37°C.

After incubation DNA was precipitated and resuspended in 20 µl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) followed by secondary digest with *ApaI* (Roche) for the *PHO5* promoter or *BglII* (Roche) for the *PHO8* promoter. The resulting DNA fragments were resolved on a 1.5 % agarose gel and blottet on a membrane (Biodyne RBO 45µ, Pall Corporation). The membrane was baked for 1-2 hours at 80°C and then washed for two hours at 68°C in 3 x SSC (900 mM NaCl, 90 mM Na-Citrate), 1 x Denhardt (0.5 % SDS, 1 mM EDTA, 0.02 % BSA, 0.02 % PVP-40, 0.02 % Ficoll). Prior to hybridization the membrane was pre-hybridized in 2 x SSC, 1 x Denhardt with carrier DNA for 1 hour at 68°C. Hybridization was carried out over night and the following day the blots were washed 3 x 30 min in 2 x SSC, 1 x Denhardt. In all DNaseI mapping experiments chromatin samples were digested with a range of DNaseI concentrations. However, due to space limitations only one or few representative lanes are shown in the figures.

### 5.2. Micrococcal nuclease digestion of in vitro generated chromatin

*In vitro* assembled chromatin was digested with a range of Micrococcal Nuclease concentrations (MNase, S7Nuclease, Roche) and DNA was precipitated using the same protocol as for DNaseI digestions but without the secondary digest with a restriction enzyme.

### 5.3. DNaseI digestion of nuclei

Nuclei were washed in DNaseI buffer (15 mM Tris, pH 7.5, 75 mM NaCl, 3 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM β-mercaptoethanol) and resuspended in the same buffer. The nuclei were digested with a range of DNaseI for 20 min at 37°C and the reaction was stopped with 0.5 % SDS, 4 mM EDTA, 50 mM Tris-HCl, pH 8.8 a final concentration. 300-600 µg Proteinase K (Roche) was added and incubated for 30 min at 37°C. DNA was extracted by phenol/chloroform extraction and ETOH precipitated. RNA was digested with RNaseA (8 % final concentration) (Roche) for 1 hour at 37°C followed by precipitation with isopropanol. Secondary digest, blotting and hybridizing was performed as described for DNaseI digestion of *in vitro* assembled chromatin.

### 5.4. Digestion of nuclei with restriction nucleases

Nuclei were washed in 1 x SSTEEM (0.15 mM Spermine, 0.5 mM Spermidine, 10 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, 0.2 mM EGTA, 10 mM $MgCl_2$, 5 mM NaCl, 5 mM β-mercaptoethanol, pH 7.4 adjusted with HCl) and resuspended in the same buffer. Nuclei were

digested with two concentrations of restriction nuclease for 30 min at 37°C and stopped with 0.5 % SDS, 4 mM EDTA, 50 mM Tris-HCl, pH 8.8. The nuclei were then digested with Proteinase K (Roche), phenol/chloroform extracted and RNase treated as descirbed for the DNaseI digest of nuclei. The rest of the procedure follows that described for DNaseI digestion of *in vitro* assembled chromatin except that secondary cleavage was performed with *HaeIII* for the *PHO5* promoter and *BglII*/*EcoRV* for the *PHO8* promoter.

### 5.5. Probes

The ApaI-BamHI fragment upstream of the *PHO5* gene was used as a probe for DNaseI mapping of the *PHO5* promoter and the *XhoI-PvuII* fragment at the beginning of the *PHO8* open reading frame (ORF) for mapping of the *PHO8* promoter. The probes hybridizing within the *PHO5* or *PHO8* promoter regions correspond to the *BstEII-DraI* fragment of the *PHO5* promoter or to a PCR fragment of the *PHO8* promoter using the following primers TGGAACTACTTGCGAATATG and ACGCCTTCTTCTAGTAGGAA, respectively.

## *6. Acid phosphatase activity*

Acid phosphatase activity was performed as described (Haguenauer-Tsapis and Hinnen, 1984). Briefly, cells were grown to an OD of around 2 to 4. For cells grown at high phosphate 4 OD cells were taken and for cells grown without phosphate 0.8 OD was taken. The cells were washed in water and resuspended in 0.1 M NaAc, pH 3.6. The exact amount of cells was determined by $OD_{600}$ and 1 ml of each sample was incubated with 1 ml of NPP solution (75 mg 4-Nitrophenylphosphate dinatrium-hexahydrate in 10 ml 0.1 M NaAc, pH 3.6) for exactly 10 min at 30°C. The reaction was stopped with 500 µl 1 M NaOH, and the phosphatase activity was measured at $OD_{410}$.

# VII. References

Abbott,D.W., Ivanova,V.S., Wang,X., Bonner,W.M., and Ausio,J. (2001). Characterization of the stability and folding of H2A.Z chromatin particles: implications for transcriptional activation. J. Biol. Chem. *276*, 41945-41949.

Adkins,M.W., Howar,S.R., and Tyler,J.K. (2004a). Chromatin Disassembly Mediated by the Histone Chaperone Asf1 Is Essential for Transcriptional Activation of the Yeast PHO5 and PHO8 Genes. Mol. Cell *14*, 657-666.

Adkins,N.L., Watts,M., and Georgel,P.T. (2004b). To the 30-nm chromatin fiber and beyond. Biochim. Biophys. Acta *1677*, 12-23.

Ahmad,K. and Henikoff,S. (2002). The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol. Cell *9*, 1191-1200.

Almer,A. and Hörz,W. (1986). Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the *PHO5/PHO3* locus in yeast. EMBO J. *5*, 2681-2687.

Almer,A., Rudolph,H., Hinnen,A., and Hörz,W. (1986). Removal of positioned nucleosomes from the yeast *PHO5* promoter upon *PHO5* induction releases additional upstream activating DNA elements. EMBO J. *5*, 2689-2696.

Almouzni,G., Mechali,M., and Wolffe,A.P. (1990). Competition between transcription complex assembly and chromatin assembly on replicating DNA. EMBO J. *9*, 573-582.

Anderson,J.D. and Widom,J. (2000). Sequence and position-dependence of the equilibrium accessibility of nucleosomal DNA target sites. J. Mol. Biol. *296*, 979-987.

Anderson,J.D. and Widom,J. (2001). Poly(dA-dT) promoter elements increase the equilibrium accessibility of nucleosomal DNA target sites. Mol. Cell. Biol. *21*, 3830-3839.

Angelov,D., Molla,A., Perche,P.Y., Hans,F., Cote,J., Khochbin,S., Bouvet,P., and Dimitrov,S. (2003). The histone variant macroH2A interferes with transcription factor binding and SWI/SNF nucleosome remodeling. Mol. Cell *11*, 1033-1041.

