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NoRC,
a novel chromatin remodeling complex involved in ribosomal RNA gene silencing

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Erklärung

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TABLE OF CONTENT

1 FIGURE INDEX

2 TABLE INDEX

3 LIST OF ABBREVIATIONS

aa	Aminoacid
ACF	ATP-utilizing chromatin assembly and remodeling factor
Acf-1	large subunit of ACF and CHRAC
ATP	Adenosine-5'-triphosphate
AM-X	Tris based buffer containing X mM KCl
BAZ	Bromodomain Adjacent to Zinc finger domain
bp	Base pairs
BRG1	Brahma-related gene product
BRM	Brahma protein
BSA	Bovine serum albumine
CEA	chicken egg albumin
CHD	Chromodomain
CHIP	Chromatin Immunoprecipitation
CHRAC	Chromatin accessibility complex
Ci	Curie
CPE	Core promoter element
Cpm	counts per minute
C-terminal	Carboxy-terminal
CV	Column volume
DEAE	Diethylaminoethyl
DEAE 280	Partial purified transcription extract
d.m.	*Drosophila melanogaster*
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonucleosidase I
DNMT 1/ 3a / 3b	DNA methyl transferase
dNTP	Deoxyribonucleoside triphosphate
DREX	*Drosophila* embryo extract
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMSA	Electrophoretic mobility shift assay
EX-X	Tris based buffer containing X mM KCl
FCS	Foetal calf serum
g	gram or relative centrifugal force
GST	Glutathione-S-transferase
H1 / H2A / H2B / H3 / H4	histone proteins
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	histone methyl transferase
HMG	High mobility group
HRP	Horse radish peroxidase
h	hour
Ig	Immunoglobulin
IGS	Intergenic spacer
IP	Immunoprecipitation
ISWI	Imitation of switch
kDa	Kilo daltons
M	Molar
MBD	methyl binding domain
min	minute(s)
MNase	Micrococcus Nuclease

mut	mutante
MW	Molecular weight
Ni-NTA	Nickel-nitroacetic acid
NoRC	Nucleolar remodeling complex
NoRC wt	recombinant NoRC, reconstituted using wild type Snf2h
NoRC mut	NoRC, reconstituted using a Snf2h KR211 point mutant
NP-40	Nonidet P-40
NRD	Negative regulatory domain
N-terminal	Amino-terminal
NURF	Nucleosome-remodeling factor
PAA	Polyacrylamide
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pol I, II, III	RNA polymerase I, II, III
pre-rRNA	precursor of ribosomal RNA
PTRF	Polymerase I transcript release factor
rDNA	Ribosomal DNA
rec	recombinant
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rounds pro minute
rRNA	Ribosomal RNA
RT	Room temperature
S	Svedberg unit
SDS	Sodium Dodecyl Sulphate
sec	second
Sf9	*Spodoptera frugiperda* 9 cells
Snf	Sucrose non-fermenter
Snf2h	Snf2 homolog protein
SWI	Mating type switching
TAF	TBP-associated factor
TBE	Tris borate EDTA buffer
TBP	TATA-binding protein
TIF-IA / B / C	Transcription initiation factors for RNA polymerase I
Tip5	TTF-I interacting protein 5
Tris	Tris(hydroxymethyl)-amino-methane
TSA	Trichostatin A
TTF-I	Transcription termination factor for RNA polymerase I
TTFΔN185	N-terminal (aa 1 to 185) truncated form of TTF-I
Tween-20	Polyoxyethylene-sorbitan monolaurate
UBF	upstream binding factor
UCE	Upstream control element
UV	Ultraviolet light
V	Volts
Vol	Volume(s)
WSTF	Williams syndrome transcription factor
WT	wild type

SI - units were used.

4 SUMMARY

Regulation of gene expression takes place in the nucleus in a highly structured and condensed nucleoprotein environment, called chromatin (Felsenfeld and Groudine, 2003; Khorasanizadeh, 2004; Vaquero et al., 2003). A broad group of factors regulates the properties of chromatin; e.g. by covalently modifying histones and / or by ATP-dependent chromatin remodeling, thereby allowing or preventing gene expression.

The mammalian genome contains hundreds of gene copies encoding precursor ribosomal RNA and the transcription of these genes is highly regulated with respect to cellular metabolism (Grummt, 2003). However, even in actively growing cells, only a subset of the rRNA genes are actively transcribed, exhibiting an accessible chromatin conformation (Conconi et al., 1989). In a chromatin context, the activation of rDNA genes involves the transcription termination factor TTF-I (Längst et al., 1998; Längst et al., 1997a). However, the silenced rDNA gene fraction remains in an inaccessible heterochromatic state throughout the cell cycle (Conconi et al., 1989). Until recently, the onset of silencing and the mechanisms that maintain the inactive state of rRNA genes were less understood.

Recent studies, including the work presented in this thesis, provide insights into the molecular mechanism of ribosomal RNA gene silencing (Lawrence et al., 2004; Németh et al., 2004; Santoro and Grummt, 2001; Santoro et al., 2002; Strohner et al., 2004; Zhou et al., 2002). Accumulating evidence indicates that the combined action of chromatin modifying mechanisms such as chromatin remodeling, histone modification and DNA methylation contribute to the process of rRNA gene silencing. Here I present data demonstrating an active role of the chromatin remodeling complex NoRC in rDNA gene silencing and propose dual functions of TTF-I in rDNA regulation in chromatin, namely involvement in both activation and silencing of rDNA transcription.

4.1 NoRC, a novel chromatin remodeling complex

In this doctoral study, a novel protein complex, composed of the nucleolar protein Tip5 and the ATPase Snf2h, was purified using convential chromatography and affinity purification methods. A detailed chromatin remodeling analysis revealed that this complex is able to induce mononucleosome movement in an ATP and histone H4 tail dependent fashion. Finally, this Tip5-Snf2h complex was termed NoRC (nucleolar remodeling complex), a novel member of the ISWI family of ATP-dependent chromatin remodeling complexes (Strohner et al., 2001).

To dissect its functions, the NoRC complex was reconstituted from its recombinant subunits Tip5 and Snf2h, using the baculo virus driven expression system. Reconstitution confirmed the direct interaction between Tip5 and Snf2h. Furthermore, recombinant and cellular NoRC display similar sizes in gel filtration columns. Recombinant NoRC exhibits chromatin stimulated ATPase activity and mobilizes nucleosomes in an energy-dependent manner. Both activities are histone H4 tail dependent. NoRC and its subunits Tip5 and Snf2h were compared in different DNA / Nucleosome binding assays. NoRC shows preferred binding to structured (bent) DNA, e.g. a region within the mouse rDNA promoter, and interacts with mononucleosomes in electrophoretic mobility shift assays (EMSA). While no stable interaction with core nucleosomes could be detected in EMSA, ATPase assays and DNase I protection assays noticeably pinpointed to NoRC / nucleosome interactions with both nucleosomal and protruding linker DNA.

4.2 NoRC specifically represses rDNA transcription in chromatin

The functional consequences of the Tip5 / TTF-I interaction were assessed and the influence on chromatin structure of the rDNA promoter in an *in vitro* system was determined. Tip5 in NoRC interacts with the N-terminal part of full length TTF-I and unmasks its DNA binding site. This interaction is required both for binding of TTF-I to its promoter-proximal target site and for the recruitment of NoRC to the promoter in chromatin. After association with the rDNA promoter, NoRC alters the position of the promoter-bound nucleosome.

To elucidate a potential role of NoRC in rDNA transcriptional regulation, we used an *in vitro* transcription system with an rDNA minigene reconstituted into chromatin. These studies revealed a specific function for NoRC in rDNA transcriptional repression on chromatin templates. In contrast, NoRC had no effect on DNA transcription. Transcription experiments were then performed with chromatin templates reconstituted from recombinant histones lacking individual histone tails. The results indicate that NoRC-mediated rDNA gene repression is dependent on the histone H4 tail, suggesting an involvement of chromatin remodeling. Further transcription experiments revealed that NoRC-mediated repression occurs prior to preinitiation complex formation and does not affect activated rDNA genes. NoRC stably associates with the silenced gene, and these early steps of rDNA repression do not depend on DNA and histone modifications (Strohner et al., 2004).

NoRC showed preferred binding to a structured (bent) region within the mouse rDNA promoter. Methylation of a single CpG dinucleotide within this region abrogated rDNA transcription in chromatin (Santoro and Grummt, 2001), but did not influence DNA binding of NoRC. Furthermore, nucleosomal DNA is less methylated than free DNA, but chromatin remodeling enhances methylation.

The results suggest an important role for the chromatin remodeling complex NoRC in the establishment of rDNA silencing. NoRC then contributes to maintenance of the silenced state throughout the cell cycle by interacting with DNA and histone modifying enzymes. Transcriptional repression by chromatin remodeling factors seems to be a common mechanism to stably inhibit gene expression.

5 INTRODUCTION

The genome of eukaryotic cells is assembled into a highly compact structure termed chromatin, consisting of DNA and associated proteins (histones and non-histone proteins). Chromatin presents the natural substrate for all kinds of DNA-dependent processes such as the control of gene expression, as well as DNA replication, recombination and repair (Felsenfeld and Groudine, 2003; Khorasanizadeh, 2004). A broad group of factors has been characterized that regulates the dynamics of chromatin, e.g. by covalently modifying histones (Fischle et al., 2003; Vaquero et al., 2003) and / or by energy-dependent alterations of the chromatin structure, a process called ATP-dependent nucleosome remodeling (Becker and Hörz, 2002; Tsukiyama, 2002). These modulations influence the chromatin structure by regulating the accessibility of nucleosomal DNA, and thereby allowing or preventing gene expression.

5.1 Chromatin structure

In human cells, approximately 2 meters of DNA are compacted into chromatin, such that the genome fits into the nucleus, with an average diameter of only 10 μm. The term chromatin (from the greek word 'chroma' for coloring) was first proposed in the 1880s when W. Flemming observed a stainable substance in the cell nucleus. To this day great discoveries are being made, which heights our understanding of the organization of DNA into a hierarchy of chromatin structures (Olins and Olins, 2003). Remarkably, the highly packaged and condensed DNA, the chromatin, maintains a dynamic nature to facilitate all kind of nuclear processes.

5.1.1 The nucleosome is the basic unit of chromatin

The fundamental building unit of chromatin is the nucleosome (Kornberg, 1974; Oudet et al., 1975). The structural details of the nucleosome core particle have been determined by X-ray crystallography with high resolution (Luger et al., 1997; Richmond and Davey, 2003). It consists of approximately 147 bp of DNA, which are wrapped in ~1.7 left-handed superhelical turns around the outside of a disc-like shaped protein core (Figure 1). This protein core (histone octamer) contains two copies each of the core histones H2A, H2B, H3 and H4. A complete histone octamer is composed of a central $(H3\text{-}H4)_2$ tetramer flanked by two H2A-H2B dimers. The four core histones are small basic proteins (11 to 16 kDa), which are highly conserved through evolution. Each contains a central 'histone fold' motif, consisting of a three-helix core domain, mediating histone-histone as well as histone-DNA interactions and a highly basic amino-terminal domain ('tail'), which extends from the surface of the nucleosome. These histone tails are targets for post-translational modifications (section 5.2), and are important for higher order chromatin structure (section 5.1.2).

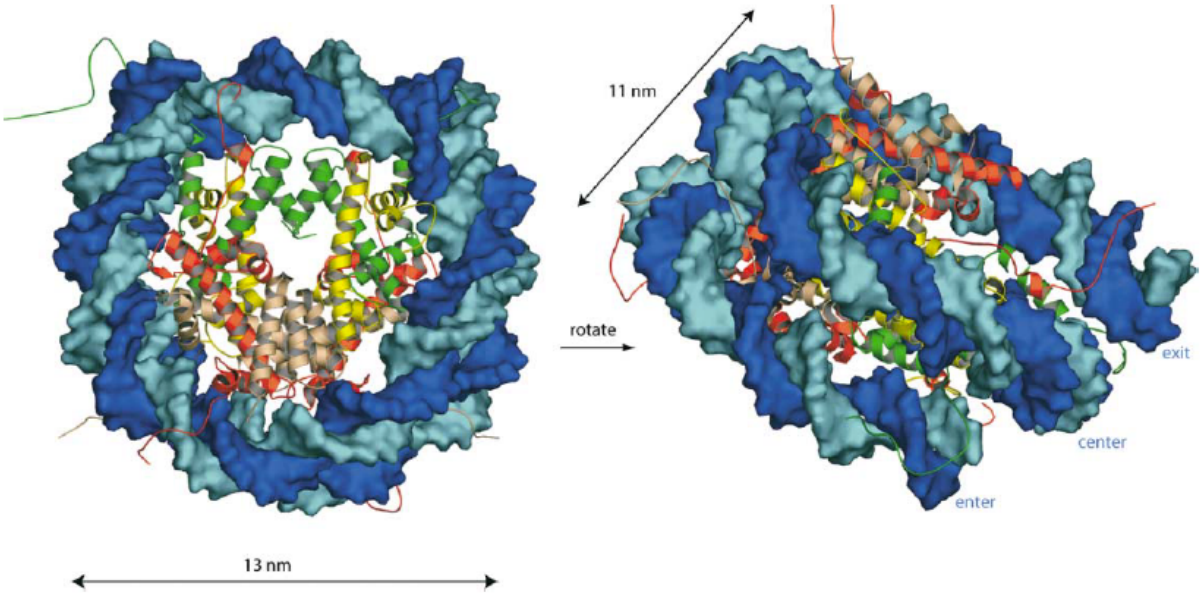

Figure 1. Structure of the nucleosome core particle (Khorasanizadeh, 2004)
147 bp of DNA (colored in two shades of blue) are wrapped around the histone octamer composed of two copies each of histone H2A (red), H2B (pink), H3 (green) and H4 (yellow) to form the nucleosome core particle with its disc-like shape.

5.1.2 From nucleosome core particle to higher order structures of chromatin

DNA packaging into chromatin can be dissected into a structural hierarchy with several levels of organization (see Figure 2) (Adkins et al., 2004; Horn and Peterson, 2002; Woodcock and Dimitrov, 2001). Neighboring nucleosome core particles are connected by short segments of linker DNA (15-80 bp in length) to form a nucleosome repeat ('array'), which can be considered as the first (primary) level of chromatin structure. This 'beads on a string' like conformation (Olins and Olins, 1974) folds at physiological ionic concentrations into more compact forms, e.g. the 30 nm chromatin fiber. Subsequently, these folded nucleosome arrays (the 30 nm fiber) define the second level of compaction.

There are two architectural concepts of how nucleosomes are arranged within this fiber. Whilst early studies suggested an arrangement of the adjacent nucleosomes into a regular 'solenoid model' (Finch and Klug, 1976), other results led to the proposal of a 'zig zag' packaging model (Bednar et al., 1998). According to this 'zig zag' formation, consecutive nucleosomes are alternating packaged into a more compact 30nm chromatin fiber (Woodcock and Dimitrov, 2001).

Compaction into the higher order structures of chromatin seems to be highly dynamic. It is thought to be facilitated and stabilized by multiple chromatin-associated proteins, e.g. linker histones (Adkins et al., 2004). Linker histones, like histone H1 or H5, associate between two nucleosomes and stabilize both intramolecular folding and fiber-fiber interactions (Carruthers et al., 1998). In addition, internucleosomal and intramolecular interactions between the histone tails and other factors are important for stable folding of the nucleosomal arrays and for fiber-fiber interactions (Hansen, 2002; Zheng and Hayes, 2003). Less is known about how these fibers are further compacted (the tertiary structures), forming a hierarchy of folding levels. There is considerable evidence to suggest that chromatin fibers are organized into large domains potentially through interaction with a 'nuclear matrix' or 'scaffold' (Fisher and Merkenschlager,

2002; Hancock, 2000). Furthermore, these condensed sections appear to be even more organized and are finally folded in an ordered manner to form the visible chromosome during mitosis. An overview of the different packaging levels gives Figure 2.

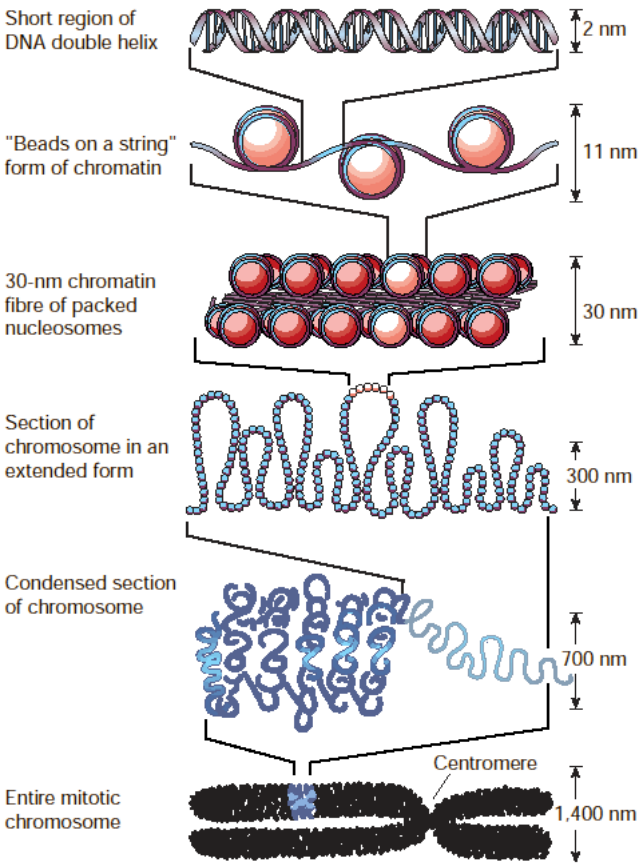

Figure 2. From the nucleosome to the higher order structure of chromatin (Felsenfeld and Groudine, 2003)
Model of the organization of DNA within chromatin. Double helix DNA is compacted into multiple structural levels of chromatin to form mitotic chromosomes.

5.1.3 'Chromatin territories' (eu- and heterochromatin)

Except during cell division, when chromatin can been seen as individual compacted chromosomes, chromatin is dispersed throughout the nucleus. However, the distributed chromatin still appears to be organized in more and less condensed regions (Pederson, 2004). The spatial arrangement of chromatin into distinct compartments within the cell nucleus is suggested to influence the functional activities within the nucleus, e.g. gene activity (Baxter et al., 2002; Cremer and Cremer, 2001). Transcriptional activation might occur if a locus is sequestered to an open, accessible chromatin domain. In contrast, transcriptional silencing might be facilitated if a locus is relocated to compact chromatin environment (Chubb and Bickmore, 2003).

The first and best-described chromatin 'territories', were initially cytological defined (Heitz, 1928): the less stained, decondensed euchromatin, and the more compact, intensively stained, heterochromatin. Heterochromatic domains are in general gene poor, less accessible to DNA binding factors,

transcriptionally inactive regions and are replicated late in the cell cycle (Fisher and Merkenschlager, 2002; Grewal and Elgin, 2002; Grewal and Moazed, 2003). In contrast, euchromatic domains are more loosely defined as the more accessible and transcriptional active regions. Most protein coding genes are located in the so-called euchromatin, which decondenses during interphase and is replicated early in S-phase.

Large parts of heterochromatin surround functional structures such as the centromeres (chromosomal organizer during mitosis) and the telomeres (at the end of chromosomes). Smaller heterochromatic parts are interspersed throughout the chromosome (Fahrner and Baylin, 2003). Heterochromatin is thought to stabilize the genome, via heterochromatin proteins associating with repetitive sequences, and plays important roles in the regulation of gene expression through development and differentiation (Grewal and Moazed, 2003). Examples for heterochromatin regions, in which genes are specifically silenced, are the inactive X chromosome in mammals, the mating type loci in yeast and genes affected by 'position effect variegation' in *Drosophila*. The last case describes the variegated expression of euchromatic genes, e.g. reporter genes, which are placed close to heterochromatic regions through chromosomal rearrangements. The basis for the stochastic on / off gene expression is unknown, but it is suggested that occasionally 'spreading' of the condensed structure of heterochromatin into the euchromatic regions changes their chromatin structure and results in gene inactivation (Reuter and Spierer, 1992; Schotta et al., 2003).

Many factors, histone modifications and special histone variants are associated with these heterochromatic domains (see following sections). In addition, inter-relationships between chromatin remodeling, histone modification, DNA methylation and the RNAi machinery seem to be important for the establishment and maintenance of the heterochromatic state of chromatin (Vermaak et al., 2003).

5.2 Posttranslational modifications of histones

Many amino acids of core histones, particularly those in the N-terminal regions (the histone 'tails') are chemically modified. Examples of the rich variety of posttranslational histone modifications are, e.g. acetylation, methylation or phosphorylation (Fischle et al., 2003; Vaquero et al., 2003). For a summary of known histone modifications, see Figure 3. Many histone modifications where already identified more than 30 years ago, e.g. histone acetylation (Allfrey et al., 1964; Phillips, 1963) or histone methylation (Byvoet et al., 1972; Murray, 1964). The impact of certain modifications on gene regulation and the discovery of factors mediating the histone modifications ('histone modifying proteins') has emerged through the last decade (Vaquero et al., 2003). Most of the covalent modifications are reversible (except for methylation). Most prominent and best studied are acetylation and methylation of lysine residues in the highly conserved amino termini of histone H3 and H4 (see following sections).

Histone modifications constitute a set of markers to the chromatin environment. They play important roles in the regulation of the chromatin structure and are implicated in the regulation of gene activity (Fischle et al., 2003; Iizuka and Smith, 2003; Khorasanizadeh, 2004). Many covalent modifications alter the electrostatic charge of the histones, most likely change the structural properties of the histones or alter the interactions of the histone tails. Some histone modifications serve as targets for protein recognition modules, like the bromodomain, which recognizes acetylated lysine (Dhalluin et al., 1999; Jacobson et al., 2000), and the chromodomain, which recognizes methylated lysine protein (Jacobs and Khorasanizadeh, 2002; Min et al., 2003).

This led to the proposal of a 'histone code' hypothesis, which suggests that a specific combinatorial sets of histone modification signals can dictate the recruitment of particular transacting factors to accomplish specific functions (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2002; Turner et al., 1992). An alternative idea, following the principles of the signal transduction pathways in cells, with its bistability, robustness and adaptability, led to the proposal of a signaling network model of histone modifications (Schreiber and Bernstein, 2002).

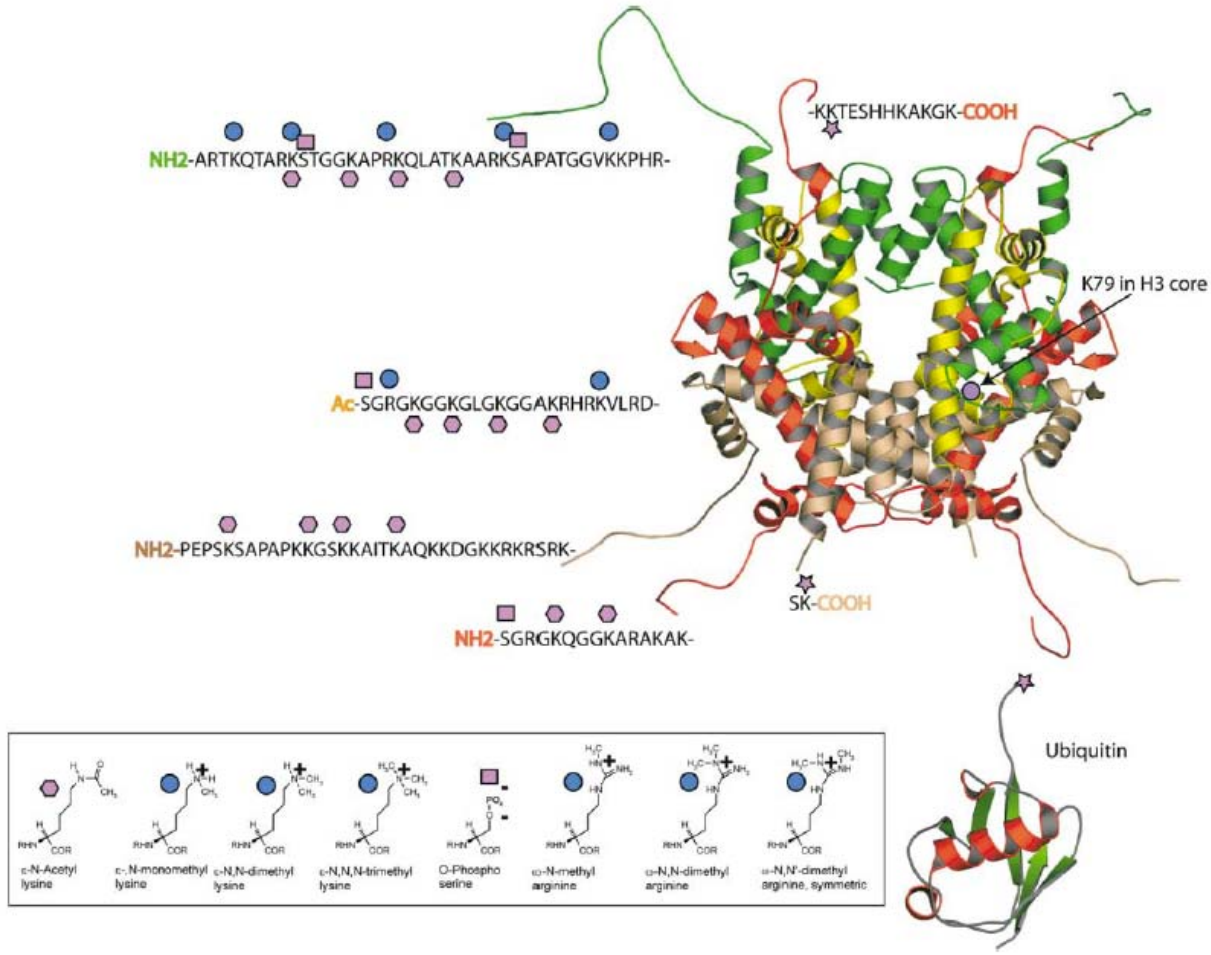

Figure 3. Posttranslational histone modifications (Khorasanizadeh, 2004)
The histone octamer of the nucleosome core particle is shown. The observed histone modifications on each histone are indicated. Color code as in Figure 1. The different covalent modifications of the amino acids are depicted in the box.

5.2.1 Histone acetylation

Histone acetylation is mediated by histone acetyltransferases (HAT), which catalyze the transfer of acetyl groups from acetyl-CoA to the ε-amino terminal groups of specific lysine residues (see box Figure 3). This reaction is reversed by specific factors, the histone deacetylases (HDAC), which remove acetyl groups from lysines. Alterations of the histone acetylation state appear to play an important role in chromatin assembly and gene regulation. Increased histone acetylation often correlates with

transcriptional activity, whereas decreased acetylation correlates with a transcriptionally repressed state (Fischle et al., 2003; Grunstein, 1997; Katan-Khaykovich and Struhl, 2002).