Angermayr,M., Oechsner,U., and Bandlow,W. (2003). Reb1p-dependent DNA bending effects nucleosome positioning and constitutive transcription at the yeast profilin promoter. J. Biol. Chem. *278*, 17918-17926.

Angus-Hill,M.L., Schlichter,A., Roberts,D., Erdjument-Bromage,H., Tempst,P., and Cairns,B.R. (2001). A Rsc3/Rsc30 zinc cluster dimer reveals novel roles for the chromatin remodeler RSC in gene expression and cell cycle control. Mol. Cell *7*, 741-751.

Archer,T.K., Cordingley,M.G., Wolford,R.G., and Hager,G.L. (1991). Transcription factor access is mediated by accurately positioned nucleosomes on the mouse mammary tumor virus promoter. Mol. Cell. Biol. *11*, 688-698.

Arents,G., Burlingame,R.W., Wang,B.C., Love,W.E., and Moudrianakis,E.N. (1991). The nucleosomal core histone octamer at 3.1 A resolution a tripartite protein assembly and a left-handed superhelix. Proc. Natl. Acad. Sci. U. S. A. *88*, 10148-10152.

Axelrod,J.D., Reagan,M.S., and Majors,J. (1993). GAL4 disrupts a repressing nucleosome during activation of GAL1 transcription invivo. Genes Dev. *7*, 857-869.

Baker,R.E. and Masison,D.C. (1990). Isolation of the gene encoding the Saccharomyces cerevisiae centromere-binding protein CP1. Mol. Cell. Biol. *10*, 2458-2467.

Baldi,P., Brunak,S., Chauvin,Y., and Krogh,A. (1996). Naturally occurring nucleosome positioning signals in human exons and introns. J. Mol. Biol. *263*, 503-510.

Bannister,A.J., Schneider,R., and Kouzarides,T. (2002). Histone methylation: dynamic or static? Cell *109*, 801-806.

Bao,Y., Konesky,K., Park,Y.J., Rosu,S., Dyer,P.N., Rangasamy,D., Tremethick,D.J., Laybourn,P.J., and Luger,K. (2004). Nucleosomes containing the histone variant H2A.Bbd organize only 118 base pairs of DNA. EMBO J. *23*, 3314-3324.

Barbaric,S., Fascher,K.D., and Hörz,W. (1992). Activation of the weakly regulated *PHO8* promoter in *S. cerevisiae*: Chromatin transition and binding sites for the positive regulator protein Pho4. Nucl. Acids Res. *20*, 1031-1038.

Barbaric,S., Münsterkötter,M., Goding,C., and Hörz,W. (1998). Cooperative Pho2-Pho4 interactions at the *PHO5* promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. Mol. Cell. Biol. *18*, 2629-2639.

Barbaric,S., Münsterkötter,M., Svaren,J., and Hörz,W. (1996). The homeodomain protein Pho2 and the basic-helix-loop-helix protein Pho4 bind DNA cooperatively at the yeast *PHO5* promoter. Nucl. Acids Res. *24*, 4479-4486.

Barbaric,S., Walker,J., Schmid,A., Svejstrup,J.Q., and Hörz,W. (2001). Increasing the rate of chromatin remodeling and gene activation - a novel role for the histone acetyltransferase Gcn5. EMBO J. *20*, 4944-4951.

Becker,P.B., Tsukiyama,T., and Wu,C. (1994). Chromatin assembly extracts from Drosophila embryos. Methods Cell Biol. *44*, 207-223.

Becker,P.B. and Wu,C. (1992). Cell-free system for assembly of transcriptionally repressed chromatin from Drosophila embryos. Mol. Cell. Biol. *12*, 2241-2249.

Belikov,S., Gelius,B., Almouzni,G., and Wrange,O. (2000). Hormone activation induces nucleosome positioning in vivo. EMBO J. *19*, 1023-1033.

Belikov,S., Gelius,B., and Wrange,O. (2001). Hormone-induced nucleosome positioning in the MMTV promoter is reversible. EMBO J. *20*, 2802-2811.

Bellard,M., Kuo,M.T., Dretzen,G., and Chambon,P. (1980). Differential nuclease sensitivity of the ovalbumin and beta-globin chromatin regions in erythrocytes and oviduct cells of laying hen. Nucleic Acids Res. *8*, 2737-2750.

Bernstein,B.E., Liu,C.L., Humphrey,E.L., Perlstein,E.O., and Schreiber,S.L. (2004). Global nucleosome occupancy in yeast. Genome Biol. *5*, R62.

Bina,M. (1994). Periodicity of dinucleotides in nucleosomes derived from simian virus 40 chromatin. J. Mol. Biol. *235*, 198-208.

Blank,T.A. and Becker,P.B. (1996). The effect of nucleosome phasing sequences and DNA topology on nucleosome spacing. J. Mol. Biol. *260*, 1-8.

Boeger,H., Griesenbeck,J., Strattan,J.S., and Kornberg,R.D. (2003). Nucleosomes unfold completely at a transcriptionally active promoter. Mol. Cell *11*, 1587-1598.

Boeger,H., Griesenbeck,J., Strattan,J.S., and Kornberg,R.D. (2004). Removal of promoter nucleosomes by disassembly rather than sliding in vivo. Mol. Cell *14*, 667-673.

Bolshoy,A. (1995). CC dinucleotides contribute to the bending of DNA in chromatin. NAT. STRUCT. BIOL. *2*, 446-448.

Bonte,E. and Becker,P.B. (1999). Preparation of chromatin assembly extracts from preblastoderm Drosophila embryos. Methods Mol. Biol. *119*, 187-194.

Buttinelli,M., Dimauro,E., and Negri,R. (1993). Multiple nucleosome positioning with unique rotational setting for the saccharomyces-cerevisiae 5s rRNA gene invitro and invivo. Proc. Natl. Acad. Sci. U. S. A. *90*, 9315-9319.

Cai,M. and Davis,R.W. (1990). Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. Cell *61*, 437-446.

Cairns,B.R., Lorch,Y., Li,Y., Zhang,M.C., Lacomis,L., Erdjument-Bromage,H., Tempst,P., Du,J., Laurent,B., and Kornberg,R.D. (1996). RSC, an essential, abundant chromatin-remodeling complex. Cell *87*, 1249-1260.

Cao,H., Widlund,H.R., Simonsson,T., and Kubista,M. (1998). TGGA repeats impair nucleosome formation. J. Mol. Biol. *281*, 253-260.

Chakravarthy,S., Gundimella,S.K., Caron,C., Perche,P.Y., Pehrson,J.R., Khochbin,S., and Luger,K. (2005). Structural characterization of the histone variant macroH2A. Mol. Cell. Biol. *25*, 7616-7624.