Allfrey and colleagues proposed already 40 years ago that acetylation of the core histone tails might regulate gene expression (Allfrey et al., 1964). Clear evidence came with the development of antibodies against specific acetylated histones (Turner et al., 1992) and the discovery of the factors mediating histone acetylation. Gcn5, the first identified histone acetyltransferase (Brownell et al., 1996), was known before to be a transcriptional regulator, and clearly linked histone acetylation to gene regulation. Indeed, Gcn5 is the catalytic subunit of the 'SAGA' transcriptional coactivator complexes (Grant et al., 1997). Later on, for several other transcriptional activators and coactivators histone acetyltransferase activity has been described e.g. p300/CBP, PCAF and TAF-I (Brown et al., 2000). A highly ordered, specific histone H3 and H4 acetylation pattern was deciphered for the activation of the interferon-beta gene (Agalioti et al., 2002). Histone acetylation plays an important role in other nuclear processes like chromatin assembly, DNA repair and apoptosis, VDJ recombination and dosage compensation in *Drosophila* ((Iizuka and Smith, 2003) and references therein). Histone acetyltransferases can also acetylate non-histone proteins (e.g. the tumor suppressor protein p53 or the transcription factor UBF) and thereby influence their functions (Gu and Roeder, 1997; Pelletier et al., 2000).

HATs are found in most if not all species, often in multiprotein complexes and display different histone tail specificities. They can be divided into several families based on their homology (see Table 1). Remarkably, acetylation can serve as a target for the binding of proteins, as the bromodomain binds to acetylated histone tails (Dhalluin et al., 1999; Jacobson et al., 2000).

HAT family	HAT enzyme	Organism known	Complex	Specificity	Function
GNAT	Gcn5	Yeast to humans	ADA2	H3, H2B	Coactivator
	PCAF	Human, mice	PCAF	H3, H4	Coactivator
	Hat1	Yeast	HatB	H4 [K5, K12], H2B	Histone deposition, silencing
	Elp3	Yeast to humans	Elongator	H3, H4	Transcriptional elongation
	Hpa2	Yeast		H3, H4	Unknown
	ATF-2	Humans, mice		H2B, H4	Sequence-specific transcription factor
MYST	Sas2	Yeast		H4 [K16]	Silencing
	Sas3	Yeast	NuA3	H3, H4, H2A	Silencing
	MORF	Humans		H4>H3	Unknown
	TIP60	Humans	TIP60	H4>>H3, H2A	HIV Tat interaction, DNA repair, Apoptosis
	Esa1	Humans	NuA4	H4, H2A	Cell cycle progression
	MOF	Drosophila	MSL	H4 [K16]	X- chromosome hyperactivation dosage compensation
	HBO1	Humans	HBO1	H3, H4	DNA replication
	MOZ	Humans	AML1	H3, H4> H2A	Transcription activation, others?
P300/CBP	p300	Multicelular organisms		H2A, H2B, H3, H4	Coactivator
	CBP	Multicelular organisms		H2A, H2B, H3, H4	Coactivator
Hormone Receptor Coactivators	ACTR	Humans, mice		H3>H4	Hormone Receptor coactivators
	SRC-1	Humans, mice		H3>H4	Hormone Receptor coactivators
	TIF2	Humans, mice			Hormone Receptor coactivators
TAFII250	TAFII250	Yeast to humans	TFIID	H3>H4,H2A	TBP-associated factor/ Cell cycle progression
TFIIIC	TFIII220	Humans	TFIIIC	H3, H4 >H2A	RNA polymerase III-mediated transcription
	TFIII110	Humans			
	TFIII90	Humans			
Nut1	Nut1	Humans	Mediator	H3>>H4	RNA polymerase II-mediated transcription

Table 1. Different classes of histone acetyltransferases (Vaquero et al., 2003)

Histone deacetylases, the enzymes that remove the acetyl groups, are generally suggested to play an important role in gene inactivation. Indeed, the first identified histone deacetylase (HDAC1), was shown to be a homolog of the yeast Rpd3p transcriptional regulator (Taunton et al., 1996). Many transcriptional repressors are associated with histone deacetylases, and their activity is necessary to repress certain genes (Peterson, 2002; Vaquero et al., 2003). These proteins are often part of large multiprotein complexes. Several groups of HDAC were defined, according to their expression pattern, homology and their sensitivity against specific inhibitors (see Table 2).

Histone deacetylation is also described for transcriptional activation (Kurdistani and Grunstein, 2003; Robyr et al., 2002; Wang et al., 2002). Recent results highlighted that both hyper- and hypoacetylation of individual lysines are associated with transcription, generating distinct patterns of acetylation that define groups of biologically related genes (Kurdistani et al., 2004).

HDAC group	HDAC members	TSA sensitivity	NAD^+ dependence	Localization	Function
Class I (Rpd3)	HDAC1, HDAC2, HDAC3, HDAC8	Yes	No	Nuclear and ubiquitous localization.	Involved in a variety of functions, such as transcriptional repression and cell differentiation.
Class II (Hda1)	HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10	Yes	No	Tissue-specific expression.	Transcriptional repression, microtubule regulation
Class III (yeast Sir2)	Sir2, HST1-4 Homologues in different organisms from yeast to humans.(Arabidopsis, C. elegans, D. Melanogaster, etc) e.g. Human Sirt1-7	No	Yes	Some are nuclear, others cytoplasmic and mitochondrial.	Involvement in silencing. Connection with aging. Function in development, gene repression and DNA repair.

Table 2. Different groups of histone deacetylases (Vaquero et al., 2003)

5.2.2 Histone methylation

Histone methylation occurs at different levels upon both lysine and arginine residues on several histones. This posttranslational modification is best described for histone H3 and H4 (Fischle et al., 2003; Vaquero et al., 2003). Histone methyltransferases (HMT) catalyze the transfer of up to three methyl groups from S-adenosyl-methionin to the ε-amino terminal group of a single lysine residues, thereby creating mono-, di- or trimethylated lysines (see box Figure 3). The protein arginine methyltransferases (PRMT) generates both mono- or dimethylate arginine residues, either symmetrically or asymmetrically by transferring methyl groups to the guanidine group (Kouzarides, 2002). Both, the particular site in an histone tail and the number of methyl groups in a given modification, play an important role in the functional consequences of histone methylation (Lachner and Jenuwein, 2002; Lachner et al., 2003).

Histone methylation has been described for gene activation and transcriptional repression (see Table 3). Examples for transcriptional activation are methylation of lysine 4, lysine 36 and lysine 79 of histone H3 (Beisel et al., 2002; Ng et al., 2003; Santos-Rosa et al., 2002). Histone methylation is also a characterized mark for repressed, silenced genes (e.g. di- and trimethylation of lysine 9 or 27 of histone H3 (Bannister et al., 2001; Cao et al., 2002; Czermin et al., 2002; Lachner et al., 2001). Indeed, a specific histone methylation pattern appears to be characteristic for different forms of heterochromatic regions

(Peters et al., 2003; Schotta et al., 2004). The di- and tri methylated form of lysine 9 of histone H3 is a 'docking station' for HP1, (heterochromatin protein 1), a small protein characteristic of inactive, heterochromatic regions (Bannister et al., 2001; Lachner et al., 2001). Similar, methylation of lysine 27 of histone H3 facilitates binding of Polycomb, a protein involved in maintaining the silencing state of homeotic genes during development. Both proteins bind to the methylated lysines through a specific recognition module, the chromodomains (Brehm et al., 2004). Recently, the crystal structure of the chromodomains together with methylated peptides has been resolved, showing precisely the required amino acid interactions (Jacobs and Khorasanizadeh, 2002; Min et al., 2003).

The first described histone methyltransferases is Suvar 3/9 (Rea et al., 2000), a SET domain containing protein, which is the catalytic domain. Subsequently, other histone methyltransferases were identified by both homology searches to the SET domain and via functional approaches (Vaquero et al., 2003). An overview of the histone methyltransferases, their specificity and functions is given in Table 3. DNA methylation and the RNAi machinery are linked to histone methylation and the process of gene inactivation (Grewal and Moazed, 2003). In contrast to histone acetylation, histone methylation appears be a stable biochemical modification, with a similar half-life as the intact histone (Bannister et al., 2002). In addition, no enzymatic activity that removes methyl groups from histones has been described yet.

HMT group	HMT	Specificity	Function
Arginine	PRMT1	H4-R3 (non-histone proteins)	Transcriptional activation (signal transduction, etc)
	PRMT2	Unknown	Coactivator of estrogen receptor
	PRMT3	Unknown	Cytoplasmic (mitosis?)
	PRMT4/ CARM1	H3-R2, -R17, -R26. (Also at the C-terminal)	Transcriptional coactivator
	PRMT5	H2A, H4 (non-histone proteins)	Transcriptional repressor and spliceosome formation
Lysine SET domain	Suv39H1, Suv39H2	H3-K9	Heterochromatin formation, silencing
	G9a	H3-K9, H3-K27	Early embryogenesis role, transcriptional repression
	ASH1	H3-K4, -K9, H4-K20	Establishment of epigenetic, active transcription patterns
	Set1	H3-K4	Silencing
	Set2	H3-K36	Silencing, transcription
	Set7	H4-K20	Development, silent chromatin. Involved in aging
	Set9	H3-K4	Transcriptional activation
	ESC-E(z)	H3-K27	Polycomb-mediated silencing
	SETDB1	H3-K9	Silencing-mediated by the corepressor KAP-1
Dot	Dot1	H3-K79	Silencing by precluding Sir binding to bulk chromatin

Table 3. Groups of histone methyltransferases (Vaquero et al., 2003)

5.2.3 Other covalent histone modifications

Histones are also subjected to other modifications such as phosphorylation of serine residues (Fischle et al., 2003). In histone H3 both serine 10 and serine 28 can be phosphorylated. This modification occurs during mitosis at metaphase and is important for mitotic chromosome condensation. Several kinases and phosphatases regulate the phosphorylation state of histones, such as Ipl1/aurora kinase and Glc7/PP1 phosphatase (Hsu et al., 2000). Histones can be ubiquitinated as well, a process wherein a 76 aa peptide is added to lysine residues. Histone ubiquitination provides an example for a crosstalk between histone modifications. Ubiquitination at lysine 123 of histone H2B is a prerequisite for methylation of histone H3 lysine 4 and 79 (Briggs et al., 2002; Sun and Allis, 2002). In addition, a variety of other histone modifications has been described, such as histone ADP-ribosylation, histone biotinylation, histone glycosylation or histone sumoylation (Vaquero et al., 2003).

5.3 Histone variants

In addition to histone modifications, nucleosomes can have core histones substituted by a histone variant, also called replacement histones. Histone variants differ in their amino acid composition, mainly in the N-terminus, and they confer specialized functions to the nucleosomes (Henikoff et al., 2004).

Several histone variants are enriched in specific chromosomal locations, e.g. centromeric chromatin contains the histone H3 variant CENP-A (centromer protein A) (Palmer et al., 1991). CENP-A is essential for centromer structure and function (Ahmad and Henikoff, 2001). MacroH2A, a histone H2A variant with a C-terminal extension of 25 kDa, is enriched in the nucleosomes of inactive X chromosomes (Costanzi and Pehrson, 1998). The presence of macroH2A in nucleosomes interferes with the DNA binding of the transcription factor NF-kappa B and impedes Swi/Snf nucleosome remodeling (Angelov et al., 2003). Other histone H2A variants such as H2A.X and H2A.Z form specialized chromatin structures that affect DNA repair, chromatin remodeling and gene silencing (Dhillon and Kamakaka, 2002; Santisteban et al., 2000). The histone H3 variant H3.3 accumulates on highly transcribed regions like active rDNA arrays and correlates with transcriptional activity (Ahmad and Henikoff, 2002). Histone variants are synthesized throughout the cell cycle and are deposited independent of DNA replication. In contrast, the bulk of histones is expressed during S-phase and they are depositioned during DNA replication.

5.4 Chromatin assembly

The deposition of the basic histone proteins onto the negatively charged DNA requires a precise process with dedicated machinery, as simple mixing of histones and DNA results in non-functional precipitates *in vitro*. Specialized factors exist that direct the exact and coordinated packaging of a piece of DNA into a nucleosome, a process called chromatin (nucleosome) assembly. These processes are mediated by histone chaperones, the 'histone-transfer vesicles', and energy-utilizing factors which facilitate the exact histone deposition, such as ACF (see section 5.5.2) (Haushalter and Kadonaga, 2003; Kadam and Emerson, 2002). The bulk of histones are expressed during S-phase and deposited during DNA replication, facilitated by histone chaperones like CAF-1 (chromatin assembly factor 1) or RCAF (replication-coupling assembly factor) (Vaquero et al., 2003). Histone variants are synthesized throughout the cell cycle and their replication-independent deposition is mediated by specialized complexes. For instance, Histone H3.3 has been found to be deposited by the HIRA complex (Tagami et al., 2004) and H2A.Z specifically by the SWR1 complex (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). Histone chaperones also mediate histone transport and are important for histone storage, examples are NAP1 and Nucleoplasmin (Akey and Luger, 2003; Wolffe, 1998).

Chromatin assembly is a staged process. First, the histone $(H3\text{-}H4)_2$ tetramer is deposited on the DNA, followed by addition of two H2A-H2B dimers ((Wolffe, 1998) and references therein). Remarkably, recent data suggests a stepwise replacement of the nucleosomal octamer by the deposition of H3/H4 dimers in the histone variants H3.3 nucleosome-assembly pathway (Korber and Hörz, 2004; Tagami et al., 2004).

5.5 ATP-dependent nucleosome remodeling

Chromatin needs to be fluid to enable DNA-dependent processes. A broad group of enzymes that uses the energy of ATP hydrolysis to alter histone-DNA interactions within the nucleosome has been discovered (Becker and Hörz, 2002). The process of catalyzing these chromatin dynamics is known as 'ATP-dependent nucleosome remodeling'. The biochemical reactions lead to the mobilization of the histone octamers and their exact positioning to DNA regulatory elements, providing or restricting regulatory factors access to their sites.

The molecular machines that rearrange the nucleosome structure are called ATP-dependent chromatin remodeling factors. All ATP-dependent chromatin remodeling factors identified so far are multiprotein complexes consisting of 2 to 12 subunits, and contain a related motor protein that belongs to the Snf2 family of ATPases (Eisen et al., 1995). Members of the chromatin remodeling enzymes are found in all eukaryotes where they participate in many DNA-mediated processes like transcriptional regulation, DNA repair, homologous recombination and chromatin assembly (Lusser and Kadonaga, 2003; Tsukiyama, 2002).

The Snf2 family belongs to the DEAD/H superfamily of nucleic acid stimulated ATPases (Eisen et al., 1995; Peterson, 2000), and can be further divided into multiple subfamilies (Figure 4A). The ATPases of chromatin remodeling complexes present different groups within the Snf2 protein family. They are classified according to protein domains outside of the ATPase region (Figure 4B). At least four major classes of catalytic subunis of chromatin remodeling complexes are distinguished: The Swi/Snf family, the Mi-2 / CHD family, the ISWI class and the Ino80 group (see also following sections). Several other Snf2-like proteins have been found or suggested to possess ATP-dependent chromatin-remodeling activity, such as Rad54, ATRX, CSB (Cockayne Syndrome protein B) or the plant protein DDM1 ((Becker and Hörz, 2002; Lusser and Kadonaga, 2003) and references therein).

Beside their ATPase subunit, the chromatin remodeling factors differ in their associated cofactors, which selectively influence their functions (see following sections). The variety of associated subunits helps to target the enzymes and integrate nucleosome remodeling into a physiological context (Lusser and Kadonaga, 2003; Tsukiyama, 2002).

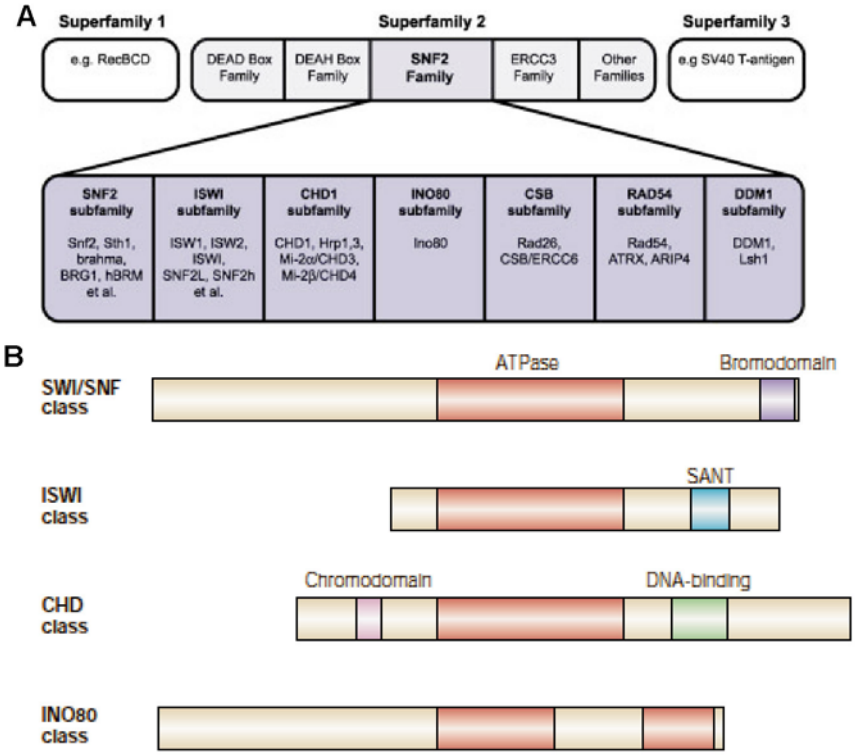

Figure 4. Snf2 family of ATPases
(**A**) The Snf2 family belongs to the DEAD/H superfamily of ATPases (Lusser and Kadonaga, 2003). (**B**) Domain structure of the four major classes of the Snf2-like ATPases, which are subunits of chromatin remodeling complexes (Tsukiyama, 2002).

5.5.1 The Swi/Snf family of remodeling machines

The yeast Swi/Snf complex is the founding member of this family of ATP-dependent chromatin-remodeling complexes. The Swi/Snf family is characterized by the Snf2 (Swi2/Snf2) type of ATPases, which contain a bromodomain in addition to the catalytic region (Figure 4B) (Horn and Peterson, 2001; Laurent et al., 1993; Tamkun et al., 1992). Members of the family have been shown to play important roles in the regulation of gene expression, at least in part by altering the chromatin structure. The Swi/Snf complexes are found from yeast to humans (see Figure 5 and (Martens and Winston, 2003; Sudarsanam and Winston, 2000)).

Several subunits of the yeast Swi/Snf complex were initially identified as gene products involved in the regulation of either the HO endonuclease gene or the SUC2 gene, which encodes for invertase (a sucrose hydrolyzing enzyme). HO is required for mating type switching, hence termed Swi, while SUC2 mutants are classified as sucrose nonfermenters, termed Snf (Sudarsanam and Winston, 2000; Winston and Carlson, 1992). Later on, Swi/Snf2 was shown to cause changes in chromatin structure *in vivo* and thereby allow transcriptional activation (Hirschhorn et al., 1992). Biochemical purification led to the characterization of a multiprotein complex of 11 known subunits, including the ATPase Snf2 (Cairns et al., 1994; Cote et al., 1994; Peterson et al., 1994). The purified yeast Swi/Snf complex was found to bind DNA and nucleosomes with high affinity and to alter the nucleosome structure in an ATP-dependent manner (Vignali et al., 2000) and references therein). For example, Swi/Snf catalyses the redistribution ('sliding') of nucleosomes along DNA in cis (Whitehouse et al., 1999). Beside local effects on the

specific genes described above, several reports present evidence for roles of the Swi/Snf complex in the transcriptional regulation of a wider subset of yeast genes (Martens and Winston, 2003; Sudarsanam and Winston, 2000). Numerous genes that are not normally dependent on the Swi/Snf complex become Swi/Snf dependent during mitosis (Krebs et al., 2000).

Many studies have established that members of the Swi/Snf family of chromatin remodeling complexes activate transcription, often in conjugation with histone acetyltransferase complexes. Swi/Snf complexes can be targeted to specific genes by interaction of individual subunits with certain transcription factors (Kadam et al., 2000). Recently, it was shown, that bromodomains within the catalytic subunits of Swi/Snf anchor these complexes to acetylated promoter nucleosomes (Hassan et al., 2002). Snf2 is also required for the direct repression of certain genes (Martens and Winston, 2002). Swi/Snf is not essential for cell viability, in contrast to the highly related RSC complex (Cairns et al., 1996).

The abundant RSC complex ('remodels structure of chromatin') contains several subunits that are homologuos of Swi/Snf subunits and the ATPase Sth1 (Snf2 (Snf two) homologous) (Martens and Winston, 2003). Again, RSC is an important transcriptional regulator that functions both in gene activation and repression (Martens and Winston, 2003). Two distinct forms of the RSC complex were identified with shared and unique functions (Cairns et al., 1999). Genome wide studies revealed that RSC is localized at many activated and repressed promoters, including RNA pol III promoters (Damelin et al., 2002; Ng et al., 2002).

Only one homolog of the ATPase Swi2/Snf2, Brahma (Brm), was discovered in *Drosophila* and shown to be part of a large multisubunit complex (Dingwall et al., 1995; Papoulas et al., 1998; Tamkun et al., 1992). The Brahma complex (BRM/BAP) contains at least eight major subunits (Figure 5) and functions as a transcriptional coactivator in the regulation of homeotic genes (Tamkun et al., 1992) (Elfring et al., 1998). Localization studies on polytene chromosomes demonstrated that the *Drosophila* Brahma complex is associated with almost all sites of active transcription. The expression of a BRM dominant-negative mutant impaired the association of RNA polymerase II with chromatin, suggesting a role for BRM in facilitating transcription (Armstrong et al., 2002). Recently, two distinct Brahma containing complexes have been isolated from *Drosophila*. These two complexes are distinguished by the presence of the subunits Polybromo and BAP170 in one, and the subunit OSA in the other, and display partially distinct distribution pattern on polytene chromosomes (Mohrmann et al., 2004).

Like yeast, mammalian cells have two Snf2-like ATPases, mammalian Brm (Brahma) and Brg1 (Brahma related gene product 1) (Tsukiyama, 2002). Multiple protein complexes have been purified from both mouse and human cells. They contain one of these proteins as the central ATPase together with several related and / or unique subunits (see Figure 5 and (Carlson and Laurent, 1994; Chiba et al., 1994; Martens and Winston, 2003; Muchardt and Yaniv, 1993; Sif et al., 2001; Wang et al., 1996a; Wang et al., 1996b) (Kwon et al., 1994)). Similar to yeast, functions for the mammalian Swi/Snf are best described for activation and repression of transcription (Martens and Winston, 2003; Sudarsanam and Winston, 2000) and references therein. With regard to their subunit composition, different *in vitro* and *in vivo* activities for mammalian Swi/Snf complexes have been described, e.g. human Brg1 and Brm complexes differ in their ability to remodel mononucleosomal core particles (Sif et al., 2001). Only Brg1 is essential in mouse

development and was found to be mutated in various tumor cells ((Tsukiyama, 2002) and references therein). It was shown that Brg1 associates with the retinoblastoma protein Rb and BRCA1, a protein linked to breast cancer (Bochar et al., 2000b; Dunaief et al., 1994). Mammalian Swi/Snf complexes are involved in cell differentiation, such as the process of MyoD mediated muscle differentiation (de La Serna et al., 2001) or in tissue specific transcriptional regulation (Armstrong et al., 1998).

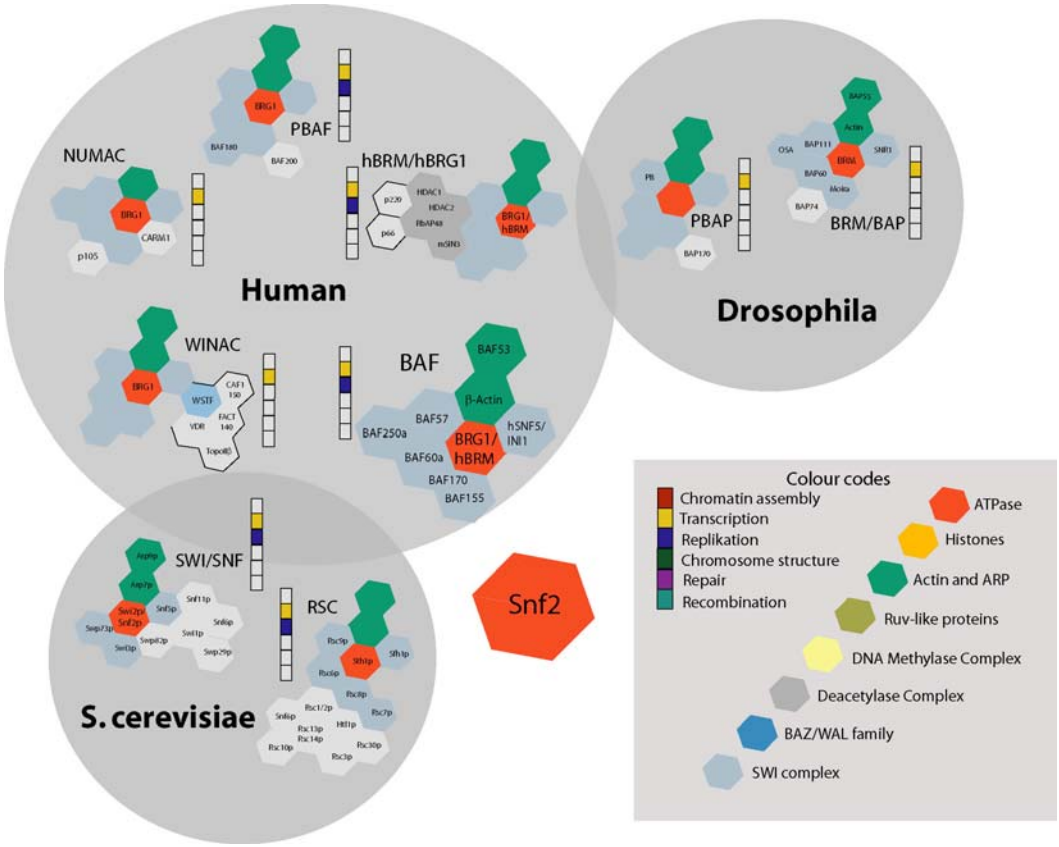

Figure 5. Swi/Snf family of chromatin remodeling complexes (Eberharter and Becker, 2004)
Different members of the Swi/Snf group of chromatin remodeling factors in yeast, *Drosophila* and mouse / human are illustrated. The color code is indicated in the box.

5.5.2 The ISWI family of nucleosome remodeling complexes

The ATPase ISWI was initially described by sequence similarity to the Swi2/Snf2 homolog Brahma in *Drosophila* and therefore called Imitation Switch (Elfring et al., 1994), and subsequently identified in most species. Characteristics of this ATPase family are two C-terminal SANT domains (Figure 4), which are suggested to interact with nucleosomes and DNA (Aasland et al., 1996; Boyer et al., 2002; Grüne et al., 2003).

Three different ISWI-containing nucleosome remodeling complexes were first discovered using *in vitro* assays in screening for nucleosome remodeling activities in *Drosophila* embryo extracts: NURF (NUcleosome Remodeling Factor), ACF (ATP-utilizing Chromatin assembly and remodeling Factor) and CHRAC (CHRomatin Accessibility Complex) (Ito et al., 1997; Tsukiyama and Wu, 1995; Varga-Weisz et al., 1997). Homologuos were identified in various species, including yeast and human. ISWI-

containing complexes are generally smaller and have less subunits (2 to 5) than the other chromatin remodeling counterparts (see Figure 6 and (Corona and Tamkun, 2004; Längst and Becker, 2001b)). Beside the ATPase, most of the complexes contain one subunit of the WAL / BAZ protein family ('WSTF, Acf1. Like' and 'Bromodomain Adjacent Zinc finger', respectively) (Jones et al., 2000; Poot et al., 2000). Members of this WAL / BAZ family are characterized by a distinct modular organization, e.g. they contain the WAKZ motif, followed by one or two PHD fingers and a bromodomain (for details about the proteins domains see section 5.7). Functions for these remodeling factors were described in a wide variety of nuclear processes, such as transcriptional regulation, chromosome organization and DNA replication (Corona and Tamkun, 2004; Tsukiyama, 2002).