Clarke,M.F., FitzGerald,P.C., Brubaker,J.M., and Simpson,R.T. (1985). Sequence-specific interaction of histones with the simian virus 40 enhancer region in vitro. J. Biol. Chem. *260*, 12394-12397.

Davey,C.A., Sargent,D.F., Luger,K., Maeder,A.W., and Richmond,T.J. (2002). Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J. Mol. Biol. *319*, 1097-1113.

Davis,C.A. and Ares,M., Jr. (2006). Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A *103*, 3262-3267.

de la Cruz,X., Lois,S., Sanchez-Molina,S., and Martinez-Balbas,M.A. (2005). Do protein motifs read the histone code? Bioessays *27*, 164-175.

Demerec,M., Adelberg,E.A., Clark,A.J., and Hartman,P.E. (1966). A proposal for a uniform nomenclature in bacterial genetics. Genetics *54*, 61-76.

Dhasarathy,A. and Kladde,M.P. (2005). Promoter occupancy is a major determinant of chromatin remodeling enzyme requirements. Mol. Cell. Biol. *25*, 2698-2707.

Dillon,N. (2004). Heterochromatin structure and function. Biol. Cell *96*, 631-637.

Dong,F. and van Holde,K.E. (1991). Nucleosome positioning is determined by the (H3-H4)2 tetramer. Proc. Natl. Acad. Sci. U. S. A *88*, 10596-10600.

Dorigo,B., Schalch,T., Bystricky,K., and Richmond,T.J. (2003). Chromatin fiber folding: requirement for the histone H4 N-terminal tail. J. Mol. Biol. *327*, 85-96.

Dorigo,B., Schalch,T., Kulangara,A., Duda,S., Schroeder,R.R., and Richmond,T.J. (2004). Nucleosome arrays reveal the two-start organization of the chromatin fiber. Science *306*, 1571-1573.

Drew,H.R. and Travers,A.A. (1985). DNA bending and its relation to nucleosome positioning. J. Mol. Biol. *186*, 773-790.

Durrin,L.K., Mann,R.K., and Grunstein,M. (1992). Nucleosome loss activates *CUP1* and *HIS3* promoters to fully induced levels in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. *12*, 1621-1629.

Eberharter,A. and Becker,P.B. (2004). ATP-dependent nucleosome remodelling: factors and functions. J. Cell Sci. *117*, 3707-3711.

Eisfeld,K., Candau,R., Truss,M., and Beato,M. (1997). Binding of NF1 to the MMTV promoter in nucleosomes: influence of rotational phasing, translational positioning and histone H1. Nucleic Acids Res. *25*, 3733-3742.

Elemento,O. and Tavazoie,S. (2005). Fast and systematic genome-wide discovery of conserved regulatory elements using a non-alignment based approach. Genome Biol. *6*, R18.

Elgin,S.C. (1981). DNAase I-hypersensitive sites of chromatin. Cell *27*, 413-415.

Englander,E.W. and Howard,B.H. (1996). A naturally occurring T14A11 tract blocks nucleosome formation over the human neurofibromatosis type 1 (NF1)-Alu element. J. Biol. Chem. *271*, 5819-5823.

Espinas,M.L., Jimenez-Garcia,E., Martinez-Balbas,A., and Azorin,F. (1996). Formation of triple-stranded DNA at d(GA.TC)n sequences prevents nucleosome assembly and is hindered by nucleosomes. J. Biol. Chem. *271*, 31807-31812.

Fan,J.Y., Rangasamy,D., Luger,K., and Tremethick,D.J. (2004). H2A.Z alters the nucleosome surface to promote HP1alpha-mediated chromatin fiber folding. Mol. Cell *16*, 655-661.

Fascher,K.D., Schmitz,J., and Hörz,W. (1990). Role of trans-activating proteins in the generation of active chromatin at the *PHO5* promoter in *S. cerevisiae*. EMBO J. *9*, 2523-2528.

Fascher,K.D., Schmitz,J., and Hörz,W. (1993). Structural and functional requirements for the chromatin transition at the *PHO5* promoter in *Saccharomyces cerevisiae* upon *PHO5* activation. J. Mol. Biol. *231*, 658-667.

Fazzio,T.G. and Tsukiyama,T. (2003). Chromatin remodeling in vivo: evidence for a nucleosome sliding mechanism. Mol. Cell *12*, 1333-1340.

Fedor,M.J., Lue,N.F., and Kornberg,R.D. (1988). Statistical positioning of nucleosomes by specific protein-binding to an upstream activating sequence in yeast. J. Mol. Biol. *204*, 109-127.

Finch,J.T., Lutter,L.C., Rhodes,D., Brown,R.S., Rushton,B., Levitt,M., and Klug,A. (1977). Structure of nucleosome core particles of chromatin. Nature *269*, 29-36.

Finch,J.T., Noll,M., and Kornberg,R.D. (1975). Electron microscopy of defined lengths of chromatin. Proc. Natl. Acad. Sci. U. S. A *72*, 3320-3322.

Fitzgerald,D.J. and Anderson,J.N. (1998). Unique translational positioning of nucleosomes on synthetic DNAs. Nucleic Acids Res. *26*, 2526-2535.

Fitzgerald,D.J., Dryden,G.L., Bronson,E.C., Williams,J.S., and Anderson,J.N. (1994). Conserved patterns of bending in satellite and nucleosome positioning DNA. J. Biol. Chem. *269*, 21303-21314.

Flaus,A., Rencurel,C., Ferreira,H., Wiechens,N., and Owen-Hughes,T. (2004). Sin mutations alter inherent nucleosome mobility. EMBO J. *23*, 343-353.

Flaus,A. and Richmond,T.J. (1998). Positioning and stability of nucleosomes on MMTV 3'LTR sequences. Journal of Molecular Biology *275*, 427-441.

Gao,J. and Benyajati,C. (1998). Specific local histone-DNA sequence contacts facilitate high-affinity, non-cooperative nucleosome binding of both adf-1 and GAGA factor. Nucleic Acids Res. *26*, 5394-5401.

Gaudreau,L., Schmid,A., Blaschke,D., Ptashne,M., and Hörz,W. (1997). RNA polymerase II holoenzyme recruitment is sufficient to remodel chromatin at the yeast *PHO5* promoter. Cell *89*, 55-62.

Gautier,T., Abbott,D.W., Molla,A., Verdel,A., Ausio,J., and Dimitrov,S. (2004). Histone variant H2ABbd confers lower stability to the nucleosome. EMBO Rep. *5*, 715-720.

Georgel,P.T., Fletcher,T.M., Hager,G.L., and Hansen,J.C. (2003). Formation of higher-order secondary and tertiary chromatin structures by genomic mouse mammary tumor virus promoters. Genes Dev. *17*, 1617-1629.

Godde,J.S. and Wolffe,A.P. (1996). Nucleosome assembly on CTG triplet repeats. J. Biol. Chem. *271*, 15222-15229.

Gordon,F., Luger,K., and Hansen,J.C. (2005). The core histone N-terminal tail domains function independently and additively during salt-dependent oligomerization of nucleosomal arrays. J. Biol. Chem. *280*, 33701-33706.

Gregory,P.D. and Hörz,W. (1999). Mapping chromatin structure in yeast. Methods Enzymol. *304*, 365-376.

Gregory,P.D., Schmid,A., Zavari,M., Lui,L., Berger,S.L., and Hörz,W. (1998). Absence of Gcn5 HAT activity defines a novel state in the opening of chromatin at the *PHO5* promoter in yeast. Mol. Cell *1*, 495-505.

Gregory,P.D., Schmid,A., Zavari,M., Münsterkötter,M., and Hörz,W. (1999). Chromatin remodelling at the *PHO8* promoter requires SWI-SNF and SAGA at a step subsequent to activator binding. EMBO J. *18*, 6407-6414.

Griffith,J.D. (1976). Visualization of prokaryotic DNA in a regularly condensed chromatin-like fiber. Proc. Natl. Acad. Sci. U. S. A *73*, 563-567.

Gruss,C., Gutierrez,C., Burhans,W.C., DePamphilis,M.L., Koller,T., and Sogo,J.M. (1990). Nucleosome assembly in mammalian cell extracts before and after DNA replication. EMBO J. *9*, 2911-2922.

Hagerman,P.J. (1988). Flexibility of DNA. Annu. Rev. Biophys. Biophys. Chem. *17*, 265-286.

Haguenauer-Tsapis,R. and Hinnen,A. (1984). A deletion that includes the signal peptidase cleavage site impairs processing, glycosylation, and secretion of cell surface yeast acid phosphatase. Mol. Cell. Biol. *4*, 2668-2675.

Han,M. and Grunstein,M. (1988). Nucleosome loss activates yeast downstream promoters *in vivo*. Cell *55*, 1137-1145.

Han,M., Kim,U.J., Kayne,P., and Grunstein,M. (1988). Depletion of histone H4 and nucleosomes activates the *PHO5* gene in *Saccharomyces cerevisiae*. EMBO J. *7*, 2221-2228.

Hansen,J.C. (2002). Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. Annu. Rev. Biophys. Biomol. Struct. *31*, 361-392.

Hayes,J.J., Tullius,T.D., and Wolffe,A.P. (1990). The structure of DNA in a nucleosome. Proc. Natl. Acad. Sci. U. S. A. *87*, 7405-7409.

Horn,P.J. and Peterson,C.L. (2002). Molecular biology. Chromatin higher order folding--wrapping up transcription. Science *297*, 1824-1827.

Huang,S. and O'Shea,E.K. (2005). A systematic high-throughput screen of a yeast deletion collection for mutants defective in PHO5 regulation. Genetics *169*, 1859-1871.

Ioshikhes,I., Bolshoy,A., Derenshteyn,K., Borodovsky,M., and Trifonov,E.N. (1996). Nucleosome DNA sequence pattern revealed by multiple alignment of experimentally mapped sequences. J. Mol. Biol. *262*, 129-139.

Jenuwein,T. and Allis,C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

Ju,Q.D., Morrow,B.E., and Warner,J.R. (1990). REB1, a yeast DNA-binding protein with many targets, is essential for growth and bears some resemblance to the oncogene myb. Mol. Cell. Biol. *10*, 5226-5234.

Kaffman,A., Rank,N.M., O'Neill,E.M., Huang,L.S., and O'Shea,E.K. (1998). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature *396*, 482-486.

Kamakaka,R.T., Bulger,M., and Kadonaga,J.T. (1993). Potentiation of RNA polymerase-II transcription by gal4-VP16 during but not after DNA replication and chromatin assembly. Genes Dev. *7*, 1779-1795.

Kaneko,Y., Hayashi,N., Toh-e,A., Banno,I., and Oshima,Y. (1987). Structural characteristics of the PHO8 gene encoding repressible alkaline phosphatase in Saccharomyces cerevisiae. Gene *58*, 137-148.

Kasten,M., Szerlong,H., Erdjument-Bromage,H., Tempst,P., Werner,M., and Cairns,B.R. (2004). Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. EMBO J. *23*, 1348-1359.

Kelley,R.I. (1973). Isolation of a histone IIb1-IIb2 complex. Biochem. Biophys. Res. Commun. *54*, 1588-1594.

Kent,N.A., Karabetsou,N., Politis,P.K., and Mellor,J. (2001). In vivo chromatin remodeling by yeast ISWI homologs Isw1p and Isw2p. Genes Dev. *15*, 619-626.

Kiyama,R. and Trifonov,E.N. (2002). What positions nucleosomes?--A model. FEBS Lett. *523*, 7-11.

Klug,A., Rhodes,D., Smith,J., Finch,J.T., and Thomas,J.O. (1980). A low resolution structure for the histone core of the nucleosome. Nature *287*, 509-516.

Kobor,M.S., Venkatasubrahmanyam,S., Meneghini,M.D., Gin,J.W., Jennings,J.L., Link,A.J., Madhani,H.D., and Rine,J. (2004). A Protein Complex Containing the Conserved Swi2/Snf2-Related ATPase Swr1p Deposits Histone Variant H2A.Z into Euchromatin. PLoS. Biol. *2*, E131.

Komeili,A. and O'Shea,E.K. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. Science *284*, 977-980.

Korber,P., Barbaric,S., Luckenbach,T., Schmid,A., Schermer,U.J., Blaschke,D., and Horz,W. (2006). The Histone Chaperone Asf1 Increases the Rate of Histone Eviction at the Yeast PHO5 and PHO8 Promoters. J. Biol. Chem. *281*, 5539-5545.

Korber,P. and Hörz,W. (2004). In vitro assembly of the characteristic chromatin organization at the yeast PHO5 promoter by a replication independent extract system. J. Biol. Chem.

Korber,P., Luckenbach,T., Blaschke,D., and Horz,W. (2004). Evidence for Histone Eviction in trans upon Induction of the Yeast PHO5 Promoter. Mol. Cell. Biol. *24*, 10965-10974.

Kornberg,R.D. (1974). Chromatin structure: a repeating unit of histones and DNA. Science *184*, 868.

Kornberg,R.D. and Lorch,Y. (1995). Interplay between chromatin structure and transcription. Curr. Opin. Cell Biol. *7*, 371-375.

Kornberg,R.D. and Thomas,J.O. (1974). Chromatin structure; oligomers of the histones. Science *184*, 865-868.