NURF was identified in biochemical studies as an ATP-dependent remodeling factor, which acts in concert with the GAGA transcription factor to alter chromatin structure. These investigations led to the purification of the nucleosome remodeling complex NURF, which is composed of four subunits (Tsukiyama et al., 1995; Tsukiyama and Wu, 1995). Beside ISWI, NURF contains a large 301 kDa protein NURF301, a 38 kDa pyrophosphatase and the WD repeat protein NURF55, a protein identical to the 55 kDa subunit of *Drosophila* chromatin assembly factor dCAF-1 (Martinez-Balbas et al., 1998). NURF301 was shown to possess dual functions within the complex (Xiao et al., 2001). It communicates with all the other subunits, thereby acting as an organizing scaffold and is necessary for efficient nucleosome sliding. NURF301 was shown to interact with sequence-specific transcription factors, providing a basis for targeted recruitment of the NURF complex to specific genes. The transcription factor GAL 4 modulates the direction of nucleosome sliding by NURF *in vitro* (Kang et al., 2002). NURF is able to facilitate transcription from repressed chromatin templates *in vitro* and is required for transcription activation of several genes *in vivo* (Badenhorst et al., 2002; Mizuguchi et al., 1997). Genetic studies in *Drosophila* demonstrate that ISWI is essential for development and cell viability in flies, but does not colocalize with RNA Pol II on polytene chromosomes (Deuring et al., 2000).

ACF was identified by the search for an activity required for the assembly of regularly spaced nucleosomal arrays *in vitro* (Ito et al., 1997). Beside ISWI as the catalytic subunit, the 170 / 185 kDa protein Acf1 was described as a component of ACF (Ito et al., 1999). Acf1 and ISWI participate synergistically in the ATP-dependent deposition of histones into periodic nucleosome arrays (Ito et al., 1999). Subsequently, other studies examined the role of ACF in nucleosome assembly in more detail, and showed that assembly is a processive process (Fyodorov and Kadonaga, 2002b; Nakagawa et al., 2001). Recent results imply, that *Drosophila* Acf1 is important in the formation of chromatin *in vivo* (Fyodorov et al., 2004). ACF is also able to mediate interaction of DNA binding factors with nucleosomal DNA and to facilitate transcription from chromatin templates *in vitro* (Ito et al., 1997).

The chromatin-accessibility complex CHRAC is highly related to ACF. *Drosophila* CHRAC was identified following an enzymatic activity that increased the accessibility to DNA in chromatin, e.g. increased restriction endonuclease cleavage (Varga-Weisz et al., 1997). Similar to ACF, CHRAC contains the ATPase ISWI and the large subunit Acf1 (Eberharter et al., 2001). Interaction of Acf1 with ISWI enhances the efficiency of nucleosome sliding and modulates the nucleosome remodeling activity qualitatively. In addition to Acf1 and ISWI, two small developmental regulated histone-fold proteins (CHRAC-14 and CHRAC-16) are present in the CHRAC complex (Corona et al., 2000). Recently it was shown that this two small subunits enhances nucleosome sliding and assembly (Kukimoto et al., 2004).

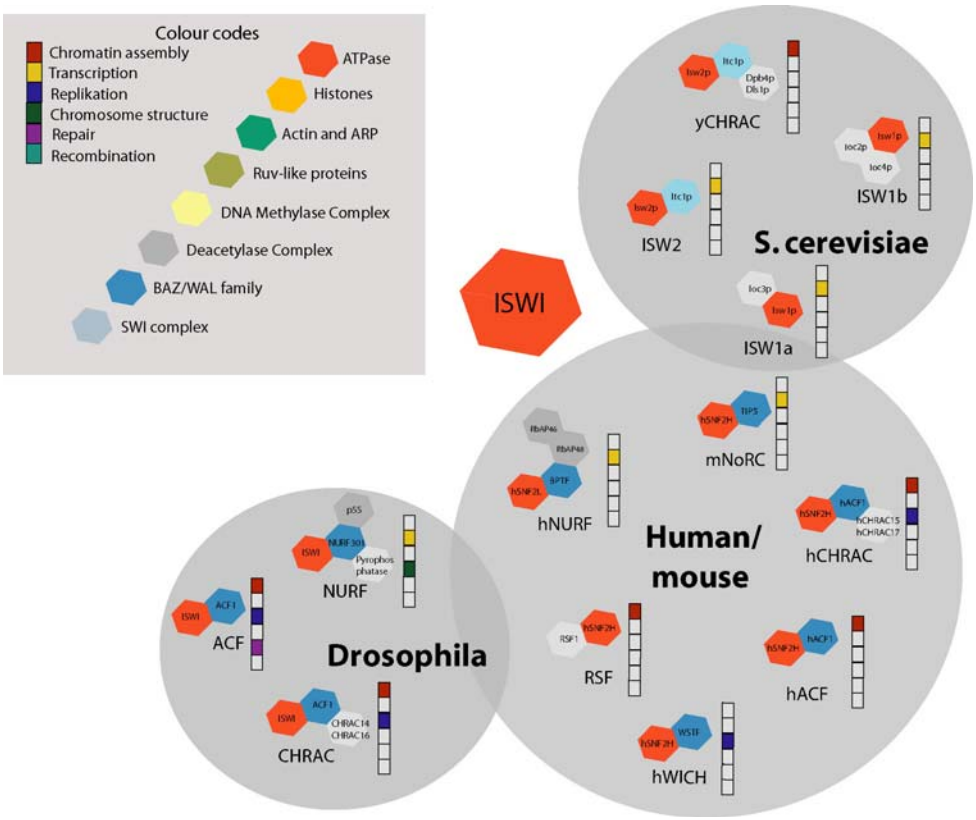

Figure 6. The ISWI family of chromatin remodeling complexes (Eberharter and Becker, 2004)
The different members of the ISWI group of chromatin remodeling factors in yeast, *Drosophila* and mouse / human are illustrated. The color code is indicated in the box.

Two homologous of *Drosophila* ISWI, Snf2l and Snf2h, are described in mammals (Aihara et al., 1998). Snf2h is mostly present in proliferating cells, whereas Snf2l is predominantly expressed in terminal differentiated cells, such as neurons. Beside the differential expression pattern during development, Snf2h is required for early mouse development (Lazzaro and Picketts, 2001; Stopka and Skoultchi, 2003). Snf2h was found to be the catalytic subunit of human CHRAC and human ACF, also known as WCRF (Bochar et al., 2000a; LeRoy et al., 2000; Poot et al., 2000). A function for an human Acf1 / Snf2h complex in replication through highly condensed regions of chromatin, e.g. heterochromatin has been described (Collins et al., 2002). Snf2l was shown to be the ATPase of human NURF (Barak et al., 2003). Herein it was suggested that human NURF plays a role in neuronal development. Additional Snf2h-containing remodeling machines with large subunits belonging to the BAZ / WAL protein family were identified in mammals (Figure 6). The WICH complex (WSTF-ISWI chromatin remodeling complex) is composed of the Williams Syndrome Transcription Factor (WSTF) and Snf2h and has a proposed role in the replication of heterochromatin (Bozhenok et al., 2002). WSTF was also found in the 'WINAC' chromatin remodeling complex (Kitagawa et al., 2003). The nucleolar remodeling complex NoRC, composed of Tip5 and Snf2h, was purified during this thesis (Strohner et al., 2001). NoRC is able to specifically repress rDNA transcription *in vitro* and *in vivo* and plays a role in rDNA gene silencing in mammalian cells (Németh et al., 2004; Santoro et al., 2002; Strohner et al., 2004; Zhou et al., 2002). RSF (remodeling and spacing factor) was purified from human cells based on its ability to facilitate

transcription from chromatin templates (LeRoy et al., 1998). Besides Snf2h, RSF contains the highly acidic protein Rsf-1, which displays histone chaperone properties (Loyola et al., 2003). Also in Xenopus several ISWI containing complexes have been described (Bozhenok et al., 2002; Guschin et al., 2000).

While all of these described ISWI containing complexes are quite similar with regard to their subunit composition, a few less related complexes have been described as well. A Snf2h / cohesin complex has been purified and is involved in chromosome segregation by direct interaction of Snf2h with the cohesin complex (Hakimi et al., 2002). Acf1 and Snf2h were also found to be recruited by Satb1, a cell-type specific nuclear protein that forms cage-like 'networks' that are suggested to regulate genes by folding chromatin into loop domains (Yasui et al., 2002).

Two ISWI-related proteins, ISW1p and ISW2p, were identified in yeast (Tsukiyama et al., 1999) and subsequently shown to be components of several chromatin remodeling complexes (see Figure 6). Two separate ISW1 complexes, ISW1a and ISW1b, and two ISW2 complexes have been described (Iida and Araki, 2004; McConnell et al., 2004; Vary et al., 2003). The subunit composition of the two described ISW2 complexes suggests that they represent the yeast orthologs of the ACF / CHRAC chromatin remodeling complexes (Figure 6). In addition to the ATPase, they share the common subunit Itc1 (ISWI two complex subunit 1). Itc1 contains a WAC domain which is also present in Acf-1 (Gelbart et al., 2001). The yeast CHRAC complex contains two additional subunits, the histone fold proteins Dpb3-like (Dls1) and Dpb4 (Iida and Araki, 2004; McConnell et al., 2004). Dls1p is required for Isw2-dependent chromatin remodeling *in vivo* on certain but not all targets (McConnell et al., 2004).

The individual ISWI proteins are not essential in yeast, but triple null mutations of the ISW1, ISW2, and CHD1 genes cause synthetic lethality (Tsukiyama et al., 1999). Further investigations with specific deletion strains led to the discovery of a variety of functions for the yeast ISWI-complexes, often displayed by direct changes of the chromatin structure at certain genes (Fazzio et al., 2001; Goldmark et al., 2000; Kent et al., 2001; Trachtulcova et al., 2004).

Roles for yeast ISWI-containing complexes have been described in many aspects of gene expression regulation. They influence directly gene activation, gene repression, gene silencing and are involved in elongation and transcription termination (Mellor and Morillon, 2004).

5.5.2.1 Biochemical properties of ISWI-containing remodeling complexes

Many *in vitro* studies on recombinant ISWI and ISWI-containing complexes have provided insights into the biochemical properties of these remodeling factors and into the mechanisms by which ATP-dependent alterations of chromatin occur (Becker and Hörz, 2002; Längst and Becker, 2001b; Lusser and Kadonaga, 2003) (for mechanism see section 5.5.5).

ATP-dependent nucleosome remodeling was studied in *in vitro* assays that monitor the relocation of a single histone octamer on a short DNA fragments. These experiments demonstrated that ISWI-containing complexes can induce ATP-dependent movement ('sliding') of histone octamers along a DNA segment in cis, without displacing them from DNA (Hamiche et al., 1999; Längst et al., 1999). The analysis of recombinant *Drosophila* ISWI showed that the enzyme alone can trigger nucleosome sliding, but the directionality of the histone octamer movement differs significantly. These differences appear to be determined by the unique properties of the large subunits. Indeed, the large subunits of NURF and ACF / CHRAC, stimulate and modulate qualitatively the remodeling activity within the complexes (Eberharter et al., 2001; Xiao et al., 2001). For instance, Acf1 the large subunit of ACF / CHRAC stimulates the activity of ISWI by an order of magnitude and reverses the directionality of the nucleosome sliding reaction. Also members of the other groups of the Snf2-like remodeling complexes catalyses the redistribution of nucleosomes along DNA, such as yeast and human Swi/Snf or *Xenopus* Mi-2 complexes (Aoyagi et al., 2003; Whitehouse et al., 1999).

The individual groups of remodeling factors display different substrate requirements in several biochemical assays. For instance, the Swi2/Snf2 ATPase activity is similarly induced by either DNA or nucleosomes and remodeling does not require histone N-termini ((Vignali et al., 2000) and references therein). The Mi-2 ATPase is only strongly activated with nucleosomal substrate and the histone tails are dispensable in remodeling reactions (Brehm et al., 2000). The ATPase activity of ISWI is weak activated by DNA and maximally stimulated in the presence of nucleosomes and requires nucleosomes with intact histone H4 N-termini for activity (Clapier et al., 2001; Corona et al., 1999).

The fact, that recombinant ISWI possesses nucleosome-remodeling activity on its own, indicates that this ATPase is capable to interact directly with nucleosomal substrates. ISWI binds poorly to nucleosome core particles, but interacts with nucleosomes containing protruding DNA (Brehm et al., 2000). High resolution analysis of nucleosome binding of ISWI, yISW2 and NURF, revealed specific interactions with nucleosomal DNA (Kassabov et al., 2002; Längst and Becker, 2001a; Schwanbeck et al., 2004). Recently, a substrate recognition domain within the C-terminal half of ISWI, composed of the SANT and SLIDE modules was identified and its structure solved by X-ray crystallography (Grüne et al., 2003).

ISWI-containing complexes can stimulate the assembly of nucleosomal arrays. Several members of the ISWI family can function as nucleosome spacing factors and induce regulatory into disturbed nuclesome arrays *in vitro* ((Haushalter and Kadonaga, 2003) and references therein). ACF was identified by a search for a factor required for the assembly of regular spaced nucleosomal arrays (Ito et al., 1997). Acf1 and ISWI function also synergistically in the assembly of chromatin (Ito et al., 1999).

5.5.3 The CHD / Mi-2 class of chromatin remodeling factors

Nucleosome remodeling complexes of the CHD / Mi-2 class are characterized by the presence of chromodomains ('chromatin organization modifier') in their ATPase subunit (Figure 4B) (Paro and Hogness, 1991). Some chromodomains can bind to methylated histone tails (see above 5.2.2), but functional analyses of different chromodomains revealed a variety of possible interaction partner targets, including histones, DNA and RNA (Brehm et al., 2004). The *Drosophila* Mi-2 chromodomains were shown to be DNA binding modules important for ATPase function and nucleosome mobilization (Bouazoune et al., 2002). While several members of the CHD family have been identified (Woodage et al., 1997), few have been studied in detail (Tsukiyama, 2002). The ATPase Chd1 is identified in several organisms, contains DNA binding activity and has a putative role in transcriptional activation (Kelley et al., 1999; Tran et al., 2000). *Drosophila* Chd1 localizes on polytene chromosomes at sites with high transcriptional activity (Stokes et al., 1996).

Mi-2 (Chd3/4) was identified as the catalytic ATPase subunit of several 'NUcleosome Remodeling and histone Deacetylase' complexes in various species (Figure 7), collectively called NuRD complexes (Tong et al., 1998; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998). In addition to the nucleosome remodeling and histone deacetylase activities, these complexes contain further subunits, such as the (putative) methylated DNA binding proteins MeCP2 and MBD3. Furthermore, they contain the Retinoblastoma associated proteins RbAp48/p46, found in several chromatin related complexes, MTA proteins (metastasis-associated) and other less characterized proteins (Bowen et al., 2004). Together with the methylated DNA binding protein MBD2, the NuRD complex can form the MeCP1 complex, which represses transcription (Feng and Zhang, 2001). Most if not all Mi-2 complexes are thought to be involved in transcriptional repression by connecting DNA methylation, histone deacetylation and ATP–dependent chromatin remodeling ((Becker and Hörz, 2002; Tsukiyama, 2002) and references therein). In agreement to this, it was shown that transcriptional repressors like Ikarus, hunchback or Tramtrack interact with Mi-2 (Kehle et al., 1998; Kim et al., 1999; Koipally et al., 1999; Murawsky et al., 2001).

5.5.4 The Ino80 group

Members of this group of chromatin remodeling factors, contain a conserved ATPase with a unique catalytic domain that is split into two subdomains (see Figure 4). *INO80* was originally found as a gene that is required for transcriptional activation of the *INO1* gene, a gene that is induced in the absence of inositol. Further studies demonstrated by gel filtration that the Ino80 protein is part of a high molecular-weight complex and showed that the Ino80p-containing complex influences the transcriptional level of several genes (Ebbert et al., 1999). Finally, an Ino80 containing complex (Ino80.com) was purified from *Saccharomyces cerevisiae* with about 12 polypeptides (see Figure 7) (Shen et al., 2000). This includes the Ino80 ATPase, two proteins related to the bacterial RuvB DNA helicase, actin and three actin-related proteins. The purified complex displayed ATP-dependent ability to alter chromatin and possessed helicase activity, consistent with the presence of the Rvb1 and Rvb2 subunits. Furthermore, the complex facilitated transcription *in vitro*. Mutants of the Ino80 ATPase showed defects in transcription and increased sensitivity to DNA-damaging agents (Shen et al., 2000). These results indicate that the Ino80 containing chromatin remodeling complex plays a role in transcription as well as in DNA damage repair.

Recently a second member of this class, the Swr1 complex, has been identified by several groups (Kobor et al., 2004; Krogan et al., 2004; Mizuguchi et al., 2004). This novel multiprotein complex contains the ATPase Swr1 (Swi2/Snf2- related 1), the specific histone variant H2A.Z and Bdf1, a two bromodomains containing protein. Notably, the Swr1 complex is able to replace histone H2A with histone variant H2AZ in nucleosome arrays and is required for the deposition of histone H2A.Z at specific chromosome locations *in vivo*. These results suggest novel roles for ATP-dependent chromatin remodeling factors.

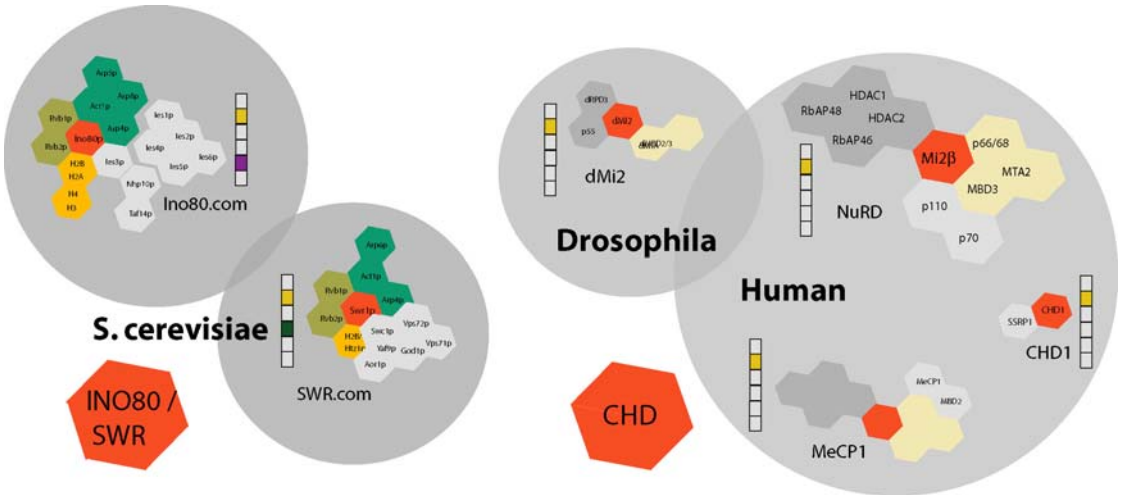

Figure 7. The CHD / Mi-2 and Ino80 chromatin remodeling complexes (Eberharter and Becker, 2004)
The different members of the CHD / Mi-2 and Ino80 group of chromatin remodeling factors in yeast, *Drosophila* and mouse / human are illustrated. Color code as in Figure 6.

5.5.5 Possible mechanism of nucleosome remodeling

All ATP-dependent nucleosome remodeling machines characterized so far perform some type of chromatin remodeling reactions, e.g. they increase the accessibility to nucleosomal DNA (Becker and Hörz, 2002; Längst and Becker, 2001b). Several biochemical studies investigated the mechanism of nucleosome remodeling mainly with Swi/Snf complexes and ISWI-containing complexes *in vitro*. These studies have provided insights into the mechanisms by which ATP-dependent chromatin alterations occur (Becker and Hörz, 2002; Flaus and Owen-Hughes, 2003; Längst and Becker, 2001b).

These analysis revealed many differences between the individual groups of nucleosome remodeling complexes (Längst and Becker, 2004; Lusser and Kadonaga, 2003), e.g. only the Swi/Snf complexes disrupt histone-DNA contacts in mononucleosomes, they alter the 'path' of the DNA ((Fan et al., 2003; Narlikar et al., 2001) and references therein). In contrast, ISWI complexes do not disrupt the DNA 'path' as they move the nucleosome (Aalfs et al., 2001; Längst et al., 1999). Furthermore, only the Swi/Snf complexes mediate the transfer of histones from one template to another, or form 'dinucleosome'-like structures from mononucleosomes (Lorch et al., 1999; Phelan et al., 2000; Whitehouse et al., 1999). These findings were initially interpreted as fundamental different mechanisms of nucleosome remodeling by these complexes. Recently the diverse phenomena of nucleosome remodeling were summarized and explained as variations of one basic remodeling reaction (Längst and Becker, 2004). The authors suggest,

that all ATP-dependent nucleosome remodeling reactions are mediated by a common mechanism: translocation of DNA segments by the 'loop recapture model' (Figure 8). According to this model, nucleosome remodeling factors detach a DNA segment from the nucleosomal surface, possible as a local loop or bulge, at sites where the DNA enters its path around the particle, and push it against the histone octamer. Directional propagation of the loop around the histone octamer will finally change the translational position of the nucleosome according to the loop size.

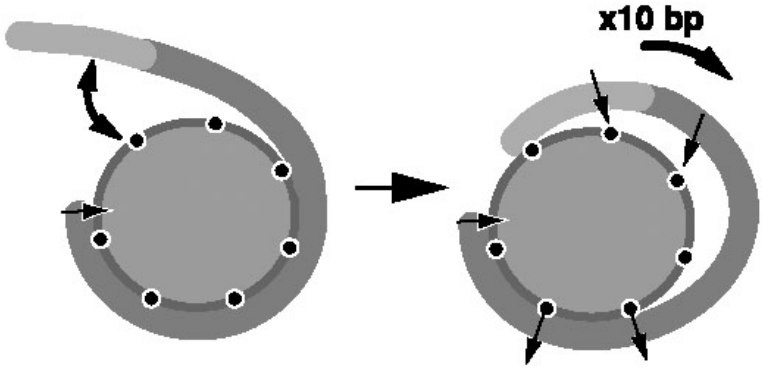

Figure 8. The 'loop recapture model' for nucleosome remodeling (Längst and Becker, 2004)
Nucleosome mobilization by the 'loop recapture model'. A schematic histone octamer with DNA wrapped around is shown. Left side: detachment of a DNA segment (light gray); right side: propagation of the DNA 'loop' over the nucleosome surface (see text).

5.6 Ribosomal RNA gene regulation

Transcription of the ribosomal RNA genes, encoding the enzymatic scaffold of the ribosome, dominates cellular transcription. Proliferating mammalian cells expend between 35 and 60% of all nuclear transcription to transcribe 18S, 28S and 5.8 S rRNA by RNA polymerase I (Pol I). In non-growing cells, however, the transcription of rRNA genes is greatly reduced.

A highly coordinated network exists that regulates rRNA synthesis, and hence ribosome production, in response to internal and external signals (Grummt, 1999; Grummt, 2003; Moss, 2004; Paule, 1998; Paule and White, 2000). Cells contain high amounts of rDNA genes to accomplish the demand for rRNA. In vertebrates, several hundreds of ribosomal RNA genes are organized in discrete clusters containing tandem arrays of rDNA. Even in actively growing cells only a subset of the genes is actively transcribed, the rest remains silenced, in a 'closed' chromatin conformation (Grummt and Pikaard, 2003; Moss, 2004). Regulation of mammalian ribosomal RNA synthesis seems not to be mediated through changes in the active gene numbers but rather at levels of transcription initiation, elongation and / or processing (see following sections). However, even if the ratio of active to silenced genes is stable throughout the cell cycle, the numbers of active transcription units can vary during the steps of cell differentiation (Haaf et al., 1991).

5.6.1 The nucleolus, the site of rRNA gene transcription and ribosome biogenesis

The nucleolus is a functional nuclear compartment visible as a morphologically distinct structure by light microscopy (Figure 9A). At this site, the precursor rRNA is synthesized, processed and then assembled into ribosome subunits (Leary and Huang, 2001). Nucleoli undergo dynamic changes within every cell cycle. They disassemble prior to mitosis and reassemble in several steps when the cell exits mitosis. The process of nucleolar assembly starts already before or at the onset of transcription (Dousset et al., 2000). Nuceloli form around clusters of rRNA gene repeats at the nucleolar organizer regions (NORs) on one or more chromosomes. Within the nucleolus, three morphological distinct subcompartments have been described by electron microscopy (Figure 9B): The fibrillar centers (FC), the dense fibrillar components (DFC) and the granular components (GC) (Paule, 1998; Scheer and Hock, 1999).

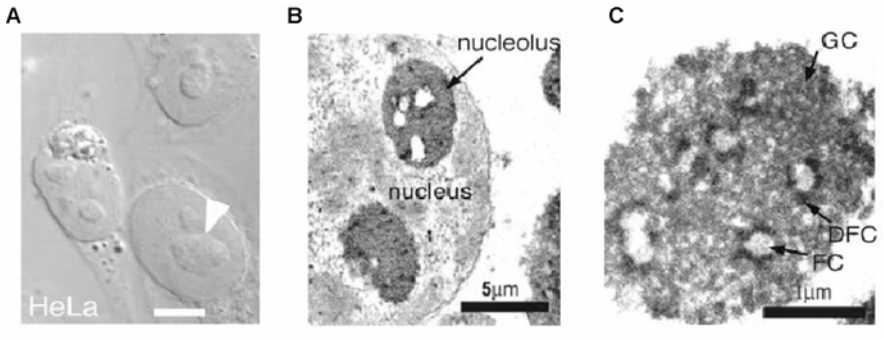

Figure 9. Ribosomal RNA genes are located in a distinct compartment: the nucleolus
(**A**) Phase contrast microscopy picture from HeLa cells. The nucleolus is visible as a distinct nuclear compartment (marked with an arrow). (**B**) and (**C**) Electron microscopic pictures of nucleoli in two magnifications. Three morphological structures can be distinguished, the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC).Figure modified (Andersen et al., 2002).

The current view is that rDNA transcription take places at the periphery of the FC, while transient accumulation, modification and processing of primary rRNA transcripts occurs in the DFC. Later processing and rRNA assembly into ribosomal subunits occurs in the GC ((Andersen et al., 2002; Lazdins et al., 1997; Mosgoeller et al., 2001) and references therein). Studies revealed that the nucleolus has additional functions, such as the synthesis of other ribonucleoprotein particles, e.g. the signal recognition particle, pre-tRNA processing and it has a prominent role in cell senescence (Comai, 1999; Olson et al., 2000; Pederson, 1998).

Two different proteomic studies analyzed the protein content of human nucleoli in detail. Nearly 400 proteins were identified in this nucleolar proteomic approach, revealing a broad range of novel or uncharacterized proteins, and factors with previously unknown nucleolar functions (Andersen et al., 2002; Scherl et al., 2002). The protein composition of this nuclear compartment has a surprisingly large complexity and is highly dynamic as it can alter in response to the metabolic state of the cell (Leung and Lamond, 2003).