Krogan,N.J., Keogh,M.C., Datta,N., Sawa,C., Ryan,O.W., Ding,H., Haw,R.A., Pootoolal,J., Tong,A., Canadien,V., Richards,D.P., Wu,X., Emili,A., Hughes,T.R., Buratowski,S., and Greenblatt,J.F. (2003). A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. Mol. Cell *12*, 1565-1576.

Kuai,L., Fang,F., Butler,J.S., and Sherman,F. (2004). Polyadenylation of rRNA in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A *101*, 8581-8586.

Längst,G. and Becker,P.B. (2004). Nucleosome remodeling: one mechanism, many phenomena? Biochim. Biophys. Acta *1677*, 58-63.

Längst,G., Blank,T.A., Becker,P.B., and Grummt,I. (1997). RNA polymerase I transcription on nucleosomal templates: the transcription termination factor TTF-I induces chromatin remodeling and relieves transcriptional repression. EMBO J. *16*, 760-768.

Längst,G., Bonte,E.J., Corona,D.F., and Becker,P.B. (1999). Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer. Cell *97*, 843-852.

Laskey,R.A., Mills,A.D., and Morris,N.R. (1977). Assembly of SV40 chromatin in a cell-free system from Xenopus eggs. Cell *10*, 237-243.

Lee,C.K., Shibata,Y., Rao,B., Strahl,B.D., and Lieb,J.D. (2004). Evidence for nucleosome depletion at active regulatory regions genome-wide. Nat. Genet. *36*, 900-905.

Lee,M.G., Wynder,C., Cooch,N., and Shiekhattar,R. (2005). An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature *437*, 432-435.

Li,Q. and Wrange,O. (1993). Translational positioning of a nucleosomal glucocorticoid response element modulates glucocorticoid receptor affinity. Genes Dev. *7*, 2471-2482.

Linxweiler,W. and Hörz,W. (1985). Reconstitution experiments show that sequence-specific histone- DNA interactions are the basis for nucleosome phasing on mouse satellite DNA. Cell *42*, 281-290.

Lohr,D. and van Holde,K.E. (1975). Yeast chromatin subunit structure. Science *188*, 165-166.

Lorch,Y., LaPointe,J.W., and Kornberg,R.D. (1987). Nucleosomes inhibit the initiation of transcription but allow chain elongation with the displacement of histones. Cell *49*, 203-210.

Lowary,P.T. and Widom,J. (1997). Nucleosome packaging and nucleosome positioning of genomic DNA. Proc. Natl. Acad. Sci. U. S. A *94*, 1183-1188.

Lowary,P.T. and Widom,J. (1998). New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning. J. Mol. Biol. *276*, 19-42.

Lu,Q., Wallrath,L.L., and Elgin,S.C.R. (1995). The role of a positioned nucleosome at the Drosophila melanogaster hsp26 promoter. EMBO J. *14*, 4738-4746.

Luger,K. and Hansen,J.C. (2005). Nucleosome and chromatin fiber dynamics. Curr. Opin. Struct. Biol. *15*, 188-196.

Luger,K., Mader,A.W., Richmond,R.K., Sargent,D.F., and Richmond,T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.

Luger,K., Rechsteiner,T.J., and Richmond,T.J. (1999). Preparation of nucleosome core particle from recombinant histones. Methods Enzymol. *304*, 3-19.

Luger,K. and Richmond,T.J. (1998). The histone tails of the nucleosome. Curr. Opin. Genet. Dev. *8*, 140-146.

Lusser,A. and Kadonaga,J.T. (2004). Strategies for the reconstitution of chromatin. Nat. Methods *1*, 19-26.

Magbanua,J.P., Fujisawa,K., Ogawa,N., and Oshima,Y. (1997). The homeodomain protein Pho2p binds at an A/T-rich segment flanking the binding site of the basic-helix-loop-helix protein Pho4p in the yeast PHO promoters. Yeast *13*, 1299-1308.

Margueron,R., Trojer,P., and Reinberg,D. (2005). The key to development: interpreting the histone code? Curr. Opin. Genet. Dev. *15*, 163-176.

Mcpherson,C.E., Horowitz,R., Woodcock,C.L., Jiang,C., and Zaret,K.S. (1996). Nucleosome positioning properties of the albumin transcriptional enhancer. Nucleic Acids Res. *24*, 397-404.

Mcpherson,C.E., Shim,E.Y., Friedman,D.S., and Zaret,K.S. (1993). An active tissue-specific enhancer and bound transcription factors existing in a precisely positioned nucleosomal array. Cell *75*, 387-398.

Meersseman,G., Pennings,S., and Bradbury,E.M. (1991). Chromatosome positioning on assembled long chromatin. Linker histones affect nucleosome placement on 5 S rDNA. J. Mol. Biol. *220*, 89-100.

Meersseman,G., Pennings,S., and Bradbury,E.M. (1992). Mobile nucleosomes--a general behavior. EMBO J. *11*, 2951-2959.

Mellor,J., Jiang,W., Funk,M., Rathjen,J., Barnes,C.A., Hinz,T., Hegemann,J.H., and Philippsen,P. (1990). CPF1, a yeast protein which functions in centromeres and promoters. EMBO J. *9*, 4017-4026.

Metzger,E., Wissmann,M., Yin,N., Muller,J.M., Schneider,R., Peters,A.H., Gunther,T., Buettner,R., and Schule,R. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature *437*, 436-439.

Mizuguchi,G., Shen,X., Landry,J., Wu,W.H., Sen,S., and Wu,C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science *303*, 343-348.

Moreau,J.L., Lee,M., Mahachi,N., Vary,J., Mellor,J., Tsukiyama,T., and Goding,C.R. (2003). Regulated displacement of TBP from the PHO8 promoter in vivo requires Cbf1 and the Isw1 chromatin remodeling complex. Mol. Cell *11*, 1609-1620.

Moreira,J.M. and Holmberg,S. (1999). Transcriptional repression of the yeast CHA1 gene requires the chromatin-remodeling complex RSC. EMBO J. *18*, 2836-2844.

Moreira,J.M. and Holmberg,S. (2000). Chromatin-mediated transcriptional regulation by the yeast architectural factors NHP6A and NHP6B. EMBO J. *19*, 6804-6813.

Moreira,J.M., Horz,W., and Holmberg,S. (2002). Neither Reb1p nor poly(dA*T) elements are responsible for the highly specific chromatin organization at the ILV1 promoter. J. Biol. Chem. *277*, 3202-3209.

Münsterkötter,M., Barbaric,S., and Hörz,W. (2000). Transcriptional regulation of the yeast *PHO8* promoter in comparison to the coregulated *PHO5* promoter. J. Biol. Chem. *275*, 22678-22685.

Muyldermans,S. and Travers,A.A. (1994). DNA sequence organization in chromatosomes. J. Mol. Biol. *235*, 855-870.