5.6.2 The organization of mammalian ribosomal RNA genes

In mammals, several hundreds copies of ribosomal RNA genes are organized in repeated clusters, arranged head-to-tail. These large tandem arrays, termed nucleolus organizer regions (NORs), are distributed among the short arms of acrocentric chromosomes (Grummt, 1999; Paule, 1998; Paule and White, 2000). A mammalian rDNA transcription unit consits of approximately 43 kb, encoding the 45S precursor ribosomal RNA (pre-rRNA). The pre-rRNA is rapidly processed into the 18S, 5.8S, and 28S rRNAs. Each rRNA gene is separated from the neighboring unit by a non transcribed intergenic spacer (IGS) (Gonzalez and Sylvester, 1995; Grozdanov et al., 2003). The IGS contains important regulating elements for transcriptional regulation, replication or recombination. Examples are the rDNA promoter, enhancer elements, a spacer promoter, an origin of replication and transcription terminator elements (see Figure 10).

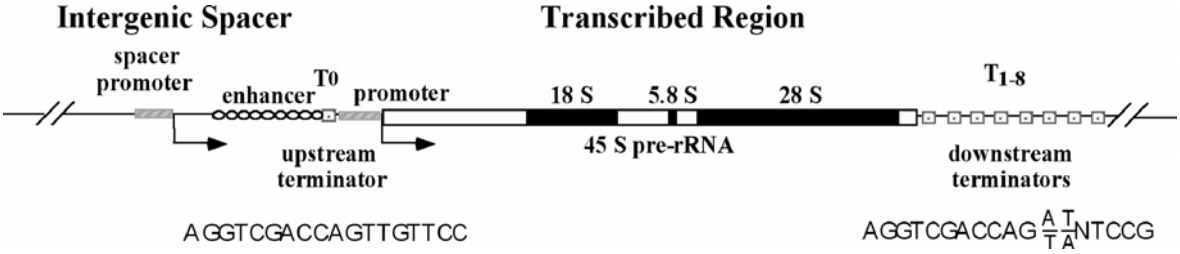

Figure 10. Organization of a mammalian ribosomal RNA gene repeat
Schematic representation of a murine rDNA transcription unit composed of the transcribed region and the intergenic spacer. The position of the promoter, enhancer elements, the coding region and the position of the upstream (T_0) and downstream (T_{1-8}) terminators are indicated (Paule, 1998).

A mammalian rDNA promoter consists of approximately 170 to 200 bp of DNA with a modular organization (see Figure 11). Characteristic elements are a core promoter (CP) upstream of the initiation site, an upstream control element (UCE) and a transcription terminator (T_0) next to the UCE (see section

5.6.6) (Paule, 1998). Most species display a similar structural organization of the rDNA transcriptional unit and share this modular promoter structure. However, there are no significant sequence similarities between rDNA promoters from different species, except an AT-rich sequence surrounding the initiation site ((Paule and White, 2000) and references therein). Hence, Pol I transcription exhibits species specificity (Heix and Grummt, 1995).

5.6.3 Regulation of ribosomal RNA transcription

Transcription of the rDNA genes requires the gene-specific RNA Pol I, a multiprotein complex of 11-13 subunits (together with one two associated factors) and a set of auxiliary factors for initiation, elongation and termination (Grummt, 1999; Grummt, 2003; Paule, 1998; Paule and White, 2000). At least four basal transcription initiation factors are involved in specific initiation of mammalian rDNA transcription: TIF-IA / Rrn3, TIF-IB / SL1, TIF-IC and UBF (Upstream Binding Factor) (see Figure 11).

The promoter selectivity factor TIF-IB / SL-I was shown to bind to the rDNA promoter and to confer promoter selectivity (Clos et al., 1986; Heix and Grummt, 1995). This binding is stabilized by the synergistic action of UBF (Kuhn and Grummt, 1992). TIF-IB / SL1 consists of four polypeptides, the TATA-box binding protein (TBP) and three Pol I specific TBP Associated Factors $TAF_I95/110$, TAF_I68 and TAF_I48 ((Grummt and Pikaard, 2003; Paule, 1998) and references therein). In contrast to Pol II transcription, promoter recognition is carried out by the TAF_Is and not by TBP.

The highly abundant UBF is composed of two 90-100 kDa polypeptides UBF1/2 and has been proposed to be an ‘architectural’ transcription factor. It has an N-terminal dimerisation domain and contains several DNA binding domains: the HMG-boxes (Grummt and Pikaard, 2003; Jantzen et al., 1990; Paule and White, 2000). UBF is a sequence-tolerant DNA binding protein that interacts with the minor groove of the DNA and binds to structured nucleic acids (Copenhaver et al., 1994; Kuhn et al., 1994). Through binding and bending of DNA dimers of UBF are able to form a loop of almost 360° once every 140 bp, a structure called the ‘enhancesome’ (Bazett-Jones et al., 1994). It is thought, that the binding properties of UBF bring the CP and UCE into close proximity and this aids TIF-IB / SL1 to recognize the promoter. Binding of UBF is not restricted to regulatory sequences within the rDNA repeat (O'Sullivan et al., 2002). Chromatin immunoprecipitation experiments demonstrated UBF binding across the entire intergenic spacer and transcribed regions, suggesting a structural role for UBF at the nucleolar organizer regions.

TIF-IA / Rrn3 was initially identified as an activity that complements transcriptionally inactive extracts obtained from quiescent mouse cells and has now been demonstrated to be a key factor in growth-dependent regulation of rDNA transcription (Bodem et al., 2000; Buttgereit et al., 1985; Milkereit and Tschochner, 1998; Schnapp et al., 1990). TIF-IA facilitates preinitiation complex formation (see below) and has been shown to interact directly with RNA Pol I and TIF-IB, thereby linking both protein complexes (Miller et al., 2001; Yuan et al., 2002).

TIF-IC is necessary for specific initiation and elongation but its precise role is still elusive (Paule, 1998; Schnapp et al., 1994).

Recently, it was demonstrated that the basal Pol II transcription factor TFII-H and CSB (Cockayne's syndrome B) are components of RNA Pol I transcription machinery and they play important roles in mediating ribosomal RNA synthesis (Bradsher et al., 2002; Iben et al., 2002). In particular, CSB was

shown to promote efficient rRNA synthesis *in vitro* and TFII-H is required for the processivity of rDNA transcription, implying a post-initiation role in transcription.

Stepwise association of the four basal transcription factors and RNA Pol I to the promoter, mediated by protein-protein and DNA-protein interactions, lead to Pol I transcription initiation complex formation (Paule, 1998). As a first step, TIFI-B and UBF bind cooperatively to the rRNA gene promoter, a process assisted by UBF, next Pol I is recruited and TIF-IA and TIF-IC associate and hence form the transcription initiation complex (Schnapp and Grummt, 1991). Recent *in vivo* experiments have demonstrated that the different components of the Pol-I machinery enter the nucleolus as distinct subunits rather than as a preassembled complex (Dundr et al., 2002). In contrast to the stepwise assembly of the preinitiation complex, it is suggested that the transcription-initiation competent Pol I exists as a preassembled complex, a 'holoenzyme', that is recruited to the rDNA promoter. In a variety of organisms large complexes have been identified, that contain RNA Pol I and most if not all components required for specific rDNA transcription (Albert et al., 1999; Fath et al., 2000; Hannan et al., 1999; Saez-Vasquez and Pikaard, 1997; Seither et al., 1998).

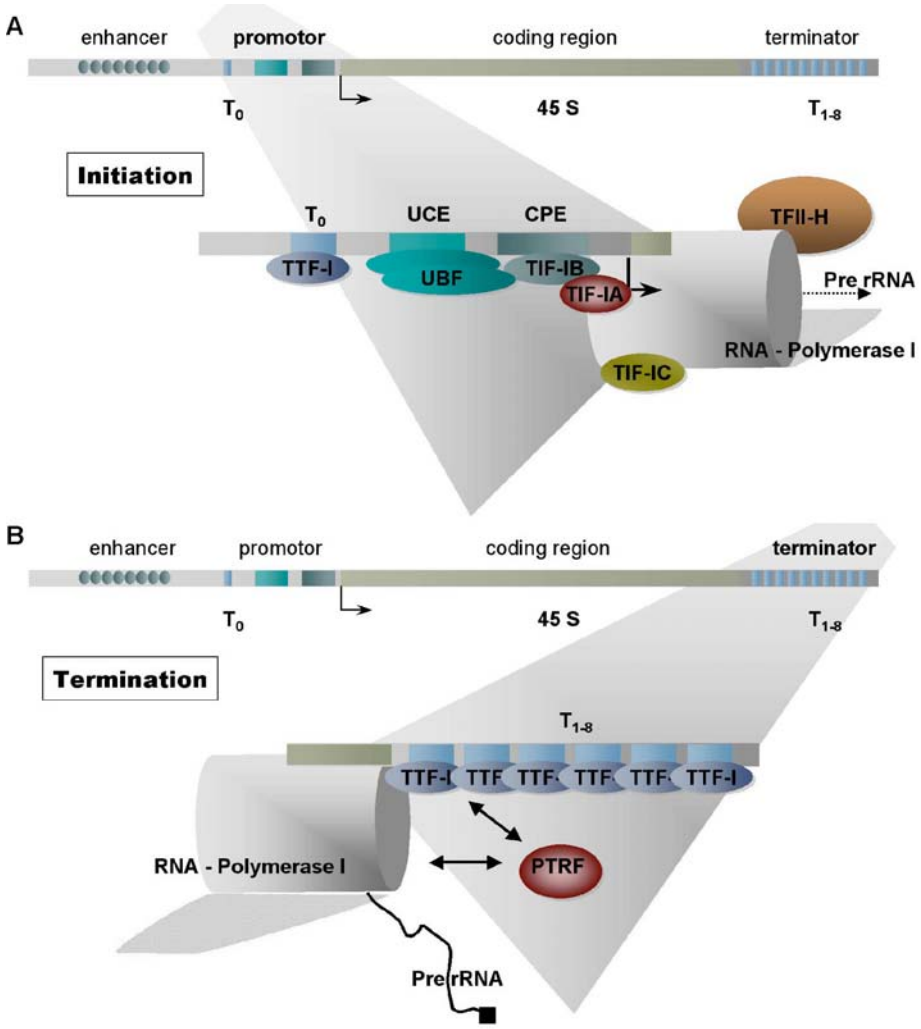

Figure 11. Basal RNA Pol I transcription transcription factors in mouse
Factors involved in ribosomal RNA synthesis during transcription initiation (**A**) and transcription termination (**B**).

Transcription of rRNA genes is highly regulated process, adapting cellular rRNA synthesis requirements to cell metabolism. Almost every protein required for Pol I transcription is a target in a regulatory pathway. For instance, TIF-IA is a target of the TOR and MAP kinase signaling pathways. Reversible phosphorylation of TIF-IA modulates its activity thereby regulating its association with Pol I and controlling rDNA transcription ((Grummt, 2003) and references therein). Acetylation of the TIF-IB subunit TAF_I68 by PCAF enhances its DNA binding activity and augments Pol I transcription, while deacetylation by Sir2 represses transcription (Muth et al., 2001). Phosphorylation and acetylation of UBF regulates its activity throughout the cell cycle (Voit et al., 1995). Furthermore, rDNA transcription is also a target for the retinoblastoma and p53 tumor suppressor proteins, which directly inhibit rRNA synthesis *in vitro* and *in vivo* ((Grummt, 2003) and references therein).

5.6.4 Termination of rDNA transcription

Mammalian rDNA transcription is initiated at the gene promoter and is specifically terminated at the terminator sites, which are located approximately 13 kb downstream of the initiation site (Figure 10 and Figure 11). The terminator sites, called T_{1-8} or'Sal-box' elements, are a series of 18 bp sequence repeats elements downstream of the 45S precursor RNA coding region (Grummt et al., 1985; Grummt et al., 1986b). Transcription termination is a multistep process, involving Pol I pausing, release of both the pre-rRNA and Pol I from the template and 3´-end processing of the primary transcript (Grummt, 1999; Paule, 1998). The Transcription Termination Factor for Pol I (TTF-I) and the Pol I and Transcript Release Factor (PTRF) are important players in this process (see Figure 11) (Bartsch et al., 1988; Evers et al., 1995; Jansa et al., 1998). TTF-I binds sequence specific to the downstream terminators and this leads to pausing of the elongating Pol I. PTRF interacts with both TTF-I and Pol I. This interaction catalyzes the dissociation of the ternary complexes, thus finishing the termination of pre-rRNA synthesis (Jansa and Grummt, 1999; Jansa et al., 1998). Notably, termination depends of the orientation of the terminator elements.

5.6.5 The transcription termination factor TTF-I

TTF-I is a multifunctional nucleolar protein that terminates Pol I transcription, but also mediates replication fork arrest, exhibits contrahelicase activity and regulates RNA polymerase I transcription on chromatin (Bartsch et al., 1988; Gerber et al., 1997; Längst et al., 1998; Längst et al., 1997a; Putter and Grummt, 2002). It is a 130 kDa protein which exhibits a modular structure, consisting of a C-terminal DNA binding domain and a central domain, to which its functions are ascribed.

More than half of the protein can be deleted without affecting its function in Pol I termination. A truncated version lacking the N-terminal 431 amino acids residues still recognizes its binding site and efficiently terminates transcription. Furthermore, a mutant lacking the N-terminal 445 amino acids is unable to support transcription termination but is able to bind to the target sequence. Hence, this 15 amino acids region must be important for transcription termination (Evers et al., 1995). Remarkably, the DNA-binding domain of TTF-I contains two SANT domains, which can be found in several transcriptional regulators (Aasland et al., 1996). At the C-terminus of TTF-I lies a 31 amino acid region which demotes the species-specific DNA binding of both human and mouse TTF-I (Evers and Grummt, 1995). Thus,

TTF-I has at least three functional domains, one that interacts with DNA, a second that is required for transcription termination and a third that is important for species specificity.

The N-terminus of TTF-I contains a negative regulatory domain (NRD) that inhibits DNA binding. While full-length TTF-I displays almost no DNA binding activity *in vitro*, limited proteolysis or deletion of 185 N-terminal amino acids of TTF-I strongly increased its DNA binding activtiy (Németh et al., 2004; Sander et al., 1996; Smid et al., 1992). Both full-length TTF-I and the deletion mutants terminate transcription efficiently *in vitro* and *in vivo*, suggesting that cellular proteins may alleviate the repression. Indeed, the DNA binding activity of full-length TTF-I is restored by interacting with Tip5 (Németh et al., 2004; Strohner et al., 2004). Notably, TTF-I is capable of forming oligomers in solution mediated by two independent domains located within the 184 N-terminal and 445 C-terminal amino acids of TTT-I, respectively (Sander and Grummt, 1997; Sander et al., 1996).

5.6.6 The role of TTF-I at the rDNA promoter

In addition to the downstream terminator elements, the rDNA promoter harbors an upstream terminator element, called T_0 (Figure 10 and Figure 11). In mouse, this promoter-proximal terminator is positioned approximately 170 bp upstream of the transcription initiation site and TTF-I binds to its target site (Grummt et al., 1986a). T_0 has characteristics of a promoter element in so much as it was shown to stimulate transcription initiation *in vivo* (Grummt et al., 1986a; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1990). Several mechanisms have been suggested to contribute to this transcriptional enhancement ((Paule, 1998; Paule and White, 2000) and references therein). For instance, by linking the enhancer and terminator elements, the end of one rDNA repeat could be brought to close proximity to the beginning of the next or same gene. Thus, the intergenic spacers would be looped out and a terminating polymerase could pass directly to the promoter ('hand over model'). Indeed, TTF-I has been shown to form oligomers and is capable of linking two separate DNA fragments (Sander and Grummt, 1997; Sander et al., 1996). Alternatively, the proximal terminator might shield the rDNA promoter from polymerases that initiated from spacer promoters.

Recent results highlighted a role for the upstream terminator element in transcription regulation in the context of a chromatin environment. *In vitro* transcription studies on mouse rDNA pre-assembled into chromatin demonstrated that binding of TTF-I to the proximal terminator T_0 mediates ATP-dependent nucleosome rearrangements. Significantly, remodeling correlates with efficient transcription initiation on the otherwise repressed nucleosomal rDNA templates, indicating that chromatin remodeling allows access for Pol I transcription factors (Längst et al., 1997a). Whereas termination by TTF-I depends on the correct orientation of a terminator, TTF-I-mediated transcription activation is independent upon the orientation of its binding site. Furthermore, transcriptional activation by TTF-I requires the precise spacing of the upstream terminator relative to the promoter (Längst et al., 1998). This indicates that TTF-I activates transcription by recruiting ATP-dependent remodeling factors to establish a specific promoter architecture that is compatible with, if not prerequisite for transcription initiation from rDNA chromatin (Längst et al., 1998).

In agreement with the results in mouse, binding of Reb1p, the yeast homologue of TTF-I, to its cognate binding site upstream of the transcription initiation site enhances rDNA transcription. Binding of Reb1p has been implicated in mediating alterations of the chromatin structure of the rDNA promoter

thereby stimulating transcription (Fedor et al., 1988; Kulkens et al., 1992; Morrow et al., 1989). Consistent with a role of Reb1p in rDNA transcription activation, chromatin remodeling precedes the activation of ribosomal gene promoters in yeast (Lucchini and Sogo, 1995).

Recent studies show that TTF-I plays a dual role in rDNA transcription regulation in chromatin: TTF-I is involved in both activation and silencing of rDNA transcription (see chapter 9, discussion).

5.6.7 Chromatin structure of ribosomal RNA genes

In eukaryotic cells, two different chromatin structures of ribosomal RNA genes coexist: one that contains nucleosomes and represents the inactive copies, and one that lacks canonical nucleosomes and corresponds to the transcribed genes (Grummt and Pikaard, 2003; Moss, 2004; Paule, 1998).

The transcribed (active) rDNA repeats can be visualized by electron microscopy as 'Miller spreads' or 'Christmas tree' structures (Miller and Beatty, 1969). The pictures revealed, that the multicopy rRNA genes are either densely packaged with RNA Pol I and nascent transcripts or are not transcribed at all (see Figure 12) (French et al., 2003; Osheim et al., 1996). Analysis of the DNA length within the active genes revealed a low compaction rate, suggesting that the eukaryotic rRNA genes might lack nucleosomes on the transcribed repeats ((Paule, 1998) and references therein). However, the chromatin structure is most likely completely disrupted during the 'Miller spreading method'. It is important to note, that these studies did not address the structure of the transcriptionally inactive genes (Paule, 1998).

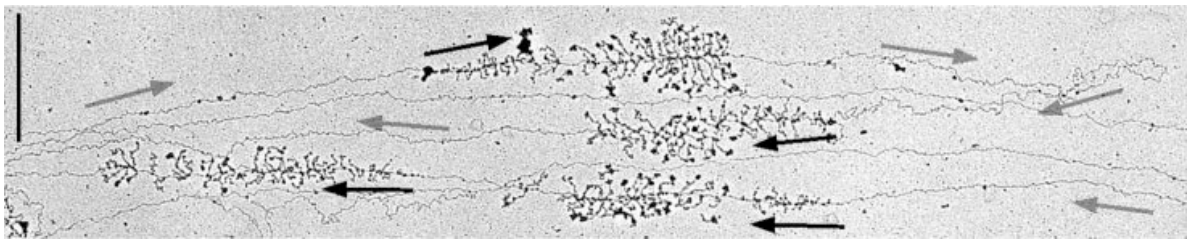

Figure 12. Miller spreads of ribosomal RNA genes (French et al., 2003)
Active transcribed rDNA genes are occupied with RNA Pol I and the nascent pre–rRNA, forming the so called 'Christmas tree' structure (black arrows). These genes are randomly separated by inactive copies (gray arrows).

Several approaches have demonstrated that the rRNA genes contain variegated forms of chromatin. Studies of the nucleosomal structure of rDNA genes in *Physarum polycephalum* investigating the accessibility of histone H3 to the sulfhydryl reagent iodoacetamidofluorescein (IAF) present evidence for two different 'accessible' nucleosomes within the transcriptionally active and inactive states of rDNA chromatin (Johnson et al., 1987; Prior et al., 1983). Remarkably, these studies demonstrated that the extended, transcribed rDNA regions contain all four of the core histones. Electron microscopy pictures showed that most of the extended subunits present unfolded nucleosomes, consisting of two roughly spherical bodies connected by a 50 bp nucleoprotein bridge. Studies on ribosomal chromatin of *Xenopus* oocytes indicated that the transcribed rRNA genes are organized into nucleosome-like arrays (Culotta and Sollner, 1988). A chromatin fractionation procedure of micrococcal nuclease-digested mouse nuclei separated rDNA genes into two chromatin components. One which contained nucleosomes and another in

which the ribosomal genes did not display a repeating structure (Davis et al., 1983). Similarly, separation of transcriptionally active from inactive chromatin by mercury affinity chromatography, where specifically 'unfolded' nucleosomes of the transcriptionally active chromatin are recovered, revealed two separate forms of rDNA chromatin (Chen et al., 1990).

The two distinct chromatin structures of rRNA genes were confirmed in several studies using the psoralen crosslinking technique (Conconi et al., 1992; Conconi et al., 1989; Dammann et al., 1993; Stancheva et al., 1997). Psoralen, an organic reagent, intercalates into DNA, and following exposure to UV light, it crosslinks one DNA strand of non-protein associated DNA to another. Thus, psoralen crosslinking occurs preferentially within free DNA and is absent in nucleosomal DNA (Sogo et al., 1984). *In vivo* psoralen crosslinking experiments in mouse cells demonstrated the existence of two rDNA gene populations, one which was psoralen-accessible, and therefore by definition canonical nucleosomes free. The other psoralen-inaccessible fraction consists of rDNA packaged into chromatin (Conconi et al., 1989). Crosslinking of nascent pre-rRNA allowed to distinguish between transcriptional active and inactive genes. It was revealed, that the transcriptionally active rDNA genes have an 'open' chromatin conformation, whereas the inactive gene copies resides in the compact 'closed' nucleosomal structure.

The ratio of the two states of chromatin is maintained irrespectively of the transcriptional activity and is stably propagated throughout the cell cycle. Even in exponentially growing mammalian cells no changes in the ratio of psoralen-accessible (active) to inaccessible (inactive) rRNA genes were observered (Conconi et al., 1989). The various levels of ribosomal RNA in mammals are not mediated through changes in the number of active genes but rather by adapting the transcription rate of particular genes. However, even if the ratio of active to silenced genes is stable propagated throughout cell cycle, the number of active transcription units might alter upon the steps of cell differentiation (Haaf et al., 1991). Notably, yeast cells do accommodate their need of rRNA by varying the number of active genes (Dammann et al., 1993; Sandmeier et al., 2002). Even in these cases approximately half of the ribosomal RNA genes stay in a closed conformation, suggesting that in yeast the inactive rDNA genes have two distinct states: one facultative and the other constitutively repressed (Moss, 2004).

The chromatin (activity) state of the ribosomal genes is transiently erased during replication, when the newly synthesized daughter strands are re-packaged into nucleosomes (Lucchini and Sogo, 1995). Regeneration of the active chromatin structure along the coding region occurs after replication and involves the disruption of pre-formed nucleosomes. Studies in yeast with an RNA Pol I deficient strain indicate, that the establishment of the open chromatin conformation on the activated gene copies requires transcribing polymerase molecules (Dammann et al., 1995).

The active and inactive rRNA gene copies are randomly distributed rather than organized or grouped within a NOR (Dammann et al., 1995; French et al., 2003). Analysis of a single, tagged transcription unit within the tandem array in yeast revealed a random distribution of active and inactive copies throughout the ribosomal rRNA gene locus (Dammann et al., 1995). The onset and the mechanisms that maintain the inactive state of rRNA genes remain to be addressed. Growing evidence indicates that several repressive components like chromatin remodeling, histone modifications and DNA methylation can cooperate in the process of rRNA gene silencing (see following section 5.6.8).

Nucleolar dominance in plants describes a similar phenomenon, where only a subset of the rRNA genes is transcribed (Pikaard, 2000). In genetic hybrids that display nucleolar dominance, the transcription by RNA polymerase I is restricted to only one parental set of ribosomal RNA genes, while the genes inherited from the other parent are repressed. Recent data suggest that nucleolar dominance uses the same mechanisms that control the ratio of active to silent rRNA genes in mammalian cells (Lawrence et al., 2004).

There are several hypotheses to suggest, why a cell might require this large number of inactive rDNA genes (Moss and Stefanovsky, 2002; Paule and White, 2000). The intensely transcribed, 'open', rDNA genes are potentially preferred subjects to various forms of DNA damage. The inactive, 'closed', genes might serve as a kind of 'somatic rRNA gene germline', necessary for the exchange of the damaged genes. The two forms of rDNA genes might be important for appropriate DNA recombination. Alternatively, the silenced genes might be crucial for the integrity or assembly of the nucleolus; they might act as a landing platform for factors involved in the diverse functions of the nucleolus or prevent the nucleolus from unwanted factors.

5.6.8 Silencing of ribosomal RNA genes

The term 'rRNA gene silencing' defines the mechanisms that establish and maintain the transcriptionally inactive rRNA genes in cells of higher eukaryotes. Notably, in yeast, 'transcriptional silencing at rDNA repeats' refers to different phenomena, like the suppression of recombination, or the inactivation of Pol II driven genes when they are incorporated into the rDNA gene locus (Huang, 2002).

Gene silencing generally describes a heritable form of gene inactivation that involves the combined effects of specific histone modifications, DNA methylation, chromatin remodeling and the RNAi machinery, collectively described as 'epigenetic regulation' (Grewal and Moazed, 2003; Jaenisch and Bird, 2003). In many cases, transcriptional silencing is accomplished through a dense packaging of the corresponding DNA into heterochromatin or heterochromatin-like structures. These specialized structures allow the maintainenance of the silent chromatin states throughout the cell cycle. DNA methylation, predominantly at the 5´-position of cytosine within CpG base pairs, is a well-documented phenomenon of gene silencing in many higher eukaryotes. This modification plays an important role in defining the inactive state of certain genes and directs the propagation of this state through mitotic cell division (Bird, 2002; Bird and Wolffe, 1999; Ng and Bird, 1999).

Similar phenomena, as distinct histone modifications and DNA methylation, are characteristic for silenced rDNA genes. The inhibition of histone deacetylation with specific inhibitors de-repressed silenced rRNA genes in plants (Chen and Pikaard, 1997). Furthermore, the inactive rRNA genes are found to be hypoacetylated (Mutskov et al., 1996) and the ribosomal RNA genes are methylated ((Bird et al., 1981b) and references therein). Until recently the role of rDNA gene methylation remained ambiguous ((Grummt and Pikaard, 2003) and references therein). Loss of rDNA methylation accompanied the onset of transcription during *Xenopus* embryonic development; in contrast even highly methylated rRNA genes isolated from sperm were transcriptionally active (Bird et al., 1981a; Macleod and Bird, 1983). Further studies with human cells identified a mosaic methylation pattern of the rDNA genes and suggested a correlation between DNA methylation and the inactive gene copies (Brock and Bird, 1997). Indeed, treatment with 5-aza-2´-deoxycytosine, an inhibitor of DNA methylation, de-

repressed silenced rDNA genes (Chen and Pikaard, 1997). Next it was shown, that CpG methylation is mainly present in the enhancer and promoter region of inactive rRNA gene copies and a single methylation site strongly correlates with transcriptional activity (Stancheva et al., 1997). Subsequently, it was elucidated that methylation of a single CpG in the mouse rDNA promoter region is sufficient for inactivation of transcription in both transfection experiments and *in vitro* assays using chromatin templates (Santoro and Grummt, 2001). DNA methylation blocks binding of the transcription factor UBF to nucleosomal rDNA, thereby preventing initiation complex formation.

Recent studies, including the work presented in this thesis, provide further insights into the molecular mechanisms of ribosomal RNA gene silencing (Lawrence et al., 2004; Németh et al., 2004; Santoro et al., 2002; Strohner et al., 2004; Zhou et al., 2002). A concert action of several mechanisms such as the recruitment of the repressive chromatin remodeling complex NoRC, DNA methylation and histone modifications is suggested to alter the rDNA chromatin structure and thereby control the ratio of active and inactive genes (see chapter 9, discussion and for review (Grummt and Pikaard, 2003).

5.7 Tip5, subunit of a nucleolar remodeling complex?

Tip5 (TTF-I interacting protein 5) was initially identified in a 'yeast two hybrid' screen for novel interaction partners of TTF-I (for TTF-I, see section 5.6.5) (Jansa et al., 1998). The cDNA of Tip5 was cloned and the protein-protein interaction region was determined to reside within the N-terminus of TTF-I and between amino acids 600 to 700 of Tip5 (Németh et al., 2004). Notably, the N-terminal interaction region of TTF-I acts as a negative regulatory domain and masks its DNA binding activity, thus ascribing important regulatory functions for this domain (Evers et al., 1995; Németh et al., 2004).

The modular organization of Tip5 resembles that of proteins which participate in chromatin structure and regulation, thereby suggesting that Tip5 belongs to the WAL / BAZ protein family (WSTF-, Acf1-like / bromodomain adjacent zinc finger, respectively) (Jones et al., 2000; Poot et al., 2000). Many members of the WAL / BAZ family are characterized as subunits of ISWI-containing chromatin remodeling complexes (see section 5.5.2). These proteins share several structural motifs like PHD fingers, bromodomains, WAKZ and BAZ domains (Figure 13A). NURF301, the large subunit of the ISWI-containing remodeling complex NURF, is highly related to this family as it shares most domains in a slightly different order.

The PHD finger is a 50-80 amino acid zinc-finger-like motif with a unique Cys4-His-Cys3 pattern that has been identified in numerous proteins, many of which are implicated in chromatin-mediated transcription control (Aasland et al., 1995).. The 110 aa bromodomain, which interacts with acetylated lysines of histone H4 tails (Dhalluin et al., 1999; Owen et al., 2000), is present in many chromatin-associated factors and most histone acetyltransferases. Many of these factors reside in large multiprotein complexes and are involved in transcriptional regulation (Horn and Peterson, 2001; Jeanmougin et al., 1997). The central segment of WAL / BAZ proteins, including the DDT domain and the BAZ motifs, has been reported to be involved in the interaction with ISWI (Eberharter, 2004; Fyodorov and Kadonaga, 2002a; Jones et al., 2000). Indeed, all characterized members of this family have been shown to interact with ISWI. The DDT domain has also been suggested to be able to bind DNA (Doerks et al., 2001). The WAKZ (WSTF, Acf1, KIAA0314, ZK783.4) motif, present in all members of this family is ill-defined (Jones et al., 2000).

WSTF, NURF301 and Acf1, but not Tip5, contain at their N-terminal region a WAC motif, which is required for the interaction between Acf1 and the small histone fold subunits within the CHRAC complex (Kukimoto et al., 2004). The WAC motif was shown to be necessary for the efficient binding of the ACF complex to DNA *in vitro* (Fyodorov and Kadonaga, 2002a) and targets proteins to pericentric heterochromatin upon being fused to it (Tate et al., 1998). Several AT hooks, a motif known to mediate binding to the minor groove in DNA, are present within NURF301 and Tip5 (Aravind and Landsman, 1998). In contrast, only Tip5 contains a TAM module (Tip5, ARBP, MBD) that is related to the MBD motif present in methyl-CpG binding proteins (Nan et al., 1993).

In previous work (diploma thesis in the laboratory of Prof I. Grummt), I started the biochemical purification of cellular Tip5 to determine potential associated factors (Strohner, 2000). As expected from its theoretical size, native Tip5 turned out to be approximately a 220 kDa protein in SDS-PAGE and localized predominantly in the chromatin fraction. Cellular Tip5 cofractionated together with Snf2h, the human homolog of ISWI (Aihara et al., 1998), over several steps of chromatographic purification (Figure 13B). Significantly, partially purified cellular Tip5 coeluted on a gel filtration column (Superose6) with a subpopulation of Snf2h at a size of approximately 800 kDa (Figure 13C), suggesting a macromolecular protein complex compound of at least these two factors. Immunofluorescence experiments analyzing the cellular localization of Tip5, showed a clear nucleolar staining for endogenous Tip5, moderately overlapping with the nucleolar protein UBF (Figure 13D).

Therefore, it was attempted to speculate that Tip5 could be a part of a novel nucleolar chromatin remodeling complex (Németh, 2002; Strohner, 2000). However, the tight association of Tip5 and Snf2h was elusive. Furthermore, it was yet to be determined how cellular Tip5 and associated factors are able to alter the chromatin structure.

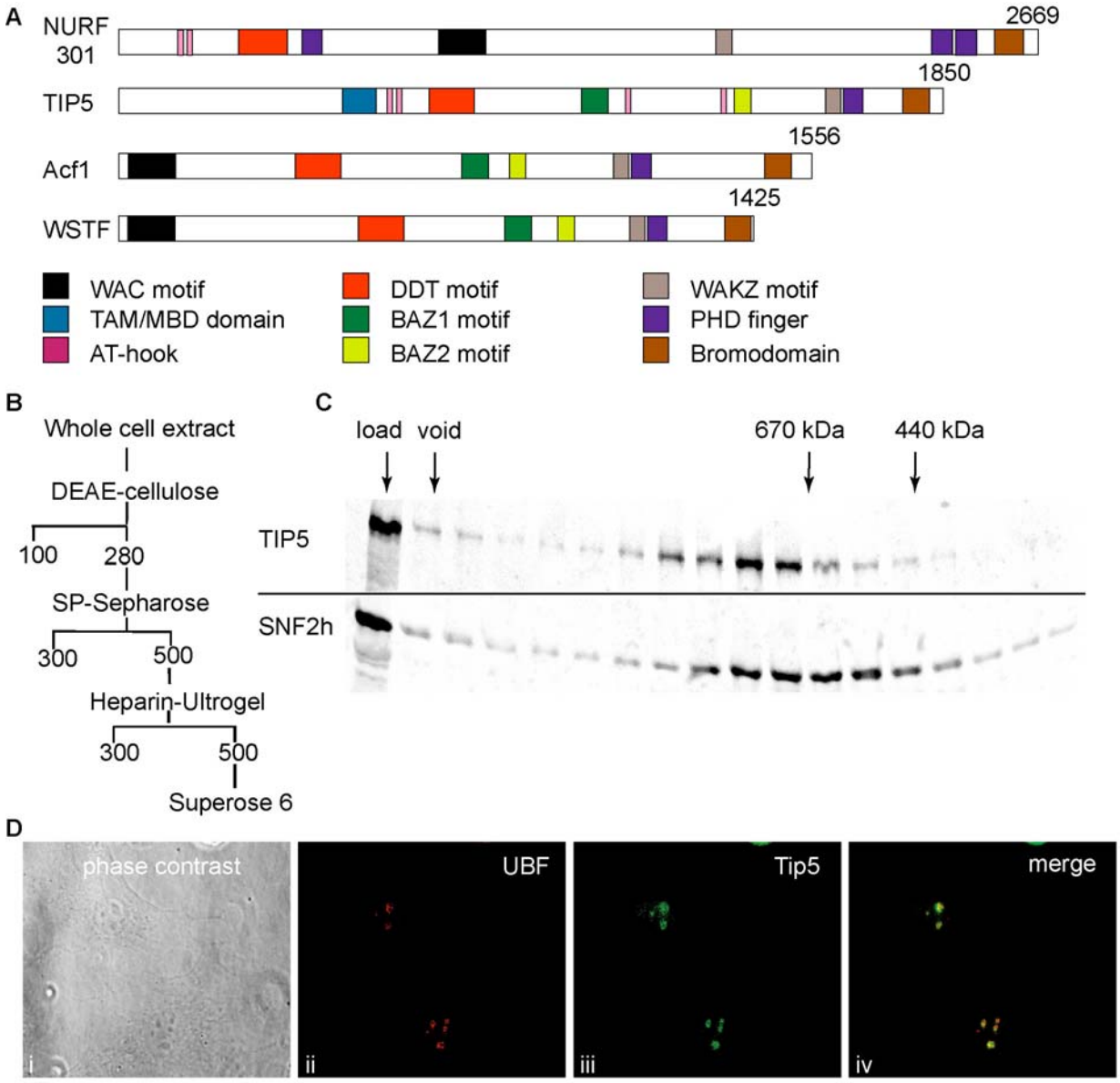

Figure 13. Tip5, subunit of a nucleolar remodeling complex?
(**A**) Tip5 (TTF-I interacting protein 5) is homolog to large subunits of ISWI-containing chromatin remodeling complexes. Schematic diagrams of Tip5 (mouse), WSTF (human), Acf1 (human) and NURF301 (*Drosophila*) show the location of conserved sequence motifs (color code below). Numbers of amino acids in each protein are indicated. (**B**) Fractionation scheme used to purify cellular Tip5 and associated proteins. (**C**) Resolution of cellular Tip5 by size exclusion chromatography. Proteins present in the H-500 fraction were size-fractionated on Superose 6 and analyzed on immunoblots using anti-Tip5 (upper panel) and anti-Snf2h (lower panel) antibodies. The positions of molecular weight standards (Thyroglobulin, 670 kDa; Apoferritin, 440 kDa) are indicated at the top of the panel. (**D**) Tip5 co-localizes with UBF in the nucleolus. Interphase NIH3T3 cells were fixed and immunostained with antibodies against UBF (ii) and Tip5 (iii). Co-localization of Tip5 and UBF are shown as yellow in the merged image (iv). The corresponding phase-contrast micrograph is shown at the left (i).

6 OBJECTIVES

The aim of this doctoral thesis was to elucidate the molecular mechanisms that regulate rDNA gene expression in chromatin. In this respect, the role of Tip5, a novel TTF-I interacting protein, was to be studied.

6.1 Ribosomal RNA gene regulation in chromatin

Mammalian cells harbor about 400 rRNA gene copies arranged in tandem repeats, and their transcription is strictly regulated (Grummt, 2003). Even in metabolically active cells, only half of the genes are actively transcribed, whereas the remaining genes are maintained in a silenced state. The ratio of active and inactive genes is stably maintained in consequent cell divisions. However, during replication the chromatin states are erased, and the newly replicated daughter strands are re-packaged into nucleosomes (Lucchini and Sogo, 1995). It was previously demonstrated that TTF-I dependent chromatin remodeling at the rDNA promoter is required to activate RNA polymerase I transcription in chromatin (Längst et al., 1998; Längst et al., 1997a). DNA methylation was shown to be directly involved in rDNA gene inactivation, as silent rDNA genes were found to be specifically methylated in the promoter region (Santoro and Grummt, 2001; Stancheva et al., 1997). The factors altering the chromatin structure of ribosomal RNA genes are not known and the mechanisms that initiate and maintain silencing of the rRNA genes are ill-defined.

6.2 Purification of a Tip5 complex and analysis of its role in rDNA transcription

The nucleolar protein Tip5 (TTF-I interacting protein 5) was initially identified in a 'yeast two hybrid' screen for novel interaction partners for TTF-I (Jansa et al., 1998). The domain and motif structure of Tip5 resembles the structure of other known chromatin modifying proteins. Furthermore, it was suggested that Tip5 together with Snf2h form a nucleolar chromatin remodeling complex (Németh, 2002; Strohner, 2000).

One aim of this work was to purify and identify the molecular composition of cellular Tip5 complexes and to determine their activity. This included the functional analysis of Tip5 containing complexes, reconstitution of recombinant Tip5 (and associated subunits) and biochemical characterization of the factors with different assays. In addition, since Tip5 interacts with TTF-I, an important regulator of rDNA transcription in chromatin, a further aim was to address the potential role of Tip5 in rDNA transcriptional regulation.

7 MATERIALS AND METHODS

7.1 Materials

7.1.1 Chemicals, radioactive material, enzymes, chromatographic material

Unless otherwise stated, all common chemicals and materials were ordered by Amersham / Pharmacia (Freiburg), E. Merck (Darmstadt), NEN / Perkin Elmer (Rodgau), Pierce (Bonn), Promega (Mannheim), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Radioactive materials were ordered either by Amersham (α-^{32}P CTP and α-^{32}P dCTP (3000 Ci/mmol, 10 mCi/ml)) or by NEN (γ-^{32}P ATP (3000 Ci/mmol, 10 mCi/ml)).

7.1.1.1 Enzymes

Restriction endonucleases	New England Biolabs, Fermentas, Promega, Roche
Klenow enzyme	New England Biolabs
Shrimp alkaline phosphatase	New England Biolabs
T4 polynucleotide kinase (PNK)	Promega
T4 DNA ligase	New England Biolabs
Taq DNA polymerase	Promega
RNasin	Promega
Endoproteinase Glu-C (V8 protease)	Roche / Boehringer Mannheim
MNase (S7 Nuclease)	Roche
DNase I	Roche
Sss-I methylase	New England Biolabs

7.1.1.2 Chromatographic material

Nickel-NTA-agarose (Ni^{2+}-beads)	Qiagen
M2-agarose (Flag-beads)	Sigma
Gelfiltration columns (Superose 6, Superdex 200)	Pharmacia
DEAE Sepharose FF	Pharmacia
SP-Sepharose FF	Pharmacia
Heparin Hi-Trap column	Pharmacia
Hydroxyl apatite resin	Bio Rad
Biorex 70 Resin	Bio Rad
PEI Cellulose F (Thin layer chromatography plates)	Merck
Chromatography systems (ÄKTA, FPLC & HPLC)	Pharmacia
Sephadex G25 spin columns	Roche

7.1.1.3 Blotting materials

Hybond N+ membrane	Amersham
Nitrocellulose membrane	Protrane
Whatman 3MM paper	Whatman
PVDF membrane	Amersham

7.1.1.4 Dialysis and filtration materials

Dialysis membranes	Spectra Por
Concentration tubes (‘Centricon’)	Amicon, Pall
Filtration units	Merck

7.1.2 Standard solutions

Stock solutions and buffers were made according to standard protocols (Ausubel, 1999; Hoffmann-Rohrer and Labaere, 2000; Sambrook et al., 1989; Sambrook and Rusell, 2001). Protease Inhibitors (either Complete® EDTA-free (Roche), or a mix of Leupeptin, Pepstatin, Aprotinin (all 1 μg/ml) and PMSF (0.2 to 1 mM)) and DTT / ß-mercaptoethanol (1 mM) were freshly added. The most common solutions are listed below.

EX-X buffers	20 mM Tris-HCl pH 7.6 1.5 mM $MgCl_2$ 0.5 mM EGTA 10% glycerol X mM KCl
AM-X buffers	20 mM Tris-HCl pH 7.9 5 mM $MgCl_2$ 0.2 mM EDTA 10% or 20% glycerol X mM KCl
Phosphate Buffered Saline (PBS)	140 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH adjusted to 7.4 with HCl
TAE-buffer	40 mM Tris 40 mM Acetate 1 mM EDTA
TBE-buffer	90 mM Tris 90 mM Boric acid 2 mM EDTA
TE buffer	10 mM Tris-HCl pH 7.6 1 mM EDTA
DNA sample buffer (10x)	50% glycerol 50 mM Tris-HCl pH 7.6 10 mM EDTA either 0.05% bromophenol blue and xylene cyanol or 0.05% Orange G
SDS-protein sample buffer (6x)	350 mM Tris-HCl pH 6.8 10% SDS 30% glycerol 5% β-Mercaptoethanol 0.2% bromphenol blue
Stacking buffer (4x)	0.5 M Tris-HCl 0.4% SDS, pH 6.8 with HCl

Resolving buffer (4x)	1.5M Tris-HCl 0.4% SDS, adjust to pH 8.8 with HCl
SDS-PAGE running buffer	192 mM glycine 25 mM Tris 0,1% (w/v) SDS

Additional buffers are described in the individual method sections.

7.1.3 Antibodies

α-hTip5 / 332-726	Polyclonal antibody (rabbit) against amino acids 332-726 of hTip5 (Jansa et al., 1998). The serum was diluted 1:2500
α-mTip5 / N1-18	Polyclonal peptide antibody (rabbit) recognizing amino acids 1-18 of Tip5 (Németh, 2002; Strohner, 2000). Purified antibodies were diluted 1:1000
α-mTTF-I C7	Polyclonal antibody (rabbit) raised against ΔN323 TTF-I (Evers et al., 1995), used 1:2500.
α-Myc 9E10	Monoclonal mouse antibody (clone 9E10), available in the department (Sigma). Purified with protein A / G beads, used 1:500.
α-hSnf2h	Polyclonal rabbit antibody. Kindly gift from P. Varga-Weisz (Poot et al., 2000) and I. Grummt (Santoro et al., 2002), used 1:2000.
α-Acf1	Polyclonal rabbit antibody. Kindly gift from P. Varga-Weisz (Poot et al., 2000), used 1:1000.
α-His	Commercial (Qiagen) penta-His antibody (mouse), used 1:1000.
α-Flag	Commercial (Sigma) antibody (mouse), used 1:1000.
α-rabbit	Commercial (Amersham) secondary antibody (anti-rabbit IgG, Horseradish peroxidase (HRP) - conjugate), used 1:10000.
α-mouse	Commercial (Amersham) secondary antibody (anti-mouse IgG, HRP-conjugate), used 1:5000.

7.1.4 Plasmids

Tip5 constructs

pcDNA3.1-ßFLAG-Tip5	full length mouse Tip5 cDNA in mammalian expression vector (N-terminal Flag-tag), kindly gift from I. Grummt
pBS HA-Tip5 fl	full length mouse Tip5 cDNA in pBluescript SK+ (Stratagene) (N-terminal HA-tag), kindly gift from I. Grummt
pESC Myc-Tip5	full length mouse Tip5 cDNA cloned into yeast expression vector pESC-Ura (Stratagene), (N-terminal Myc-tag)
pFastBac Myc-Tip5	full length mouse Myc-Tip5 cDNA cloned into Baculo expression vector pFast Bac1 (Life Technologies), (N-terminal Myc-tag)
pFastBac His-Myc-Tip5	full length mouse Myc-Tip5 cDNA cloned into Baculo expression vector pFast Bac HTc (Life Technologies), (N-terminal His_6- and Myc-tag)

TTF-I constructs

pRset-mTTF-I	full length mouse TTF-I cDNA, in bacterial expression vector (N-terminal Flag and His_6-tag), kindly provided by I. Grummt
pRset-mTTFΔN185	N-terminal truncated (aa 1-185) form of TTF-I, in bacterial expression vector (N-terminal Flag and His_6-tag), kindly provided by I. Grummt
pFastBac His-TTF-I	full length mouse TTF-I cDNA cloned into Baculo expression vector pFast Bac HTc (Life Technologies), (N-terminal His_6-tag)
p FastBac N185 TTF-I	N-terminal part of TTF-I (aa 1-185), cloned into Baculo expression vector pFast Bac HTc (Life Technologies), (N-terminal His_6-tag)

Snf2h constructs

pFastBac:Snf2h (Flag)	full length Snf2h cDNA clone in Baculo expression vector pFast Bac1 (Life Technologies), (N-terminal Flag-tag), kindly provided by R.E. Kingston (Aalfs et al., 2001)
pBS Flag-Snf2h	Flag-Snf2h cDNA cloned into pBluescript SK+ (Stratagene) (N-terminal Flag-tag)
pESC Snf2h-Flag	Snf2h cDNA cloned in yeast expression vector pESC-Ura (Stratagene), (C-terminal Flag-tag)
pcDNA Snf2hKR211	Snf2h KR211 point mutant (ATPase mutant), in mammalian expression vector, (C-terminal Flag tagged), kindly provided by R. Shiekhattar (Hakimi et al., 2002)
pFastBac Snf2hKR211-Flag	Snf2h KR211 point mutant cloned into Baculo expression vector pFastBac1 (Life Technologies), (C-terminal Flag tagged)

mouse rDNA constructs

pMr 974	pUC9 plasmid containing a 11.3 kb genomic EcoRI fragment from mouse rDNA, extending from -5635 to +5646 with the respected to the transcription start site of RNA Pol I.
pMrWT-T	Artificial 'rDNA minigene'. It contains the mouse rDNA promoter sequences from -170 to +155 including the upstream terminator T_0 at position -170 and a 3.5 kb 3´-terminal rDNA fragment with 8 terminator elements (T_1-T_8), spaced by 686 bp of plasmid sequences (pUC plasmid).
pMr SP- / +BH	Template for Pol I transcription reaction, containing a murine 5´- rDNA promoter fragment (-292 to +155) fused to a 3´-terminal rDNA fragment with 8 terminator elements (T_1-T_8). rDNA elements are spaced by 686 bp of plasmid DNA (pMr SP-) and 856 bp (pMr SP+BH), respectively. pMr SP+BH contains an 170 bp fragment insertion ('BH fragment') in the transcribed region.

For additional information see DNA libraries of the groups of P.B. Becker, I. Grummt and G. Längst and the manufacturer's descriptions.

7.1.5 Recombinant baculo viruses

Recombinant viruses, encoding the following proteins, were available in the department:

Flag - ISWI
Acf1 (untagged)
Flag - Acf1
His - TTFΔN185

Viruses, that encode for the following recombinant proteins, were made during these studies, using the Bac-to-Bac system (Life Technologies):

Flag - Snf2h
Snf2h - KR211 - Flag
Myc - Tip5
His - Myc Tip5
His - full length TTF-I
His - N185 TTF-I

For details about cDNA clones, and how to produce recombinant baculovirus with the Bac-to-Bac system: see sections 7.1.4 and 7.2.4.

7.1.6 Oligonucleotides within the mouse rDNA promoter

A

```
-231      -222      -212      -202      -192      -182
   GAAAGCTATGGGCGCGGTTTTCTTTCATTGACCTGTCGGTCTTATCAGTT
   CTTTCGATACCCGCGCCAAAAGAAAGTAACTGGACAGCCAGAATAGTCAA

-181      -172      -162      -152      -142      -132
   CTCCGGGTTGTCAGGTCGACCAGTTGTTCCTTTGAGGTCCGGTTCTTTTC
   GAGGCCCAACAGTCCAGCTGGTCAACAAGGAAACTCCAGGCCAAGAAAAG

-131      -122      -112      -102      -92       -82
   GTTATGGGGTCATTTTTGGGCCACCTCCCCAGGTATGACTTCCAGGTATT
   CAATACCCCAGTAAAAACCCGGTGGAGGGGTCCATACTGAAGGTCCATAA

 -81       -72       -62       -52       -42       -32
   CTCTGTGGCCTGTCACTTTCCTCCCTGTCTCTTTTATGCTTGTGATCTTT
   GAGACACCGGACAGTGAAAGGAGGGACAGAGAAAATACGAACACTAGAAA

 -31       -22       -12       -2        +9     +16
   TCTATCTGTTCCTATTGGACCTGGAGATAGGTACTGACACGCTGTCC
   AGATAGACAAGGATAACCTGGACCTCTATCCATGACTGTGCGACAGG
```

B

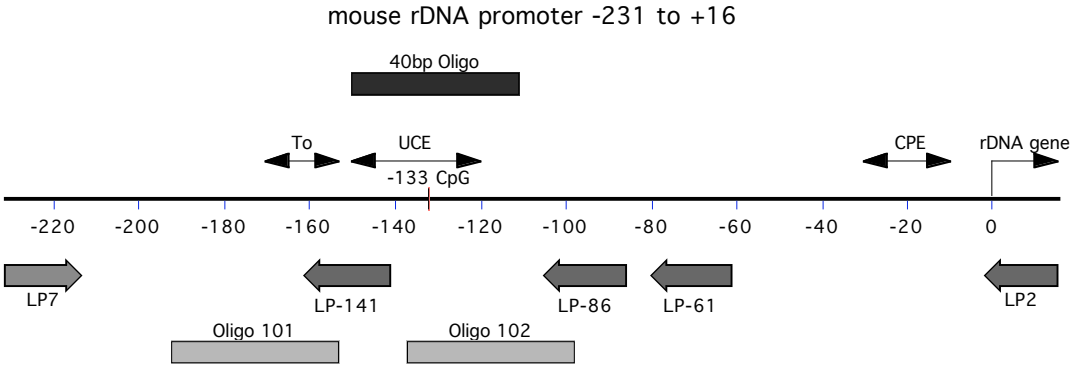

Figure 14. Sequence of the mouse rDNA promoter and positions of oligonucleotides within
(**A**) Sequence of the mouse rDNA promoter from -231 to +16, relative to the transcription start site. (**B**) Diagram of the oligonucleotides utilized from the mouse rDNA promoter. The indicated primers (gray arrows) were used for synthesis of 90 bp (LP7 / LP-141), 146 bp (LP7 / LP-86), 171 bp (LP7 / LP-61), and 247 bp (LP7 / LP2) DNA fragments. Oligonucleotides spanning regions from / to: LP7 (-231 to -210), LP2 (+16 to -4), LP-61 (-61 to -80), LP-86 (-86 to -105), LP-141 (-141 to -161). The indicated double stranded oligonucleotides (solid bars) were used in electromobility shift assays. The start site (rDNA gene), the core promoter element (CPE), the upstream control element (UCE), the -133 CpG methylation site and the TTF-I binding site (T_0) are shown.

7.1.7 Bacteria, cells & cell extracts

XL1-Blue, TG1, DH5α and JM110 *E. coli* strains were used for DNA plasmid amplifications. Cloning of the recombinant Bacmid DNA was performed with DH10α competent cells (Gibco BRL). Sf9 (*Spodoptera frugiperda*) insect cells were used for Baculovirus-directed protein expression. The DEAE 280 fraction (Schnapp and Grummt, 1996) and mouse whole cell extracts (from FM3A cells) were kindly provided by I. Grummt.

7.1.8 *Drosophila melanogaster*: maintenance, embryo collection and extracts

Fly maintenance and embryo collection was performed according to the rules established for the fly facility in the institute. *Drosophila* embryo extracts for chromatin assembly were kindly provided by G. Längst.

7.1.9 Recombinant histones

Recombinant histones were a common reagent, produced routinely in the department. Recombinant octamer were also a kind gift from K. Nightingale.

7.2 Methods

7.2.1 Working with DNA: standard procedures

Preparation of competent bacteria, transformation of electro-, or chemically-competent bacteria with DNA, amplification of plasmid DNA in bacteria, purification, concentration determination, restriction enzyme digestion, ligation of DNA fragments, analysis of DNA on agarose and polyacrylamide gels, and amplification of the DNA by the polymerase chain reaction (PCR) were performed according to the standard protocols (Ausubel, 1999; Hoffmann-Rohrer and Labaere, 2000; Sambrook et al., 1989; Sambrook and Rusell, 2001). In addition, plasmid DNA was prepared with plasmid purification kits (Qiagen) for different amounts of DNA. Isolation of DNA fragments from agarose gels was performed using the Qiagen Gel Extraction kit.