Neef,D.W. and Kladde,M.P. (2003). Polyphosphate loss promotes SNF/SWI- and Gcn5-dependent mitotic induction of PHO5. Mol. Cell. Biol. *23*, 3788-3797.

Negri,R., Buttinelli,M., Panetta,G., De,A., V, Di Mauro,E., and Travers,A. (2001). Sequence dependence of translational positioning of core nucleosomes. J. Mol. Biol. *307*, 987-999.

Nelson,H.C., Finch,J.T., Luisi,B.F., and Klug,A. (1987). The structure of an oligo(dA).oligo(dT) tract and its biological implications. Nature *330*, 221-226.

Neubauer,B., Linxweiler,W., and Hörz,W. (1986). DNA engineering shows that nucleosome phasing on the African green monkey alpha-satellite is the result of multiple additive histone-DNA interactions. J. Mol. Biol. *190*, 639-645.

Nickol,J., Behe,M., and Felsenfeld,G. (1982). Effect of the B--Z transition in poly(dG-m5dC) . poly(dG-m5dC) on nucleosome formation. Proc. Natl. Acad. Sci. U. S. A *79*, 1771-1775.

Nonet,M., Scafe,C., Sexton,J., and Young,R. (1987). Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol. Cell. Biol. *7*, 1602-1611.

Olins,A.L. and Olins,D.E. (1974). Spheroid chromatin units (v bodies). Science *183*, 330-332.

Olson,W.K., Gorin,A.A., Lu,X.J., Hock,L.M., and Zhurkin,V.B. (1998). DNA sequence-dependent deformability deduced from protein-DNA crystal complexes. Proc. Natl. Acad. Sci. U. S. A *95*, 11163-11168.

Oshima,Y. (1997). The phosphatase system in Saccharomyces cerevisiae. Genes Genet. Syst. *72*, 323-334.

Oudet,P., Gross-Bellard,M., and Chambon,P. (1975). Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. Cell *4*, 281-300.

Park,Y.J., Dyer,P.N., Tremethick,D.J., and Luger,K. (2004). A new fluorescence resonance energy transfer approach demonstrates that the histone variant H2AZ stabilizes the histone octamer within the nucleosome. J. Biol. Chem. *279*, 24274-24282.

Pazin,M.J., Bhargava,P., Geiduschek,E.P., and Kadonaga,J.T. (1997). Nucleosome mobility and the maintenance of nucleosome positioning. Science *276*, 809-812.

Pazin,M.J., Kamakaka,R.T., and Kadonaga,J.T. (1994). ATP-dependent nucleosome reconfiguration and transcriptional activation from preassembled chromatin templates. Science *266*, 2007-2011.

Pennings,S., Allan,J., and Davey,C.S. (2005). DNA methylation, nucleosome formation and positioning. Brief. Funct. Genomic. Proteomic. *3*, 351-361.

Pennings,S., Meersseman,G., and Bradbury,E.M. (1991). Mobility of positioned nucleosomes on 5 S rDNA. J. Mol. Biol. *220*, 101-110.

Pfaff,S.L. and Taylor,W.L. (1998). Xenopus TFIIIA gene transcription is dependent on cis-element positioning and chromatin structure. Mol. Cell. Biol. *18*, 3811-3818.

Pina,B., Barettino,D., Truss,M., and Beato,M. (1990). Structural features of a regulatory nucleosome. J. Mol. Biol. *216*, 975-990.

Pokholok,D.K., Harbison,C.T., Levine,S., Cole,M., Hannett,N.M., Lee,T.I., Bell,G.W., Walker,K., Rolfe,P.A., Herbolsheimer,E., Zeitlinger,J., Lewitter,F., Gifford,D.K., and Young,R.A. (2005). Genome-wide map of nucleosome acetylation and methylation in yeast. Cell *122*, 517-527.

Raisner,R.M., Hartley,P.D., Meneghini,M.D., Bao,M.Z., Liu,C.L., Schreiber,S.L., Rando,O.J., and Madhani,H.D. (2005). Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. Cell *123*, 233-248.

Reagan,M.S. and Majors,J.E. (1998). The chromatin structure of the GAL1 promoter forms independently of Reb1p in Saccharomyces cerevisiae. Mol. Gen. Genet. *259*, 142-149.

Reeves,R. (1984). Transcriptionally active chromatin. Biochim. Biophys. Acta *782*, 343-393.

Reinke,H. and Hörz,W. (2003). Histones are first hyperacetylated and then lose contact with the activated *PHO5* promoter. Mol. Cell *11*, 1599-1607.

Reinke,H. and Hörz,W. (2004). Anatomy of a hypersensitive site. Biochim. Biophys. Acta *1677*, 24-29.

Rhodes,D. (1979). Nucleosome cores reconstituted from poly (dA-dT) and the octamer of histones. Nucleic Acids Res. *6*, 1805-1816.

Rice,J.C. and Allis,C.D. (2001). Code of silence. Nature *414*, 258-261.

Richmond,T.J. and Davey,C.A. (2003). The structure of DNA in the nucleosome core. Nature *423*, 145-150.

Robinson,K.A. and Lopes,J.M. (2000). SURVEY AND SUMMARY: Saccharomyces cerevisiae basic helix-loop-helix proteins regulate diverse biological processes. Nucleic Acids Res. *28*, 1499-1505.

Roth,S.Y., Shimizu,M., Johnson,L., Grunstein,M., and Simpson,R.T. (1992). Stable nucleosome positioning and complete repression by the yeast alpha 2 repressor are disrupted by amino-terminal mutations in histone H4. Genes Dev. *6*, 411-425.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989). Molecular Cloning: A Laboratory Manual (2nd Edition). (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schild,C., Claret,F.X., Wahli,W., and Wolffe,A.P. (1993). A nucleosome-dependent static loop potentiates estrogen- regulated transcription from the Xenopus vitellogenin B1 promoter in vitro. EMBO J. *12*, 423-433.

Schultz,M.C. (1999). Chromatin assembly in yeast cell-free extracts. Methods *17*, 161-172.

Schultz,M.C., Hockman,D.J., Harkness,T.A., Garinther,W.I., and Altheim,B.A. (1997). Chromatin assembly in a yeast whole-cell extract. Proc. Natl. Acad. Sci. U. S. A *94*, 9034-9039.

Schwarz,P.M., Felthauser,A., Fletcher,T.M., and Hansen,J.C. (1996). Reversible oligonucleosome self-association: dependence on divalent cations and core histone tail domains. Biochemistry *35*, 4009-4015.

Sekinger,E.A., Moqtaderi,Z., and Struhl,K. (2005). Intrinsic histone-DNA interactions and low nucleosome density are important for preferential accessibility of promoter regions in yeast. Mol. Cell *18*, 735-748.