7.2.1.1 Radioactive labeling of DNA

DNA was either radioactive labeled by incorporation of a radioactive dNTP during PCR ('body labeling') or labeling of an oligonucleotide with T4 polynucleotide kinase ('endlabeling'). For bodylabeling, a standard PCR reaction was performed, to which α-^{32}P dCTP was typically added. For large amounts of DNA, a 1 ml PCR reaction was performed, containing 100 ng template, 500 pmole of each primer, 100 nmol of dATP, dGTP and dTTP, 20 nmole dCTP and 16.7 pmole (α-^{32}P)dCTP. Purification and removal of non-incorporated nucleotides was done by ethanol precipitation and subsequent gel isolation (section 7.2.1.2). Alternatively, oligonucleotides were endlabeled with γ-^{32}P-ATP using T4 polynucleotide kinase according to the manufactor's protocol. Non-incorporated nucleotides were separated from the labeled DNA using Sephacryl G25 spin columns (Roche). These oligonucleotides were used as primers in PCR reactions.

7.2.1.2 Precipitation and isolation of radioactive DNA fragments

DNA fragments were precipitated from the supernatant by adding 1/3 volumes of 7.5 M ammonium-acetate (pH 7.7) and 2.5 volumes of 100% ethanol, vortexed briefly and incubated on ice (10 min). Precipitates were pelleted (+4°C, 13200 rpm, 15 min.), washed with 70% ethanol and dissolved in 100 μl of EX-100. Depending on the purpose and purity of the amplified DNA, it was either used directly or gel purified. For gel purification, DNA was separated by polyacrylamide gel electrophoresis in 0.4 X TBE.

The wet gel was exposed on an X-Ray film, and the DNA fragment was excised from the gel. The gel piece was incubated with 1000 µl EX-300 and the DNA fragment eluted by vigorous shaking for at least 3 h at room temperature. Pieces of the gel were pelleted in an Eppendorf mini-centrifuge (RT, 16000 g, 1 min.). The eluted DNA (supernatant) was precipitated again and finally dissolved in 100 μl of EX-100. This DNA was subsequently used for nucleosome assembly reactions.

7.2.1.3 Annealing of double stranded oligonucleotides

Similar quantities of complementary single strand oligonucleotides were mixed in EX-50, denatured in a thermoblock (95°C for 5 min) and slowly (1-2 hours) chilled to room temperature (by switching the thermoblock off) to allow complete oligonucleotide annealing.

7.2.1.4 DNA methylation

DNA was methylated using Sss-I methylase (NEB) according to the manufactor's instructions. Briefly, 1 μg of DNA was incubated with 5U enzyme for 1 h at 37°C. For larger DNA amounts, the incubation time was prolonged. DNA methylation experiments in chromatin were performed with minor modifications. The reaction buffer contained only 2 mM $MgCl_2$, the incubation temperature was 26°C and the reaction was incubated for 60-75 min. In initial experiments, the optimal amount of Sss-I methylase resulting in complete DNA methylation was determined and subsequently used. Mock reactions did not contain the substrate S-adenosyl-methionine (SAM). Methylation efficiency was analyzed by DNA digestion with a methylation sensitive restriction enzyme (e.g. Hpa II).

7.2.2 Protein analysis: standard procedures

Protein analysis was performed according to the standard protocols (Ausubel, 1999; Hoffmann-Rohrer and Labaere, 2000; Sambrook et al., 1989; Sambrook and Rusell, 2001). Generally, proteins were kept on ice (4°C), in the presence of protease inhibitors (either complete® (Roche), or a mix of Leupeptin, Pepstatin, Aprotinin (all 1 μg/ml), PMSF (0.2 to 1 mM)) and reducing agents (DTT or ß-mercaptoethanol: 1 mM).

7.2.2.1 Determination of protein concentrations

Protein concentrations were determined using the colorimetric assay described by Bradford (Bradford, 1976). The concentration of purified proteins was also estimated according to protein standards with a known concentration (e.g. BSA) in SDS-PAGE followed by Coomassie blue staining.

7.2.2.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Pouring and electrophoresis of SDS-polyacrylamide gels was performed either using the Novex system (Pre-assembled gel cassettes) or the Biorad gel system. Resolving and stacking gels were prepared according to standard protocols using ready-to-use polyacrylamide solutions from Roth (Rotigel, 30%, 49:1 (for buffers see also section 7.1.2)). For electrophoresis, protein samples were mixed with SDS-PAGE sample buffer, heat-denatured for 5 min at 95°C and directly loaded onto the gel. Proteins were separated at 90 V until the samples had passed through the stacking gel and then at 150 V until the dye front had reached the end of the gel. The molecular weight of proteins was estimated by running pre-

stained or non-stained marker proteins (Bio-Rad, broad range and Peqlab, peqgold protein marker) in parallel. Following electrophoresis, proteins were stained with either Coomassie Brilliant Blue, Silver or subjected to Western blotting.

7.2.2.3 Coomassie blue staining of protein gels

Polyacrylamide gels were fixed for at least 30 min in fixation solution (50% methanol / 10% acetic acid) and stained for approximately 60 min on a slowly rocking platform with Coomassie staining solution (0.025% Coomassie Blue R in 10% Acetic acid). To visualize proteins, gels were destained in 10% acetic acid (tissue added, to accelerate destain). After documentation, the gels were dried onto a Whatman paper at 80°C for 2 h on a gel dryer (BioRad).

7.2.2.4 Silver staining of protein gels

The staining of protein gels with silver nitrate solution was carried out according to the protocol of Blum (Blum, 1987). The gel was fixed in 40% ethanol / 10% acetic acid for at least 2 h and washed 3 times in 30% ethanol (20 min each), incubated for 1 min in 0.02% $Na_2S_2O_3$ (sodium thiosulfate), washed 3 times with water (ddH_2O, 20 sec) and stained with 0.2% $AgNO_3$ solution for 1 hour. Afterwards, the gel was washed with water (3 times, 20 sec each) and developed with the developing solution (3% Na_2CO_3, 0.05% H_2CO, 0.0004% $Na_2S_2O_3$) until the desired proteins were visible (typically, after 5 to 10 min). After a short wash in water (1 min) the reaction was stopped by incubating the gel in 0.5% glycine stop solution (more than 5 min). After a final water wash (>30 min), the gel was documented and dried onto a Whatman paper at 80°C for 2 h on a gel dryer (BioRad).

7.2.2.5 Silver staining of histones

This fast method was preferable for visualizing basic proteins (histones). Histones were separated by standard SDS PAGE (17.5% PAA). Afterwards, the gel was soaked in 50% methanol for at least 3 h in order to remove the glycine. Staining solution: Solution A (0.4 g $AgNO_3$ dissolved in 2 ml H_2O) was added dropwise into solution B (10.5 ml of 0.36% NaOH (freshly prepared) (constant stirring) and mixed with 0.7 ml of 14.8 M Ammonium hydroxide (Sigma)). The staining solution was adjusted to a final volume of 50 ml with water. The gel was incubated for 15 min in staining solution and washed for 5 minutes in water (several changes). The gel was incubated for 5-10 min until the histones became visible in 100 ml developing solution (500 μl 1% citric acid, 50 μl 37% formaldehyde). Subsequently, the gel was rinsed with water and the reaction was quenched with 45% methanol / 10% acetic acid. After a final wash (>30 min) the gel was documented and dried.

7.2.2.6 Semi dry Western analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters or PVDF membranes using the Bio-Rad 'Trans-Blot SD Apparatus' for 1 h at 14 V. For larger proteins transfer was prolonged (up to 2 h) and the voltage was raised (up to 17 V). For protein transfer, the gel was sandwiched between gel-sized Whatman papers, soaked in anode or cathode buffers (3 pieces in each buffer). After transfer, nitrocellulose filters were incubated for 1 h in blocking solution (1x PBS, containing 5% dried milk and 0.2% Tween-20) in order to reduce the non-specific background. Filters were sealed in a plastic bag and incubated for 1 h with an appropriate dilution of the primary antibody directed against the protein of

interest. Filters were washed three times in PBS-Tween (10 min each) and incubated for one additional hour with horseradish peroxidase-coupled secondary antibody. After 3 washes (10 min each, in PBS-Tween) antigen-antibody complexes were detected using Enhanced Chemi-Luminescence Kit (ECL, Amersham) and autoradiography according to the instructions given. All steps were performed at RT.

Anode buffer I	Anode buffer II	Cathode buffer
300 mM Tris	25 mM Tris	70 mM CAPS, pH 10.5
15% methanol	15% methanol	15% methanol

7.2.2.7 Wet Blot Western analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters or PVDF membranes using the Bio-Rad 'Wet Blot system'. The gel was placed onto a membrane and sandwiched between gel-sized Whatman papers soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The proteins were then transferred onto the membrane for 1 h (120 V constant) at 4°C (cold room). The transfer reaction was cooled by the addition of an ice block into the transfer chamber. For larger proteins, the transfer was performed over night at 50 V (cold room). The immunoblot was continued (blocking, washing, incubation with antibodies and detection) as described (section 7.2.2.6).

7.2.3 Purification of cellular Tip5-Snf2h complex (NoRC)

Tip5-containing protein complexes were purified from mouse cell extracts using a combination of conventional and immunoaffinity chromatography. A whole cell extract (800 mg protein) was first fractionated on a DEAE-Sepharose™ Fast Flow column (Pharmacia) in AM-100 buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 1 mM DTT, 10% glycerol, and protease inhibitors (Complete™ (Roche)). Proteins were eluted with AM-280 buffer, Tip5-containing fractions were applied on a SP-Sepharose™ Fast Flow column (Pharmacia). Fractions eluting at 500 mM KCl (SP-500) were further purified on a Heparin-Ultrogel column. After washing with AM-300 buffer, cellular Tip5 was step-eluted with 500 mM KCl. Tip5 fractions were precipitated with 70% ammonium sulfate, resuspend in AM-300 buffer containing 0.01% NP-40, and size-fractionated on a Superose 6 HR 10/30 (Pharmacia) gel filtration column (see also (Strohner, 2000)). After concentration on a SP-Sepharose™ Fast Flow column, Tip5 containing fractions were incubated with 70 μl protein G-bound anti-Tip5 antibodies (2 h at 4°C), transferred into columns and washed with 20 column volumes of AM-300 and 5 column volumes of AM-500. Tip5 (complex) was eluted with the epitope peptide (0.3 μg/μl) in 80 μl of AM-300 / 0.1% NP40 buffer in the presence of protease inhibitors. Nucleosome mobility reactions were performed with immunopurified bead-bound cellular NoRC. The complex was purified by incubating whole cell extracts with bead-bound anti-Tip5N1-18 antibodies (3-5 μg IgGs per 10 μl protein A agarose) in buffer AM-300 containing 0.1% NP40 and protease inhibitors. A control reaction (mock beads, incubated with pre-immune serum) was performed in parallel.

7.2.4 Expression of recombinant proteins with the baculo virus system

The baculo-virus system allows high expression levels of large eukaryotic proteins. Furthermore, this system reflects eukaryotic cells in so much as the proteins are processed and posttranslationally modified.

7.2.4.1 Construction of recombinant baculo viruses

The Bac-to-Bac system (Life technology) was used to obtain fast and efficient recombinant baculo viruses. Briefly, the cDNA was cloned into a suitable pFastBac vector containing the polyhedrin promoter and flanking transposition elements (tn7). The pFastBac-(gene-of-interest) plasmid was transformed into *E. coli* DH10bac competent cells, which contained the bacmid DNA and a helperplasmid (encoding a transposase). Triple antibiotic and blue white selection resulted in the selection of the recombinant bacmid DNA (transposition of the gene-of-interest into the bacmid DNA). The presence of the desired cDNA in the high molecular weight bacmid DNA was checked by PCR (with gene-of-interest and bacmid primer). The recombinant bacmid DNA was transfected into Sf9 cells ($9x10^5$ cells in one well of a 6-well plate) with Cellfectin (Gibco BRL) to produce a first viral stock. This virus stock was amplified and used for protein expression (see below).

7.2.4.2 Virus amplification

Amplification was undertaken to preserve the virus stock and to gain a higher titer of virus (typical 10^7 to 10^8 plaque forming units (pfu/ml)) of the initial virus stock. A 15 cm diameter plate with $1.2x10^7$ Sf9 cells (attached) was infected with 0.5 to 1 ml of virus and incubated at 26°C. The plate was sealed with parafilm (NAS) to prevent dehydration. The supernatant was collected after 5 to 7 days of cultivation (check for high level of virus infection by comparing to mock transfected cells) and kept at 4°C in the dark. Infected cells appear granular, enlarged and contain puffed-up nuclei. The cells stopped growing and began cell lysis.

7.2.4.3 Infection of insect cells and expression of recombinant proteins

Sf9 cells were infected with the recombinant baculovirus and harvested after 48 to 72 hours. Optimal virus concentrations for high levels of heterologous gene expression were determined by test infections. $1,2x10^7$ Sf9-cells / 15 cm plate were seeded and allowed to attach in 5 ml of media. Several dilutions of the virus stocks were made (typically in the range between 50 μl and 1000 μl) and added. After incubation for 1 h on a rocking platform, fresh medium was added to a final volume of 20 ml and cells were incubated at 26°C for 48 h and 72 h. The cells were harvested, washed once with PBS and analyzed for protein expression (see section 7.2.5). Each time a virus was newly amplified, test expressions were performed. For expression of ACF and NoRC, first expression of the individual each subunits was performed. An optimal ratio for coexpression was established by mixing different amounts of the viruses. (constant amounts of ISWI / Snf2h and variable amounts of Acf1 / Tip5) before adding them to the cells. The optimal conditions established in these test expressions were then used for scaling up the infections (typically 15 to 30 times). After 48 to 72 h incubation, cells were harvested, washed once with PBS and stored at -80°C, or used direct for purification (see 7.2.5).

7.2.4.4 Culturing of Sf9 cells

Sf9 cells were cultivated in Sf-900 II medium (Invitrogen) supplemented with 4 mM N-acetyl-L-alanyl-L-glutamine, 63 mg/l penicillin, 50 mg/l streptomycin and 10% fetal calf serum (inactivated by incubation at 56°C for 20 min). Sf9 cells were grown in suspension or as monolayers at 26°C. Cell density of spinner cultures was kept between $5x10^5$ and $2x10^6$ cells/ml.

7.2.5 Purification of recombinant proteins

Figure 15 shows a combined result of the most commonly purified proteins during this thesis. Expression and purification was optimized for each protein as described in the following sections.

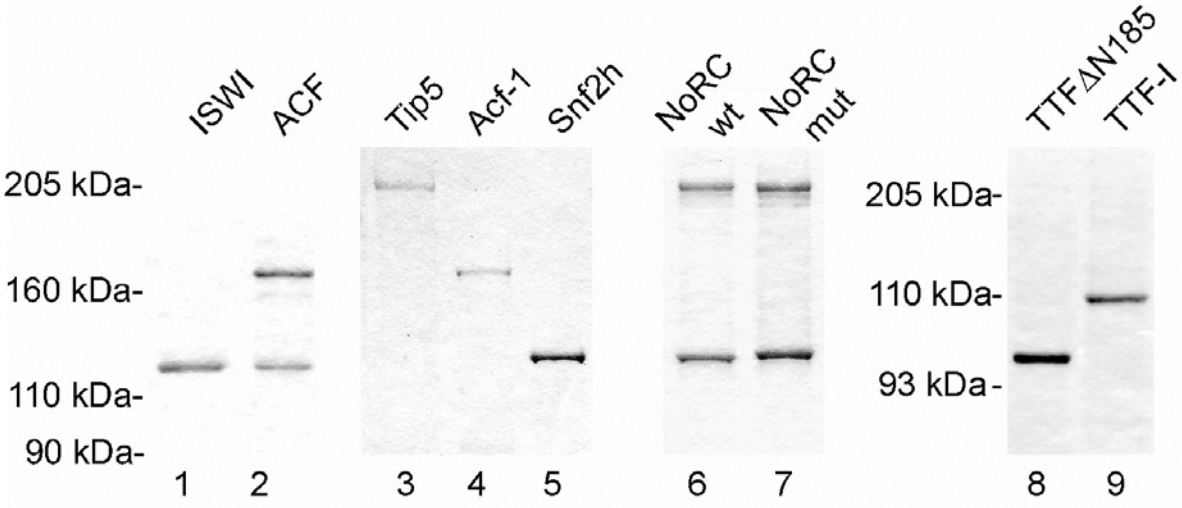

Figure 15. Purified recombinant proteins
The indicated proteins were expressed in Sf9 cells using the baculo-virus system, purified and analyzed by SDS-PAGE. Proteins were visualized by Coomassie blue staining. Relative protein sizes are indicated.

7.2.5.1 Purification of Histidine-tagged TTF-I (two steps)

Histidine-tagged TTF-I and TTFΔN185 were expressed in Sf9 cells and purified in two steps, by convential chromatography (Heparin column, Pharmacia) followed by Ni-NTA Agarose (Qiagen) purification (Figure 15, lanes 8 and 9). A typical purification started from $2.4x10^8$ infected Sf9 cells. Purification was performed in the cold room and samples were always kept on ice. Sf9 cell pellets were washed once with PBS and resuspended in 10 ml of lysis buffer K200 (200 mM KCl, 50 mM phosphate buffer pH 8.0, 10% glycerol), substituted with 0.2% NP40, 1 mM β-mercaptoethanol and protease inhibitors. Cells were opened by the freeze-and-thaw method (3 times rapidly frozen in liquid nitrogen and thawed again). The lysed cells were sonicated (3 times, each 30 sec, amplitude 50%, Branson: digital sonifier 250D) and cell debris was pelleted by centrifugation (Sorvall, SS34) for 30 min at 15.000 rpm and 4°C. The supernatant presents the whole cell extract and was used for protein purification.

Heparin chromatography:

TTF-I and TTFΔN185 bind to Heparin at low salt (~200 mM KCl) and elute between 400 mM and 700 mM KCl in phosphate-buffer. The cleared cell lysate was loaded on a 5 ml Hi-Trap Heparin column

equilibrated with K200. The column was washed with 5-10 column volumes (CV) K200 and TTF-I was eluted with a salt gradient, reaching from 200 to 1000 mM KCl over 4 column volumes. 2.5 ml fractions were collected and 10 µl samples of each fraction were analyzed on 7.5% Coomassie Gel and by western analysis. Fractions containing TTF-I were pooled, supplied with fresh protease inhibitors, β-mercaptoethanol (1 mM), 0.05% NP40 and 5 mM imidazole, and purified with Ni-NTA Agarose immediately.

Ni-NTA Agarose purification:

500 µl of Ni-NTA Agarose beads (Qiagen) were equilibrated in batch with K500 (500 mM KCl, 50 mM phosphate buffer pH 8.0, 10% glycerol), substituted with 0.05% NP40 and 5-10 mM imidazole. The equilibrated resin was incubated with the pooled TTF-I fractions (4°C, for at least 3 h, overhead shaker). The resin was washed 3 times with each 10 ml K500 (containing 10-20 mM imidazole) and loaded into a 2.5 ml column. The flow through was collected and reloaded once. Bound proteins were eluted from the Ni-NTA-Agarose by stepwise addition of elution buffer (7 times 200 µl of K500 containing 0.05% NP40 and 250 mM imidazole). Individual fractions were collected and purification efficiency was analyzed by SDS-PAGE (Coomassie blue staining). TTF-I containing fractions were pooled and dialyzed against buffer AM-100 / EX-100 containing DTT and PMSF (1 mM each). Samples were aliquoted, snap frozen in liquid nitrogen and stored at –80°C. Protein concentration was determined as described (7.2.2.1).

7.2.5.2 Purification of Snf2h, ISWI, Acf1 and ACF (via Flag-tag)

The recombinant proteins (Flag-Snf2h, Snf2h KR211-Flag, Flag-ISWI and Flag-Acf1) were purified with M2-agarose beads (Sigma) (Figure 15, lanes 1, 2, 4 and 5). ACF (coexpression of Flag-ISWI & Acf1) was purified similarly by its Flag-tagged ISWI subunit (modified from (Aalfs et al., 2001; Eberharter et al., 2001)). After optimizing protein expression and initial test purifications, a typical large-scale expression started from $2.4x10^8$ insect cells. Sf9 cell pellets were washed once with PBS and resuspended in 10 ml lysis buffer EX-500 (500 mM KCl, 20 mM Tris buffer pH 8.0, 10% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EGTA), substituted with 0.2% NP40, 2 mM β-mercaptoethanol and protease inhibitors. Cells were lysed by the freeze-and-thaw method (3 times rapidly frozen in liquid nitrogen and thawed again) and sonicated (3-4 times, 30 sec, amplitude 50%). The cleared cell lysate (centrifugation for 30 min at 15.000 rpm (Sorvall, SS34) at 4°C) was incubated with equilibrated M2-beads (typically 150 to 300 µl, the amount of M2 beads depended on the protein expression level) for at least 3 hours (4°C, overhead shaker). The beads were washed twice with 10 ml EX-1000 (ACF only with EX-500) and two times with 10 ml EX-500 (all supplemented with 0.05% NP40). Each washing step included 10 to 20 min incubation (overhead shaker, at 4°C); between the individual wash steps the beads were pelleted by centrifugation (2 min, 1000 rpm, 4°C). After the last wash step, the resin was transferred into a 1.5 ml tube and wash once with EX-300 / 0.05% NP40. The proteins were eluted from the M2-agarose with 1 bed volume of buffer EX-300, containing Flag peptide (200 ng/µl). Elution was repeated 2 to 3 times for several hours (once o/n) at 4°C (overhead shaker). Eluted proteins were dialyzed against EX-100 / EX-200 buffer containing 1 mM DTT and 1 mM PMSF, concentration was determined, samples aliquoted and stored at -80°C.

7.2.5.3 Purification of recombinant NoRC (two steps)

NoRC was prepared by coexpression of Tip5 and Flag-tagged Snf2h in Sf9 cells (Figure 15, lanes 6 and 7). The optimal expression ratio of Snf2h and Tip5 was determined in small-scale test-expressions and Flag-purification. Large-scale expressions were performed with $2.4x10^8$ Sf9 cells. To obtain stoichiometric amounts of Tip5 and Snf2h, the purification protocol combined a two-step purification. Tip5 (and associated Snf2h) binds to Biorex 70 chromatographic material (BioRad) at 300 mM KCl and elutes between 400-700 mM KCl. In contrast, free Snf2h does not bind to the column and is therefore present in the flow through. Alternatively, purification of the Flag-tagged Snf2h with M2-Agarose ('Flag-purification') is used to copurify associated Tip5 protein. Hence, the cell lysate was either first applied on a BioRex70 column and afterwards purified with M2 beads, or initially by Flag-purification followed by Biorex70 chromatography.

Biorex 70 chromatography followed by Flag-purification:

The cell pellet (1x PBS washed) was resuspended in 20 ml EX-300 / 0.2% NP40 and 1 mM β-mercaptoethanol and proteins extracted as described (7.2.5.1). The Tip5 / Snf2h containing supernatant was loaded onto a Biorex 70 column (column volume 10 ml, equilibrated with EX-300) and washed until the base line reached below a specific absorbance. Proteins were either step eluted with EX-700 or by a salt gradient (4CV up to EX-1000). Fractions were analyzed on 6% protein gels (10 μl samples) by Coomassie blue staining and western analysis against Tip5 and Snf2h. Tip5 (NoRC) containing fractions were pooled and supplied with fresh protease inhibitors, β-mercaptoethanol (1 mM) and 0.05% NP40. Flag-tag purification of Snf2h was performed similarly to the ACF purification (see section 7.2.5.2). Briefly, binding to equilibrated (EX-500) M2-agarose (50 to 100 μl) was performed o/n at 4°C. Beads were washed with buffer EX-500 containing 0.05% NP40 (3 times, each 15 min), transferred into 1.5 ml tube and washed once with EX-300, 0.05% NP40. The proteins were eluted several times with one bead volume of EX-300, 0.05% NP40, 200 ng/μl Flag peptide. Elution efficiency and protein concentration was analyzed by SDS-PAGE and Coomassie blue staining. Eluates were pooled, dialyzed against EX-200, aliquoted and stored at -80°C.

Flag purification followed by Biorex 70 chromatography:

Sf9 cell pellets were washed once with PBS, resuspended in 10 ml lysis buffer EX500 and lysed as described above. The cleared, Tip5 / Snf2h containing lysate was incubated with equilibrated M2-beads (typically 200-300 μl) for at least 3 h (4°C, overhead shaker). The beads were washed 3 times for 15-30 min with 10 ml EX500 (+0.05% NP40). After the last wash step, the resin was transferred into a 1.5 ml tube and washed once with EX-300 / 0.05% NP40. The proteins were eluted from the M2-agarose beads with one bed volume of buffer EX300, containing Flag peptide (200 ng/μl). Elution was repeated 2 to 3 times for several hours at 4°C (overhead shaker). Purification efficiency was analyzed by SDS-PAGE (Coomassie blue staining). Eluted proteins were pooled and used in a small-scale Biorex 70 chromatography (volumes of the small-scale chromatography dependent on the yield of the Flag purification). Proteins were bound to equilibrated (EX300) Biorex material in batch (2 hours, 4°C), washed with EX-300 and eluted with EX-700 (stepwise at 4°C). Finally, samples were dialyzed against buffer EX-200 (1 mM DTT and 1 mM PMSF), aliquoted, snap frozen in liquid nitrogen and store at -80°C.

7.2.5.4 Purification of recombinant Myc-tagged Tip5 (two steps)

Myc-epitope tagged Tip5 was purified by a two-step protocol (Biorex70 chromatography and Myc-affinity purification) (Figure 15, lane 3).

Biorex 70 chromatography

As described for the NoRC purification. Briefly, infected Sf9 cells ($2.4x10^8$ cells) were harvested, lysed in EX-300 / 0.1% NP40 and the cell lysate applied onto a Biorex 70 column. Tip5 was bound and consequently elutated at 700 mM KCl. Fractions were analyzed by SDS- PAGE (Coomassie blue staining and western analysis) and Tip5 containing fractions were pooled and subjected to Myc-tag affinity purification.

'Myc- tag affinity purification'

Purified α-Myc antibody (9E10) was bound to equilibrated Protein G beads, washed with EX-500 / 0.05% NP40 and stored at 4°C. Tip5 containing Biorex 70 fractions were incubated with the 'Myc-beads' o/n (4°C, overhead shaker). The beads were washed 3 times with EX-500 / 0.05% NP40 (15 min each), transferred into 1.5 ml tube and washed once more. Tip5 was eluted with EX-500 containing Myc-peptide (200 ng/μl) and fresh protease inhibitors at room temperature (2 times 1 hours). A third elution was performed o/n at 4°C. Elution efficiency and protein concentration was analyzed as described (7.2.5.4), proteins were dialyzed and stored at -80°C.

7.2.6 Superose 6 gelfiltration of recombinant proteins

Recombinant Snf2h, Tip5 and NoRC complex were loaded onto a Superose6 gelfiltration column (HR 10/30, Pharmacia) in EX-300. Individual fractions were analyzed by SDS-PAGE (western analysis or Coomassie blue staining). For size estimation the elution behavior was compared to protein standards from Pharmacia (thyroglobulin (670 kDa), apoferritin (440 kDa), aldolase (158 kDa) and BSA (67 kDa)).

7.2.7 Protein - protein interaction assay ('Pull-down' assay)

Flag-tagged proteins were immobilized on M2-agarose and control beads were saturated with the Flag-epitope peptide. To monitor the interaction of Tip5 with the N-terminal part of TTF-I, the beads (20 μl) were incubated for 4 h at 4°C in EX-300 buffer (0.1% NP-40) with similar amounts of purified TTFΔN185 and full length TTF-I. Beads were washed with 10 volumes of EX-300 (0.1% NP-40) and bound proteins were eluted with SDS-PAGE sample buffer. Usually 10% of the loaded (L), 10% of the non-bound (FT: flow-through) proteins, and 50% of the eluted (E) proteins were visualized by western blot.