Shi,Y., Lan,F., Matson,C., Mulligan,P., Whetstine,J.R., Cole,P.A., Casero,R.A., and Shi,Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

Shi,Y.J., Matson,C., Lan,F., Iwase,S., Baba,T., and Shi,Y. (2005). Regulation of LSD1 histone demethylase activity by its associated factors. Mol. Cell *19*, 857-864.

Shimamura,A., Tremethick,D., and Worcel,A. (1988). Characterization of the repressed 5S DNA minichromosomes assembled in vitro with a high-speed supernatant of Xenopus laevis oocytes. Mol. Cell. Biol. *8*, 4257-4269.

Shrader,T.E. and Crothers,D.M. (1989). Artificial nucleosome positioning sequences. Proc. Natl. Acad. Sci. U. S. A. *86*, 7418-7422.

Shrader,T.E. and Crothers,D.M. (1990). Effects of DNA sequence and histone-histone interactions on nucleosome placement. J. Mol. Biol. *216*, 69-84.

Simon,R.H. and Felsenfeld,G. (1979). A new procedure for purifying histone pairs H2A + H2B and H3 + H4 from chromatin using hydroxylapatite. Nucleic Acids Res. *6*, 689-696.

Simpson,R.T. and Shindo,H. (1979). Conformation of DNA in chromatin core particles containing poly(dAdT)-poly(dAdT) studied by 31 P NMR spectroscopy. Nucleic Acids Res. *7*, 481-492.

Snyder,M., Buchman,A.R., and Davis,R.W. (1986). Bent DNA at a yeast autonomously replicating sequence. Nature *324*, 87-89.

Staffelbach,H., Koller,T., and Burks,C. (1994). DNA structural patterns and nucleosome positioning. J. Biomol. Struct. Dyn. *12*, 301-325.

Stein,A. and Bina,M. (1999). A signal encoded in vertebrate DNA that influences nucleosome positioning and alignment. Nucleic Acids Res. *27*, 848-853.

Stein,A. and Kunzler,P. (1983). Histone H5 can correctly align randomly arranged nucleosomes in a defined in vitro system. Nature *302*, 548-550.

Straka,C. and Hörz,W. (1991). A functional role for nucleosomes in the repression of a yeast promoter. EMBO J. *10*, 361-368.

Stunkel,W., Kober,I., and Seifart,K.H. (1997). A nucleosome positioned in the distal promoter region activates transcription of the human U6 gene. Mol. Cell. Biol. *17*, 4397-4405.

Suto,R.K., Clarkson,M.J., Tremethick,D.J., and Luger,K. (2000). Crystal structure of a nucleosome core particle containing the variant histone H2A.Z. NAT. STRUCT. BIOL. *7*, 1121-1124.

Svaren,J. and Hörz,W. (1997). Transcription factors vs nucleosomes: Regulation of the *PHO5* promoter in yeast. Trends Biochem. Sci. *22*, 93-97.

Tanaka,S., Zatchej,M., and Thoma,F. (1992a). Artificial nucleosome positioning sequences tested in yeast minichromosomes: a strong rotational setting is not sufficient to position nucleosomes in vivo. EMBO J. *11*, 1187-1193.

Tanaka,S., Zatchej,M., and Thoma,F. (1992b). Artificial nucleosome positioning sequences tested in yeast minichromosomes: a strong rotational setting is not sufficient to position nucleosomes in vivo. EMBO J. *11*, 1187-1193.

Tanaka,Y., Tachiwana,H., Yoda,K., Masumoto,H., Okazaki,T., Kurumizaka,H., and Yokoyama,S. (2005). Human centromere protein B induces translational positioning of nucleosomes on alpha-satellite sequences. J. Biol. Chem. *280*, 41609-41618.

Terrell,A.R., Wongwisansri,S., Pilon,J.L., and Laybourn,P.J. (2002). Reconstitution of nucleosome positioning, remodeling, histone acetylation, and transcriptional activation on the PHO5 promoter. J. Biol. Chem. *277*, 31038-31047.

Thastrom,A., Lowary,P.T., Widlund,H.R., Cao,H., Kubista,M., and Widom,J. (1999). Sequence motifs and free energies of selected natural and non-natural nucleosome positioning DNA sequences. J. Mol. Biol. *288*, 213-229.

Tomaszewski,R. and Jerzmanowski,A. (1997). The AT-rich flanks of the oocyte-type 5S RNA gene of Xenopus laevis act as a strong local signal for histone H1-mediated chromatin reorganization in vitro. Nucleic Acids Res. *25*, 458-466.

Travers,A.A. and Klug,A. (1987). The bending of DNA in nucleosomes and its wider implications. Philos. Trans. R. Soc. Lond B Biol. Sci. *317*, 537-561.

Travers,A.A. and Muyldermans,S.V. (1996). A DNA sequence for positioning chromatosomes. J. Mol. Biol. *257*, 486-491.

Tsukiyama,T., Becker,P.B., and Wu,C. (1994). ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. Nature *367*, 525-532.

van Holde,K. (1989). Chromatin. (New York: Springer).

van Hoof,A., Staples,R.R., Baker,R.E., and Parker,R. (2000). Function of the ski4p (Csl4p) and Ski7p proteins in 3'-to-5' degradation of mRNA. Mol. Cell. Biol. *20*, 8230-8243.

Varga-Weisz,P.D., Blank,T.A., and Becker,P.B. (1995). Energy-dependent chromatin accessibility and nucleosome mobility in a cell-free system. EMBO J. *14*, 2209-2216.

Venditti,P., DiCroce,L., Kauer,M., Blank,T., Becker,P.B., and Beato,M. (1998). Assembly of MMTV promoter minichromosomes with positioned nucleosomes precludes NF1 access but not restriction enzyme cleavage. Nucl. Acids Res. *26*, 3657-3666.

Venter,U., Svaren,J., Schmitz,J., Schmid,A., and Hörz,W. (1994). A nucleosome precludes binding of the transcription factor Pho4 in vivo to a critical target site in the *PHO5* promoter. EMBO J. *13*, 4848-4855.

Vogel,K. and Hinnen,A. (1990). The yeast phosphatase system. Mol. Microbiol. *4*, 2013-2017.

Wada-Kiyama,Y. and Kiyama,R. (1996b). An intrachromosomal repeating unit based on DNA bending. Mol. Cell Biol. *16*, 5664-5673.

Wada-Kiyama,Y. and Kiyama,R. (1996a). An intrachromosomal repeating unit based on DNA bending. Mol. Cell Biol. *16*, 5664-5673.

Wada-Kiyama,Y., Suzuki,K., and Kiyama,R. (1999). DNA bend sites in the human beta-globin locus: evidence for a basic and universal structural component of genomic DNA. Mol. Biol. Evol. *16*, 922-930.