7.2.8 Chromatin – preparation of histone octamers

7.2.8.1 Expression and purification of recombinant histones

Recombinant histone expression and purification was undertaken according to Luger and colleagues (Luger et al., 1999). Briefly, individual recombinant histones were expressed in *E. coli* and inclusion bodies containing the histones were prepared. The proteins were unfolded in urea buffer and the histones were purified via SP-Sepharose chromatography. The histones were dialyzed against water, lyophilized and stored at -80°C. Recombinant histones were a common reagent, produced routinely in the department.

7.2.8.2 Reconstitution of recombinant histone octamers

The histone octamer reconstitution was undertaken according to Luger with minor modifications (Luger et al., 1999). Lyophilized histones were resuspended in unfolding buffer (7 M Guanidinium HCl, 20 mM Tris pH 7.5, 10 mM DTT) in a final concentration of about 1 mg/ml. The exact protein concentration was calculated from the UV-absorption at 275 nm using the specific absorption coefficient of each histone (Luger et al., 1999). Equal amounts of histones were mixed and a similar volume of unfolding buffer was added. This mixture was dialyzed 3 times (twice 1 hour and once o/n) against one liter of refolding buffer (2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol). Aggregates were removed by a short centrifugation step (10 min, 15000 rpm, SS34 rotor (Sorvall)). The supernatant was concentrated to less than 2 ml by centrifugation in concentration tubes (Centricon 30 K; 7000 rpm, SS34 rotor (Sorvall), at 4°C successive 20 min). The concentrated sample was purified over a Superdex 200 gel filtration column in order to separate the refolded octamer from incomplete forms and precipitates (flow 1 ml/min, 2 ml-fractions, running in refolding buffer). Individual fractions were analyzed on a 17.5% SDS gel (see Figure 16A) and the suitable fractions containing stoichiometric histone octamers were finally concentrated by centrifugation (conditions as above) down to 1 ml. One volume of glycerol was added and the recombinant histone octameres were stored at -20°C (examples see Figure 16).

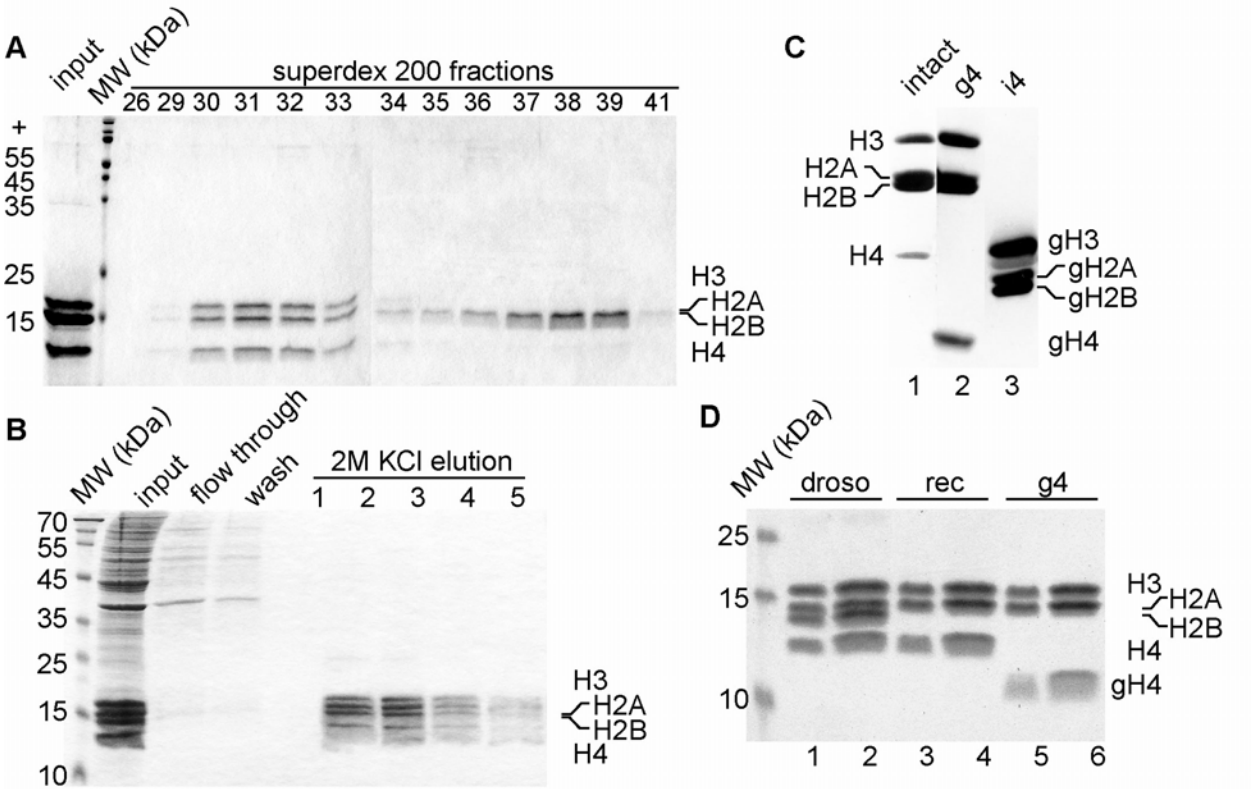

Figure 16. Purification of *Drosophila* and recombinant *Xenopus* histones
(**A**) Reconstitution of a recombinant histone octamer. Individual recombinant intact *Xenopus laevis* histones were expressed in *E. coli*, purified from inclusion bodies and reconstituted to an octamer as described. Shown is a Coomassie gel with fractions from a Superdex 200 gelfiltration, used to separate the octamer (fraction 30 to 33) from incomplete forms (e.g. fraction 35 to 39). Note that *Xenopus* histone H2A and H2B run similarly. (**B**) Purification of *Drosophila melanogaster* histones by High-salt buffer and Hydroxyl apatite chromatography. A Coomassie blue stained 17.5% SDS gel is shown, where individual fractions of the Hydroxyl apatite column where loaded. (**C**) Recombinant octamers kindly provided by K. Nightingale. Histone octamers were reconstituted from appropriate combinations of full-length and tail-less recombinant histones as indicated. Recombinant octamers with all histone tails (intact, lane 1), lacking the tail of histone H4 (globular: g4, lane 2) and containing only the histone H4 tail (intact: i4, lane 3) are shown. (**D**) Purified *Drosophila* histones (droso, lanes 1 and 2) and two different reconstituted recombinant histone octamer with all histone tails (rec, lanes 3 and 4) or lacking the tail of histone H4 (g4, lanes 5 and 6) are shown. Two different amounts of each proteins were resolved by 17.5% SDS-PAGE and stained with Coomassie blue.

7.2.8.3 Histone octamer purification from *Drosophila* embryos

Purification was done as established in the lab with Hi-salt buffer and hydroxyl apatite chromatography (Simon and Felsenfeld, 1979). The protocol is adapted from P. Korber. A typical result is displayed in Figure 16B and D.

Isolation of histones from nuclei:

- resuspend embryos (50 - 100g) in 40 ml of Lysis-buffer (15 mM Hepes pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 1mM DTT and PMSF)
- homogenization with Yamamoto homogenisator: 1000 rpm, coldroom, 6 times
- spin 10 min, 8000 rpm, HB-6 rotor, use two 30 ml Corex tubes with the thin rubber adaptors
- you get three phases: dark and very solid bottom, soft and light brown jelly on top of that (the nuclei which you want) and a liquid supernatant with a lipid skim layer at the meniscus)
- take off the supernatant as complete as possible without removing any of the nuclei

- fill a fresh 10 ml pipette with Suc-buffer (15 mM Hepes pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1.2% (v/v) sucrose, 1mM DTT an PMSF) and wash out the nuclei by pipetting up and down (the dark bottom phase will not be disturbed, sits there like a rock), transfer these resuspended nuclei into fresh, cold Corex tubes, use 50 ml Suc-buffer in total and spin again as above
- wash in 50 ml Suc-buffer as above (avoid carrying over remnants of the dark bottom pellet)
- resuspend nuclei in Suc-buffer: 30 ml final volume
- add 90 μl 1 M $CaCl_2$
- warm up resuspended nuclei for 5 min at 26° C (waterbath)
- add 125 μl of 50 u/μl MNase and protease inhibitors in addition to PMSF, e.g. aprotinin, pepstatin, leupeptin.
- incubate 10 min at 26°C [In theory one should test with small aliquots the right amount and / or time for this MNase I digest. The aim is to obtain as many mononucleosomes as possible without further degradation to subnucleosomal particles. With our MNase I stock the above conditions work just fine. Our MNase I stock is made in the following way: Add 300 μl of 20 mM TrisHCl, pH 7.6, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10% glycerol, 80 (or 50) mM KCl to 1 vial of Nuclease S7 with 15000 units (Roche) Let dissolve by light twirling around in the vial and flash freeze in 50 μl aliquots, store at -20° C.]
- stop: 600 μl 0.5 M EDTA and spin immediately as above
- resuspend pellet in 6 ml TE pH 7.6, 1 mM DTT, 0.2 mM PMSF (added freshly)
- lysis of nuclei: rotate in coldroom for 30-45 min; while doing that: wash 30 ml Biorex hydroxylapatite (You can process 100 g embryos per 30 ml hydroxylapaptite.) in 50 ml Greiner tube with 0.63 M KCl, 0.1 M K-phosphate (K_2HPO_4 / KH_2PO_4) buffer (rotating wheel, 5 times for 5 min, fill up the tube each time with fresh buffer, spin down hydroxylapaptite in Heraeus centrifuge, short spin ca. 25 sec., (< 1400 rpm, otherwise pellet is to tightly packed)
- spin the lysed nuclei: 30 min, 12000 rpm, HB-6, 4°C in 30 ml Corex tube
- keep supernatant (rather big pellet to be seen)
- adjust salt to 0.63 M KCl by adding the 2 M KCl 0.1 M K-phosphate buffer, estimate volume with 10 ml glass pipette (Volume to be added = Vol of SN / [2/0.63-1])
- add lysed nuclei to washed hydroxylapatite in 50 ml Greiner tube, resuspend and rotate for at least 60 min (coldroom) to bind the mononucleosomes; keep aliquot for documentation gel
- wash in batch as above with the 0.63 M KCl buffer, keep aliquots of the supernatants for documentation gel, include protease inhibitors with the last wash

Hydroxylapatite column:

- load the washed hydroxylapatite with the bound material into a FPLC-column (rinse with some 0.63 M buffer (0.63 M KCl, 0.1 M K-phosphate pH 7.2)
- have the 0.63 M buffer in pump A and the 2 M buffer (2 M KCl, 0.1 M K-phosphate, pH 7.2) in pump B
- wash (1 ml/min) the column with 0.63 M KCl for at least one column volume or till steady UV-baseline, autozero the baseline
- elute with 100% pump B, 0.5 ml/min, 3 ml fractions (small 4 ml-plastic tubes), fractions can be safely kept in the coldroom over night
- test 15 μl each of peak and surrounding fractions on 17.5% SDS-PAGE, run at high voltage (200 V) to reduce the smeariness of the lanes due to the high salt, have histone-prep in comparison on the gel
- pool fractions, concentrate with Filtrons (10 kD cut off, 3.5 ml or 20 ml scale, 7000 rpm, SS34, 4°C, takes some hours, don't add glycerol before the concentration step)
- adjust to 40-50% glycerol final concentration, store at -20°C with protease inhibitors and DTT
- estimate concentration by comparison with old histone preparation and / or BSA on SDS-PAGE, make sure if H3 (uppermost band of the four) is intact or degraded.

7.2.9 Chromatin – assembly and analysis of arrays

7.2.9.1 Chromatin assembly using the *Drosophila* embryo extract (DREX)

Drosophila embryo extract (DREX) assembly was performed as described (Becker and Wu, 1992). A standard assembly reaction contained 900 ng of circular DNA, 12 μl McNAP (30 mM $MgCl_2$, 10 mM DTT, 30 mM ATP, 300 mM creatine phosphate, 10 μg/ml creatine phosphate kinase) and varying amounts of *Drosophila* embryo extract (20-70 μl, depending on extract). The volume was increased with EX-80 to a final volume of 120 μl. Chromatin assemblies were performed in 0.5 ml PCR tubes in a Perkin Elmer PCR machine for 6 hours at 26°C. The quality of the assembled chromatin was analyzed by MNase digestion.

7.2.9.2 Chromatin assembly using the salt gradient dialysis technique

Nucleosomes were assembled from DNA and histones by the salt gradient dialysis technique according to Rhodes (Rhodes and Laskey, 1989). The assembly reaction was performed in the lid of siliconized 1.5 ml tubes (Biozym). Preparation of the assembly tubes (see also Figure 17): 3.5 kDa dialysis membranes (Spectrapor) were pre-wet for 5 min in High salt buffer (Hi-buffer). This membrane was placed over the lid of a 1.5 ml tube with a big hole (O-ring). The membrane was fixed with a second tube, where the bottom was cut and the lid removed. The tubes were placed (in a Styrofoam floater) into a 3 l beaker filled with 300 ml Hi-buffer (containing a magnetic stirrer). Air bubbles below the membrane were removed with a bent pasteur pipette. Finally the assembly reaction (see below) was pipetted into the lid.

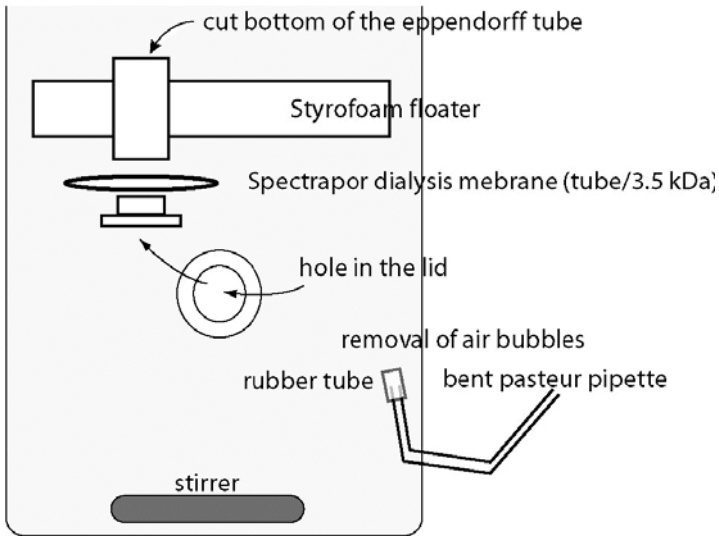

Figure 17. Diagram of chromatin assembly by the salt dialysis technique (kindly provided by G. Längst)

Assembly reaction: A typical reaction contained 1 μg DNA with varying amounts of histone octamer in 40 to 50 μl total volume. To estimate an optimal histone to DNA ratio, different amounts of histones were tested compared to a fixed amount of DNA (test assemblies, titration around DNA to histones ratio of 1:1.3). Assembly was done in High salt buffer (adjust the salt concentration with 5 M NaCl) supplemented with 200 ng/μl CEA. For larger assemblies the optimized histone / DNA ratio from the test assembly was changed by reducing the histone amount by 10%.

Salt gradient dialysis: The assembly mix was added into the tubes and stirred for approximately 30 min in High salt buffer. Salt gradient dialysis was done by continuous addition of low salt buffer (a total of 3 l) into the beaker over a period of 16-20 hours at room temperature. Finally, the assembly reaction was dialyzed against low salt buffer (300 ml) for 1 hour, characterized and stored at 4°C.

High salt buffer	Low salt buffer
10 mM Tris/HCl pH 7.6	10 mM Tris/HCl pH 7.6
2 M NaCl	50 mM NaCl
1 mM EDTA	1 mM EDTA
0,05% NP40	0,05% NP40
2 mM β-mercaptoethanol	1 mM β-mercaptoethanol

7.2.9.3 Chromatin analysis by Micrococcal Nuclease (MNase) digestion

Micrococcal nuclease (MNase) cleaves DNA preferentially in the linker region between individual nucleosomes. Partial MNase digestion generates a so-called nucleosomal ladder, thereby allowing qualitative analysis of the obtained grade of chromatin assembly (examples are shown in Figure 18). Typically, 900 ng of chromatin was partially digested with MNase (MNase concentration has to be optimized for each assembly method) for 30, 60 and 300 sec in the presence of 3 mM $CaCl_2$. The reaction was stopped by the addition of 0.2 vol of stop solution (4% SDS, 100 mM EDTA). Prior to deproteinization, DREX assembled chromatin was incubated with RNase for 1 hour at 37°C. All reactions were supplemented with proteinase K (10 μg / reaction) and glycogen (10 μg) and deproteinized for at least 1 hour at 40°C. The DNA was purified by ethanol precipitation (0.5 vol 7,5M NH_4Ac and 2.5 vol 100% ethanol were added, incubated for 10 min on ice, centrifuged for 15 min at 13000 rpm and washed once with 70% ethanol), air dried, dissolved in 10 μl loading buffer and analysed on 1.3% agarose gels (stained afterwards with ethidium bromide).

7.2.9.4 Purification of chromatin by a sucrose gradient

Chromatin was purified by ultracentrifugation (SW-41, 45 krpm, 14 h) in a 15-30% sucrose gradient. Individual fractions were then analysed by MNase digestion and supercoiling assay in collaboration with Gernot Längst.

7.2.9.5 Chromatin analysis: Measuring of DNA supercoiling

For DNA supercoiling analysis, chromatin (1 μg) was incubated for 1 hour with 10 U of topoisomerase I (Promega), deproteinized with 50 μg proteinase K for 1 h at 50°C. DNA was separated by electrophoresis at 100 V for 24 h in 1.2% Tris-glycine agarose gels containing 3.3 mM chloroquine, and visualized by ethidium bromide staining. Chromatin fractions exhibiting a high density of DNA supercoils were used for chromatin transcription assays (see Figure 18).

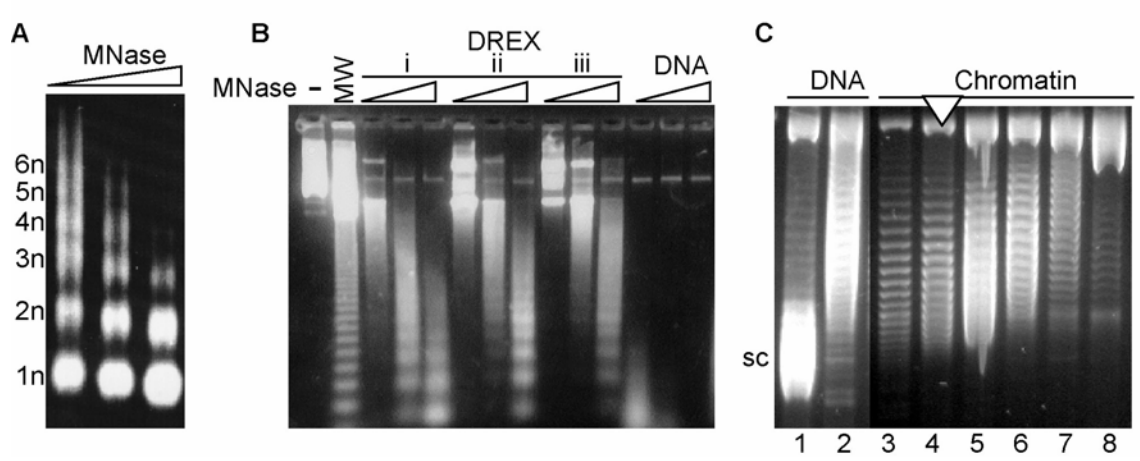

Figure 18. Assembly and analysis of chromatin arrays
(**A**) Reconstitution of chromatin arrays using gradient salt dialysis. Different ratios of histone to DNA were used in salt assembly and tested for nucleosome occupancy. Nucleosome arrays were analyzed using partial MNase digestion for different times (10, 30, 300 sec). Purified DNA was visualized by agarose gel electrophoresis and ethidium bromide staining. The regular fragment ladder indicative of the nucleosomal array is shown (1n-6n). (**B**) Assembly of chromatin using *Drosophila* embryo extracts (DREX). Different ratios of DREX (increasing from i to iii) to DNA were tested. Analysis as described in A. (**C**) Supercoiling assay to analyze chromatin quality. Nucleosomes were assembled on circular plasmid DNA (pMrWT-T) by salt dialysis and purified in a sucrose gradient. Individual fractions were incubated with topoisomerase I and the topoisomer distribution of the purified DNA was visualized on agarose gels containing chloroquine. Supercoiled DNA (sc, lane 1), partially relaxed DNA (lane 2) and fractions with decreasing nucleosome density (lanes 3 to 8) are shown. The nucleosomal fraction used for the experiments is indicated by a white triangle (Strohner et al., 2004).

7.2.10 Chromatin – preparation of positioned mononucleosomes

Mononucleosomes were reconstituted on rDNA promoter fragments of 146, 171 and 247 bp in length. Nucleosomes assembled on the 146 and 171 bp DNA fragments preferentially occupied one visible position. In contrast, nucleosomes assembled on the 247 bp rDNA promoter fragment displayed two major distinct positions that could be separated by native gel electrophoresis (Eberharter et al., 2003; Längst et al., 1999). Faster migrating nucleosomes are located at the periphery of the DNA fragment, whereas the slow migrating nucleosomes occupy positions at the center of the DNA. These positioned mononucleosomes were separated by native gel electrophoresis, purified from the gel and further used. All nucleosome containing reactions were performed in siliconized tubes (Biozym).

7.2.10.1 DNA fragments

The DNA fragments spanned sequences between -231 and +16 relative to the transcription start site of the mouse rDNA promoter (247 bp: -231 to +16; 171 bp: -231 to -61; 146 bp: -231 to -86). The DNA fragments were prepared by PCR. For radioactive labeling, α-^{32}P dCTP was added to the PCR reaction. Alternatively, one primer was endlabeled with T4 polynucleotide kinase and used in PCR reactions (see also 7.2.1.1). Purified DNA fragments were subsequently used for nucleosome assembly reactions.

7.2.10.2 Assembly of mononucleosomes (via HP-Mix)

Mononucleosomes were reconstituted using the polyglutamic acid chaperone method (Stein et al., 1979). Either recombinant histone octamers or purified *Drosophila* histones were mixed in a 1:2 ratio

with polyglutamic acid (Sigma, P4886) in 0.1-0.15 M NaCl (30-60 μg histones in 1 ml) and incubated for 90 min at room temperature. Precipitates were removed by centrifugation at RT (5 min at 13.000 rpm). The supernatant was aliqouted and stored at -20°C (= HP-Mix). A typical assembly included test assemblies with different ratios of DNA and HP mix to reveal optimal conditions and then a preparative nucleosome assembly. 0.1-0.2 μl of labeled DNA (in 10 μl EX-100, 200 ng/μl CEA) was incubated with increasing amounts of HP-Mix for 90 min at 30°C and analyzed by electrophoretic mobility shift assays (see Figure 19A). The optimal condition of the test assembly reaction (e.g. lane 6 in this experiment) was scaled up for a large preparative assembly (100 to 200 fold) and the different translational positioned nucleosomes were isolated (see below).

7.2.10.3 Isolation of positioned mononucleosomes

Nucleosomes were assembled in preparative scale (section 7.2.10.2) and the different translational positions were separated on 4.5% polyacrylamide gels in 0.4x TBE (see Figure 19B), either run at RT for 3 to 4 hours (120 V) or o/n at 4°C (60 V). The wet gel was exposed for 15 min to an X-Ray film and the different translational positioned nucleosomes (and free DNA) were isolated. Nucleosome positions were precisely marked on the autoradiogram, and the gel slices corresponding to the separated nucleosomes were cut out. Gel pieces were transferred into a siliconized 1.5 ml tube and 400 to 500 μl of EX-50 buffer containing 200 ng/μl CEA, 1 mM DTT and PMSF was added. The nucleosomes were shake-eluted from the gel pieces (occasionally crushed), once for 90 min and once o/n (all at 4°C) and stored at 4°C.

7.2.10.4 Preparation of non-labeled positioned nucleosomes

In addition to HP-mix assembly, salt gradient dialysis was performed to obtain non-labeled positioned nucleosomes. Salt gradient dialysis was performed as described above (section 7.2.9.2) with minor modifications. Briefly, approximately 100 ng of purified 247 bp DNA was assembled into chromatin with different amounts of histones (test assembly). Nucleosomes were separated on 4.5% polyacrylamide gels in 0.4x TBE and stained with ethidium bromide or SYBR green (Roche). The optimal ratio of histones to DNA was consequently used for a preparative assembly (20 to 40 fold). Different positioned nucleosomes were separated, stained and gel slices corresponding to the positioned nucleosomes were excised. Elution was performed as described in 7.2.10.3.

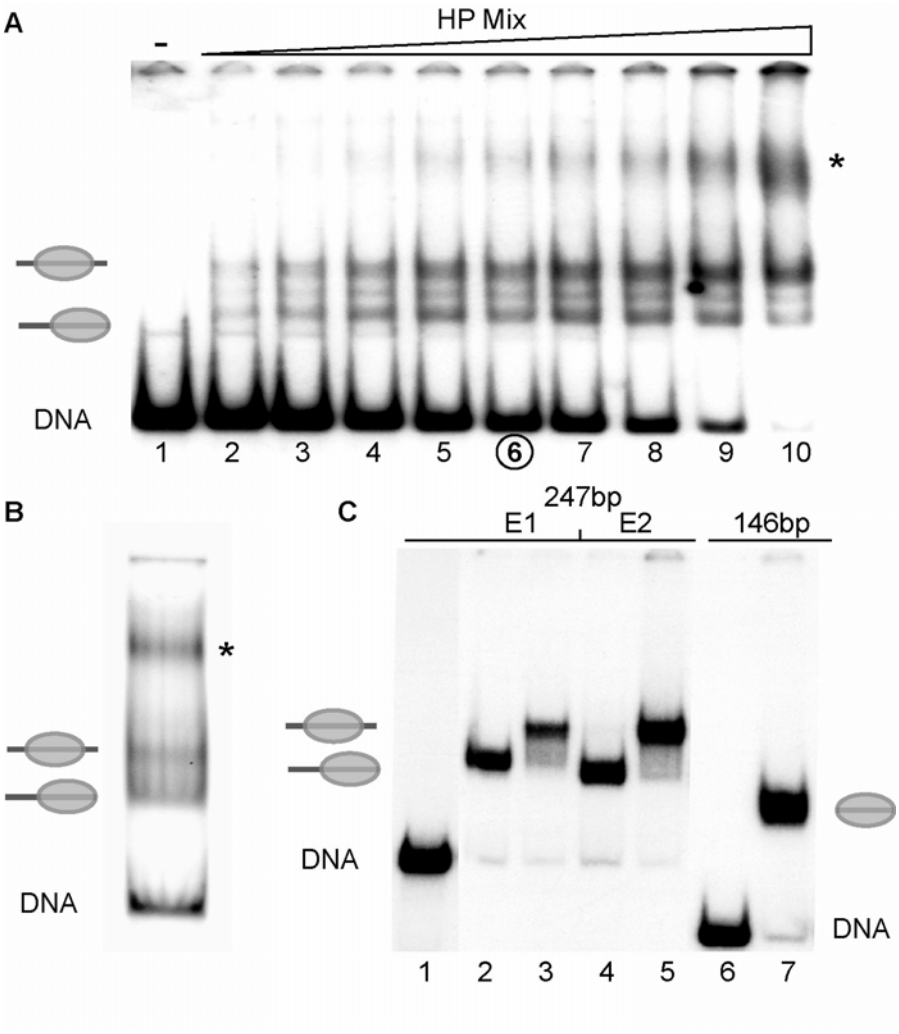

Figure 19. Reconstitution of positioned mononucleosomes
(**A**) Test assembly. Nucleosomes were assembled on a radioactively labeled 247 bp DNA fragment using an histone / polyglutamic acid mix (HP-mix). The assembly reactions were separated on a 4.5% polyacrylamide gel in 0.4x TBE and visualized by autoradiography. The free DNA and the nucleosomal positions are indicated on the left. Aggregates are marked by an asterisk. The circle marks the ratio, which is subsequently used for the preparative assembly. (**B**) Preparative assembly: The appropriate ratio between DNA and HP mix determined in the test reaction (A, lane 6) was scaled up (150 fold) to obtain preparative amounts of the nucleosomal substrate. The assembly is separated on native gel electrophoresis as described. Free DNA and the nucleosomal positions are indicated on the left. Aggregates are marked by an asterisk. (**C**) Isolated positioned mononucleosomes. The different translational positioned nucleosomes of the preparative assembly (B) were cut out of the gel and eluted. 2 μl of each eluted nucleosome was separated on a 4.5% PAA gel and visualized through autoradiography. E1 and E2 represent the two subsequent elutions of the central (lanes 3 and 5) and end positioned nucleosomes (lanes 2 and 4). The purified 247 bp DNA is shown in lane 1. In addition, a purified 146 bp nucleosome (lane 7) and the respective DNA (lane 6) are shown. The positions of the nucleosomes and free DNA are indicated.