Wall,G., Varga-Weisz,P.D., Sandaltzopoulos,R., and Becker,P.B. (1995). Chromatin remodeling by GAGA factor and heat shock factor at the hypersensitive Drosophila hsp26 promoter in vitro. EMBO J. *14*, 1727-1736.

Wang,J.C. (1982). The path of DNA in the nucleosome. Cell *29*, 724-726.

Wang,Y.H., Gellibolian,R., Shimizu,M., Wells,R.D., and Griffith,J. (1996). Long CCG triplet repeat blocks exclude nucleosomes: a possible mechanism for the nature of fragile sites in chromosomes. J. Mol. Biol. *263*, 511-516.

Wang,Y.H. and Griffith,J. (1995). Expanded CTG triplet blocks from the myotonic dystrophy gene create the strongest known natural nucleosome positioning elements. Genomics *25*, 570-573.

Wasylyk,B. and Chambon,P. (1979). Transcription by eukaryotic RNA polymerases A and B of chromatin assembled in vitro. Eur. J. Biochem. *98*, 317-327.

Wasylyk,B., Thevenin,G., Oudet,P., and Chambon,P. (1979). Transcription of in vitro assembled chromatin by Escherichia coli RNA polymerase. J. Mol. Biol. *128*, 411-440.

Weintraub,H. and Groudine,M. (1976). Chromosomal subunits in active genes have an altered conformation. Science *193*, 848-856.

Westin,L., Blomquist,P., Milligan,J.F., and Wrange,O. (1995). Triple helix DNA alters nucleosomal histone-DNA interactions and acts as a nucleosome barrier. Nucleic Acids Res. *23*, 2184-2191.

Widlak,P., Gaynor,R.B., and Garrard,W.T. (1997). In vitro chromatin assembly of the HIV-1 promoter. ATP-dependent polar repositioning of nucleosomes by Sp1 and NFkappaB. J. Biol. Chem. *272*, 17654-17661.

Widlund,H.R., Cao,H., Simonsson,S., Magnusson,E., Simonsson,T., Nielsen,P.E., Kahn,J.D., Crothers,D.M., and Kubista,M. (1997). Identification and characterization of genomic nucleosome-positioning sequences. J. Mol. Biol. *267*, 807-817.

Widlund,H.R., Kuduvalli,P.N., Bengtsson,M., Cao,H., Tullius,T.D., and Kubista,M. (1999). Nucleosome structural features and intrinsic properties of the TATAAACGCC repeat sequence. J. Biol. Chem. *274*, 31847-31852.

Widom,J. (1998). Structure, dynamics, and function of chromatin in vitro. Annual Review Of Biophysics And Biomolecular Structure *27*, 285-327.

Widom,J. (2001). Role of DNA sequence in nucleosome stability and dynamics. Q. Rev. Biophys. *34*, 269-324.

Wilhelm,F.X., Wilhelm,M.L., Erard,M., and Duane,M.P. (1978). Reconstitution of chromatin: assembly of the nucleosome. Nucleic Acids Res. *5*, 505-521.

Woodcock,C.L. and Dimitrov,S. (2001). Higher-order structure of chromatin and chromosomes. Curr. Opin. Genet. Dev. *11*, 130-135.

Woodcock,C.L., Frado,L.L., Hatch,C.L., and Ricciardiello,L. (1976). Fine structure of active ribosomal genes. Chromosoma *58*, 33-39.

Wyers,F., Rougemaille,M., Badis,G., Rousselle,J.C., Dufour,M.E., Boulay,J., Regnault,B., Devaux,F., Namane,A., Seraphin,B., Libri,D., and Jacquier,A. (2005). Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. Cell *121*, 725-737.

Wysocka,J., Milne,T.A., and Allis,C.D. (2005). Taking LSD 1 to a new high. Cell *122*, 654-658.

Yuan,G.C., Liu,Y.J., Dion,M.F., Slack,M.D., Wu,L.F., Altschuler,S.J., and Rando,O.J. (2005). Genome-scale identification of nucleosome positions in S. cerevisiae. Science *309*, 626-630.

Zhang,H., Roberts,D.N., and Cairns,B.R. (2005). Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. Cell *123*, 219-231.

Zhang,Z. and Reese,J.C. (2004). Ssn6-Tup1 requires the ISW2 complex to position nucleosomes in Saccharomyces cerevisiae. EMBO J. *23*, 2246-2257.

# VIII. Appendix

## 1. Abbreviations

| | |
|---|---|
| aa | Amino acid |
| ATP | Adenosine-5'-triphosphate |
| bp | Base pairs |
| BSA | Bovine serum albumine |
| CEA | chicken egg albumin |
| Ci | Curie |
| ChIP | Chromatin immunoprecipitation |
| CK | Creatine kinase |
| CP | Creatine phosphate |
| Cpm | counts per minute |
| C-terminal | Carboxy-terminal |
| DNA | Deoxyribonucleic acid |
| DNase I | Deoxyribonucleosidase I |
| DREX | *Drosophila* embryo extract |
| DTT | Dithiothreitol |
| E. coli | Escherichia coli |
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Ethylene glycol-bis(ℵ-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| H1/H2A/H2B/H3/ H4 | Histone proteins |
| HAT | Histone acetyltransferase |
| HDAC | Histone deacetylase |
| HMT | Histone methyl transferase |
| h | hour |
| kDa | Kilo daltons |
| M | Molar |
| min | Minute(s) |
| MNase | Micrococcus Nuclease |
| NP-40 | Nonidet P-40 |
| NPP | Nitrophenylphosphate dinatrium-hexahydrate |
| OD | Optical density |
| ORF | Open reading frame |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PMSF | Phenylmethylsulphonylfluoride |
| RNA | Ribonucleic acid |
| RNA PolII | RNA polymerase II |
| rpm | Rounds pro minute |
| RT | Room temperature |
| SDS | Sodium Dodecyl Sulphate |
| sec | Second |
| TBE | Tris borate EDTA buffer |
| Tris | Tris(hydroxymethyl)-amino-methane |
| Tween-20 | Polyoxyethylene-sorbitan monolaurate |
| wt | wild type |

## 2. Curriculum vitae

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| | |
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### Publications

Hertel,C.B., Langst,G., Horz,W., and Korber,P. (2005). Nucleosome stability at the yeast PHO5 and PHO8 promoters correlates with differential cofactor requirements for chromatin opening. Mol. Cell. Biol. *25*, 10755-10767.

Hertel, C.B., Zhou,X.G., Hamilton-Dutoit,S.J., and Junker,S. (2002). Loss of B cell identity correlates with loss of B cell-specific transcription factors in Hodgkin/Reed-Sternberg cells of classical Hodgkin lymphoma. Oncogene *21*, 4908-4920.