7.2.10.5 Analyzing the histone composition within nucleosomes

The histone composition of positioned nucleosomes was analysed by a two dimensional approach, combining 4.5% native gel electrophoresis (for separating nucleosomes, Figure 20A) and 17.5% SDS-PAGE (for separating histones, Figure 20B). Gel slices containing the nucleosomes were excised and placed directly into the loading well of a protein gel. Gel pieces were covered with protein sample buffer, histones separated by SDS-PAGE and visualized by silver staining.

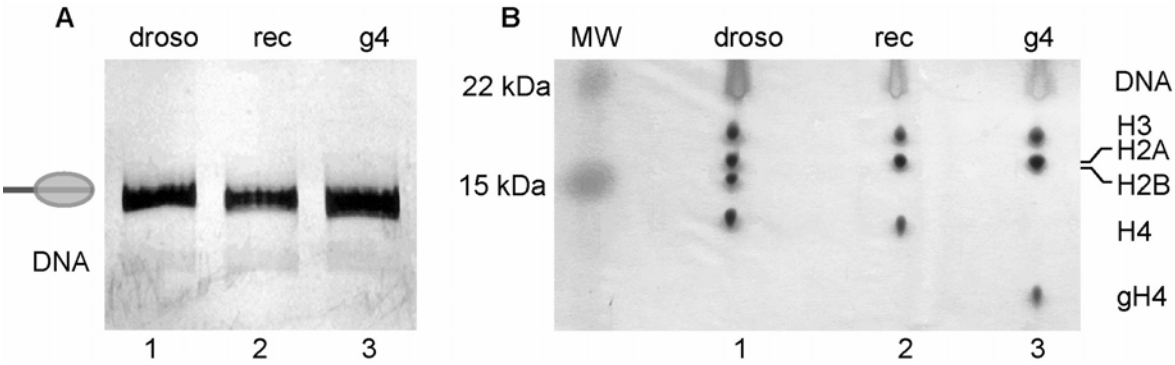

Figure 20. Histone composition of positioned mononucleosomes
(**A**) End positioned nucleosomes (~100 ng), assembled from different histone sources, were separated on a 4.5% PAA gel and visualized through ethidium bromide staining. Either purified *Drosophila* histones (droso, lane 1) or recombinant *Xenopus* histone octamer containing all tails (rec, lane 2) or lacking the histone H4 tail (g4, lane 3) were used as histone source for the assembly. The nucleosome positions and free DNA are indicated. (**B**) SDS-PAGE of the positioned nucleosomes in A. Gel slices, containing the nucleosomes were excised out of the gel, placed directly into the loading well of a 17.5% protein gel and covered with protein sample buffer. After running the gel, histones were visualized by silver staining. Histone octamer sources as described in A. The individual histones and the DNA are depicted at the right. Note that recombinant *Xenopus* histones H2A and H2B run at a similar size in these gels.

7.2.11 Chromatin – functional assays

7.2.11.1 ATPase assay

ATPase assays were performed in the presence of γ^{32}P-ATP (3000 Ci/mmol, 10 mCi/ml, NEN) with various substrates to analyze the specific activity. A typical reaction contained 150 ng of either DNA or chromatin in 10 to 15 μl reaction volume in following buffer conditions: 20 mM Tris pH 7.6, 50-75 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 1mM ß-mercapto ethanol, 10% glycerol, 200 ng/μl CEA, in the presence of 10 μM ATP together with 0.1 μl of γ^{32}P-ATP. The reaction was kept on ice and incubated at 26°C after the addition of the analyzed proteins. At different time points (typically after 30 and 60 min) 1 μl of the reaction was spotted on a thin layer chromatography cellulose plate (Merck) and air-dried. The hydrolyzed phosphate was separated from unreacted ATP by thin layer chromatography in 0.5 M LiCl / acetic acid buffer. Samples were separated until the buffer reached the top of the plate (approximately 20 min). The plates were dried at 60°C for 5 min and exposed on Phospho Imager (FujiFilm BAS-1500). ATP and hydrolyzed phosphate spots were quantified using the AIDA software. The percentage of hydrolyzed ATP was calculated according to the following equation: (amount of hydrolyzed phosphate) divided by (amount of left ATP and hydrolyzed phosphate). Reactions were repeated several times, given similar results even if the absolute numbers were different.

7.2.11.2 HDAC assay

The DEAE fraction used for *in vitro* transcription was incubated for 60 min at 30°C with 2 µl ^{3}H-marked chicken histones (25000 cpm/µl, kindly provided by A. Brehm) in the presence or absence of 500 nM TSA under transcription condition. The reaction was supplied with 230 μl of AM100 and 65 μl of 1 M HCl / 0.16M acetic acid solution. 700 µl of ethyl acetate was pipetted on top, reaction mixed by

vortexing and the different phases separated by short centrifugation. Afterwards 500 µl of the upper phase, containing the free, labeled acetate, was taken and analyzed by scintillation counting.

7.2.11.3 TTF-I footprinting

Primer extension experiments, indirect endlabeling (southern blot) for TTF-I footprinting and analyzing the remodeling event at the rDNA promoter were done in collaboration with Gernot Längst as described (Längst et al., 1998; Strohner et al., 2004).

7.2.11.4 Nucleosome mobility assay

The nucleosome mobility assay ('nucleosome sliding assay') assay allows the visualization of single nucleosome movements, catalyzed by ATP dependent nucleosome remodeling factors (Längst et al., 1999). All reactions were performed in siliconized tubes (Biozym). A typical reaction contained 30 to 60 fmol of radioactive labeled mononucleosomes with defined translational positions in a total volume of 12 µl in EX-75 containing 1 mM ATP, 1 mM DTT and 200 ng/µl CEA. The nucleosomes were incubated with the proteins indicated in the individual figures for 60-90 min at room temperature (or 26°C). The reaction was stopped by the addition of 200-400 ng competitor DNA (plasmid DNA or the non-radioactive 247 bp PCR fragment) and further incubation for 5 min. Nucleosome positions were resolved by native gel electrophoresis in 4.5% polyacrylamide gels in 0.4x TBE. Gels were pre-electrophoresed for 1 hour and run for 3 hours at 100-130 V. Optimal running time was controlled by using orange G as a marker. After separation of the nucleosomes, gels were dried (1 h at 80°C) and nucleosome positions visualized by autoradiography. Reactions with non radioactive nucleosomes ('cold sliding') were performed similarly, with slight modifications for detection. After separation of the nucleosomes positions by native gel electrophoresis, the nucleosomal DNA was visualized by staining with ethidium bromide. Documentation was performed with the gel documentation system (Pharmacia). The amount of nucleosomes used was approximately 20 times higher as compared to the radioactive assay.

7.2.12 Protein / DNA and protein / nucleosome interaction studies

7.2.12.1 Electrophoretic mobility shift assays (EMSA)

Radioactivly labeled DNA and positioned mononucleosomes were used to study protein-DNA and protein-nucleosome interactions. The interactions were analyzed by electrophoretic mobility shift assays (EMSA). A typical reaction contained 10 to 50 fmol (2000-5000 cpm) of DNA or nucleosomes in a total volume of 12 µl. Reaction were typically performed in EX-50 to EX-100, containing 200 ng/µl CEA and competitor DNA if indicated. Incubation time and temperature was dependent on the proteins used (see below). After incubation, protein / DNA (protein / nucleosome) complexes were separated from free DNA (free nucleosomes) by native gel electrophoresis. The reactions were either loaded on pre-electrophoresed 3.3-5% PAA gels in 0.4x TBE and run for 3 h at 100-140 V at RT, or alternatively on 1.3% agarose gel in 0.4x TBE at 4°C (120 V, 90 min). Optimal running time was controlled by using orange G as a marker. Gels were dried and analyzed by autoradiography. The DNA binding activity of recombinant TTF-I was analyzed in EMSA as described (Smid et al., 1992) with slight modifications. A purified rDNA promoter fragment containing a specific binding site (the proximal terminator T_0) was

used. A typical reaction contained 10 fmol of DNA in EX-100, in a total volume of 12 µl, supplemented with 200 ng/µl CEA and 100 ng/µl poly dI/dC. Incubation with the indicated TTF-I polypeptides was performed at 30°C for 30 min except for the V8 Protease treated reactions. After 15 min incubation, 1 µl of 100 ng/µl of V8 protease was added and incubation was continued for another 15 min. DNA and nuclesome binding activities of Tip5, Snf2h, NoRC, Acf1, ISWI and ACF were studied with DNAs and nucleosomes differing in their linker DNA, as described in the figure legends. Briefly, proteins were incubated with the radioactive labeled probe for 10 to 20 min on ice. Indicated antibodies and DNA (in DNA competition assays) were added prior to the reaction. Protein / DNA- and protein / nucleosome - complexes were resolved on 3.5% to 5% polyacrylamide gels or on 1.3% agarose gels. The gels were dried and analyzed by autoradiography. The cruciform DNA was a kind gift from F. Hartlepp and was prepared as described in (Bianchi, 1988; Bianchi et al., 1989).

7.2.12.2 DNase I protection assays 'DNase I footprinting'

Protein - DNA and protein - nucleosome interactions were studied in DNase I protection assays ('DNase I footprinting'). Endlabeled DNA was either used directly or assembled into nucleosomes. Purified DNA and positioned nucleosomes were incubated in EX-75 containing CEA and DTT and mildly treated with DNase I for 10, 30 and 90 sec. DNase I was inactivated by the addition of EDTA to a final concentration of 5 mM. The reaction was proceeded (not for NoRC, see below) by proteinase K digestion and precipitation of the DNA as described in section 7.2.1.2. The purified DNA was dissolved in 80% formamide and analyzed on sequencing gels (7% polyacrylamide, 40% urea, 1x TBE). DNase I footprinting with TTFΔN185 was performed with purified, endlabeled 146 bp DNA (rDNA promoter -231 to -86) containing a specific binding site (proximal terminator T_0). After incubation for 15 min, allowing DNA / protein complex formation, the samples were mildly treated with DNase I, stopped and analyzed. DNase I protection assays with NoRC were performed as described for ISWI (Längst and Becker, 2001a) with minor modifications. A 247 bp purified rDNA promoter fragment, labeled at either position -231 or +16 relative to the rDNA transcription start site, was assembled into mononucleosomes. Central positioned nucleosomes were isolated and used for DNase I footprint assays. Optimal binding conditions (ratio of NoRC to nucleosome) were estimated in gel retardation assays (5 min incubation at 4°C). The EMSA reaction was scaled up (approximately 50 times) and treated with DNase I for different time points. Reactions were stopped by addition of EDTA and directly loaded on 4% PAA gels containing 0.4x TBE. Free nucleosomes and nucleosome / protein complexes were isolated from the gel, processed and analyzed as described above.

7.2.13 *In vitro* Pol I transcription assay

Pol I *in vitro* transcription experiments were performed in general on DNA constructs, containing fusion of mouse rDNA promoter and terminator sequences (rDNA minigene). pMrWT-T contains mouse rDNA promoter sequences from -170 to +155 including the upstream terminator T_0 at position -170 and a 3.5 kb 3'-terminal rDNA fragment containing 10 terminator elements (T_1-T_8). The promoter and the terminator elements are separated by a transcribed spacer region of 686 bp (plasmid DNA). pMr SP- and pMr SP+BH contain a longer murine 5´-rDNA promoter fragment (-292 to +155) fused to the 3´-terminal rDNA fragment. pMr SP+BH contains an 170 bp fragment insertion (BH fragment) in the transcribed region. The rDNA elements are spaced by 686 bp of plasmid DNA (pMr SP-) and 856 bp (pMr SP+BH), respectively.

A typical transcription assay was performed in a volume of 25μl, containing 12 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 0.5 mM DTT, 2 mM $MgCl_2$, 80 mM KCl, 10 mM creatine phosphate, 12% glycerol, 0.66 mM each of ATP, UTP, GTP, 12.5 μM CTP and 1.5 μCi of a-^{32}P CTP with either 20 ng of naked DNA or chromatinized template. As a source for transcription factors 5 μl nuclear extract proteins, that have been partially purified by chromatography on DEAE-Sepharose CL-6B (DEAE-280 (Schnapp and Grummt, 1996)), were added. Transcription assays were incubated for 60 min at 30°C with purified TTF-I, NoRC, Snf2h, Tip5 and ACF added prior transcription extract. Transcription reactions were positively influenced through the presence of Rammstein during the incubation period (Rammstein, 1995; Rammstein, 2001). Reactions were stopped by the addition of 25 μl stop buffer (10 μg glycogen, 2% SDS, 10 μg proteinase K, 100 mM EDTA) and incubated for 1 h at 40°C. Transcripts were precipitated by the addition of 25 μl 7.5 M NH_4Ac and 200 μl pure ethanol and incubation on ice for 15 min. Transcripts were pelleted and purified from the vast of free nucleotides by centrifugation (4°C, 15 min, 13000 rpm (Eppendorf table centrifuge 5415R)). After washing once with 70% ethanol, the pellet was dissolved in 9 μl 80% formamide (+ bromphenol blue) and 5 min shaked at RT. Transcripts were separated on 4.5% polyacrylamide gels in 0.4x TBE (at 250V for approximately 1 h). After 10 min, the upper buffer was changed to avoid background of free, radioactive nucleotides in the gel.

8 RESULTS

8.1 Purification of a cellular Tip5 - Snf2h complex, termed NoRC

Tip5 was identified as a novel interaction partner for TTF-I (Jansa et al., 1998), a factor playing an important role in the regulation of rDNA transcription in chromatin (Längst et al., 1998; Längst et al., 1997a). The modular organization of Tip5 resembles that of proteins that have been associated with functions in chromatin structure and chromatin regulation, suggesting that Tip5 is part of a chromatin remodeling complex. Furthermore, initial results demonstrated that Tip5 coeluted together with a significant part of Snf2h at a native molecular mass of ~800 kDa, implying that both are contained within a macromolecular protein complex (see introduction 5.7 and (Strohner, 2000)). Notably, Snf2h is the mammalian homologue of *Drosophila* ATPase ISWI (Aihara et al., 1998), the catalytic subunit of several chromatin remodeling complexes (Längst and Becker, 2001b).

To confirm the association of Snf2h with Tip5, the biochemical purification of cellular Tip5 was continued by affinity chromatography to obtain a homogenous purified complex (see scheme, Figure 21A). After the final gelfiltration (Superose 6) step, Tip5 containing fractions were pooled and concentrated on SP-Sepharose. This highly enriched Tip5 fraction, which contained Tip5, Acf1 and Snf2h, was incubated with α-Tip5 / N1-18 antibodies, washed and eluted with the Tip5-specific epitope peptide. Proteins were separated by SDS-PAGE and analyzed by Western (Figure 21B).

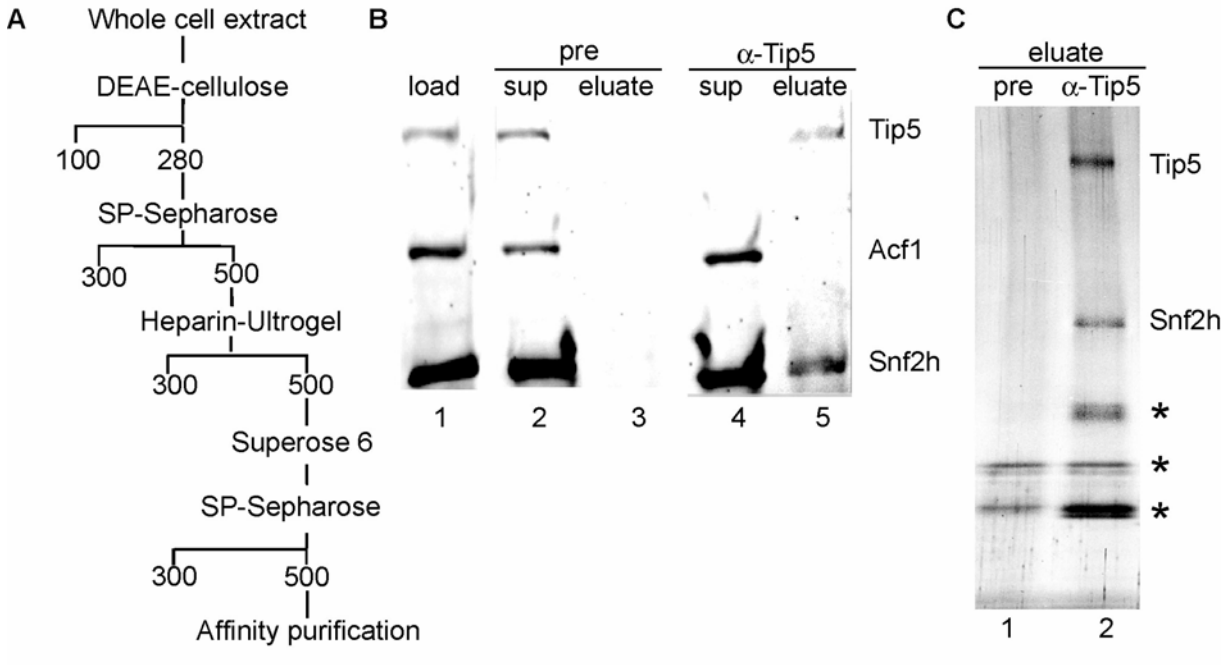

Figure 21. Purification of a cellular Tip5 - Snf2h complex, termed NoRC
(**A**) Fractionation scheme used to purify the murine Tip5 complex (NoRC). (**B**) Western analysis. NoRC was precipitated from a partially purified fraction (SP-500) with affinity-purified α-Tip5 / N1-18 antibodies and eluted with the epitope peptide. As control preimmune serum (pre) was used. 2.5% of the load (lane 1), the supernatants (lanes 2 and 4), and 15% of the eluate (lanes 3 and 5) were subjected to Western blot analysis using antibodies against Tip5, Snf2h and Acf1. (**C**) Subunit composition of NoRC. A silver-stained 4-12% PAA gel of immuno purified NoRC is shown (lane 2). Tip5 and Snf2h were identified by mass spectroscopy. The three indicated proteins (*) are contaminations by the rabbit antibody. Lane 1 shows the result of a control immunoprecipitation (pre).

After precipitation with α-Tip5 antibodies, Acf1 remained in the supernatant (lane 4), whereas both Tip5 and Snf2h were present in the eluate (lane 5). In contrast, a control precipitation with preimmune serum showed no enrichment of these proteins in the eluted fraction (lane 3). Thus, Snf2h was co-precipitated with anti-Tip5 antibodies, showing that Tip5 and Snf2h form a tight association in a multiprotein complex. The affinity-purified complex was subjected to SDS-PAGE and visualized by silver staining to define the subunit composition (Figure 21C). Five prominent bands turned out to be present in the specific eluate (lane 2). Mass spectrometry confirmed Tip5 and Snf2h, while the other three were rabbit proteins, probably antibody contaminants.

This result demonstrated that Tip5 associates with Snf2h and forms a novel protein complex, distinct from ACF. As Tip5 localized within the nucleolus, and the cellular Tip5 / Snf2h complex catalyzed nucleosome movement (see section 8.2), the complex was termed NoRC (Nucleolar Remodeling Complex).

8.2 Cellular NoRC exhibits chromatin remodeling activity

The structural similarity of Tip5 to Acf1, as well as the presence of the ISWI homologue Snf2h in the purified complex, suggested that NoRC is a novel chromatin remodeling complex. However, a nucleosome remodeling activity was yet not confirmed. To assess the ability of NoRC to alter nucleosome positions, a mononucleosome mobility assay ('nucleosome sliding assay') was used, that has been previously established to demonstrate ISWI and ACF / CHRAC mediated nucleosome movement (Längst et al., 1999).

8.2.1 The nucleosome mobility assay ('nucleosome sliding assay')

The nucleosome mobility assay allows the visualization of single nucleosome movements, catalyzed by ATP-dependent nucleosome remodeling factors. The principle relies on the fact, that mononucleosomes reconstituted on short DNA fragments frequently exhibit distinct translational positions, e.g. on a 247 bp fragment, derived from the mouse rDNA promoter, nucleosomes locate predominantly either on the end of the DNA fragment, or are more centrally positioned (Längst et al., 1999). These positioned nucleosomes can be separated by native gel electrophoresis, due to their different migration behavior. Endpositioned nucleosomes migrate relatively fast compared to nucleosomes, which are located at the center of the DNA fragment. Subsequently, these positioned nucleosomes can be purified from native gels and are used to study nucleosome movement ('remodeling') in the presence of chromatin remodeling factors.

In the experiment shown in Figure 22, purified mononucleosomes, either positioned at the end or at the center of the 247 bp DNA fragment, were incubated with increasing amounts of recombinant ACF and ISWI (Figure 22A), two factors known to alter nucleosomal positions (Eberharter et al., 2001; Längst et al., 1999). The nucleosome remodeling reactions were performed for 75 min, in the presence or absence of ATP as indicated, stopped and the nucleosome positions subsequently analyzed by native PAA gel electrophoresis (Figure 22B and C). Consistent with previous results, ACF catalyzed an ATP-dependent conversion of the fast migrating nucleosome to the slow migrating form (Figure 22B, lanes 1 to 6), which is due to the relocation of the histone octamer from the peripheral to the central position of the DNA fragment. ACF did not alter the positions of nucleosomes localized at the center of the DNA

fragment (lanes 7 and 8). In contrast, ISWI mobilized only the centrally positioned nucleosomes towards the end of the DNA fragment (Figure 22C, lanes 1 to 4). The translational position of the octamer was not affected if ISWI was incubated with nucleosomes positioned at the border of the DNA fragment (lanes 7 and 8). Again, this change from slow migrating to fast migrating nucleosomes was energy-dependent (lane 6).

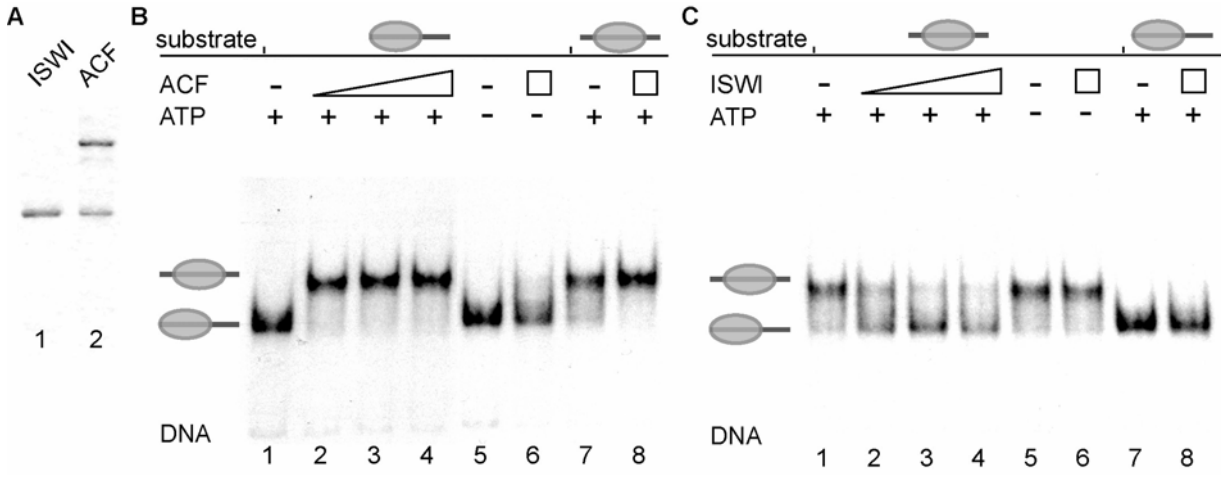

Figure 22. The nucleosome mobility assay ('nucleosome sliding assay')
(**A**) Recombinant Flag-ISWI and ACF (Flag-ISWI / Acf-1). The proteins were expressed in Sf9 cells, purified, resolved by SDS-PAGE and stained with Coomassie blue. (**B**) Nucleosome mobilization by ACF. Approximately 50 fmoles of radioactive labeled, positioned mononucleosomes were incubated with increasing amounts (2.5, 5 and 10 fmoles) of ACF in the presence or absence of ATP as indicated. The reactions were stopped after 75 min, separated on a native 4.5% polyacrylamide gel and visualized by autoradiography. The positions of the two isolated nucleosomal particles and of the free DNA are shown on the left side. The positioned nucleosomes used in the individual reactions are indicated on the top. (**C**) Nucleosome mobilization by ISWI. Affinity purified ISWI (2.5, 5 and 10 fmoles) was incubated with 50 fmoles of positioned nucleosomes in the presence or absence of ATP as indicated. Nucleosome positions were analyzed by electrophoresis on native polyacrylamide gels as described above.

8.2.2 NoRC triggers ATP-dependent movement of mononucleosomes

Next, the activity of cellular NoRC in the nucleosome mobility assay was investigated. Purified nucleosomes, either positioned at the border or at the center of the 247 bp DNA fragment, were incubated with immunopurified NoRC or control beads in the presence or absence of ATP. Purified CHRAC and recombinant ISWI were used as controls (Figure 23). Similar to ACF, purified CHRAC catalyzed an energy-dependent shift of fast migrating to slow migrating nucleosomes (Figure 23, lanes 1 to 6). Significantly, immunopurified NoRC behaved like ACF / CHRAC, i.e. it induced movement of peripheral nucleosomes to the center of the DNA fragment (lanes 7 to 9). This reaction was dependent on the amount of NoRC and required ATP (lanes 13,14). NoRC driven nucleosome mobilization was restricted to end-positioned nucleosomes (lanes 18 and 19) while ISWI on the other hand mobilized the centrally positioned nucleosome to the end of the DNA fragment (lanes 14 to 16, see also Figure 22). Bead bound control antibodies incubated with whole cell extract, did not support nucleosome sliding (lanes 10 to 12).

This assay showed that cellular NoRC exhibits ATP-dependent chromatin remodeling activity. Similar to ACF / CHRAC, NoRC mobilizes exclusively end-positioned nucleosomes in an energy-dependent fashion.

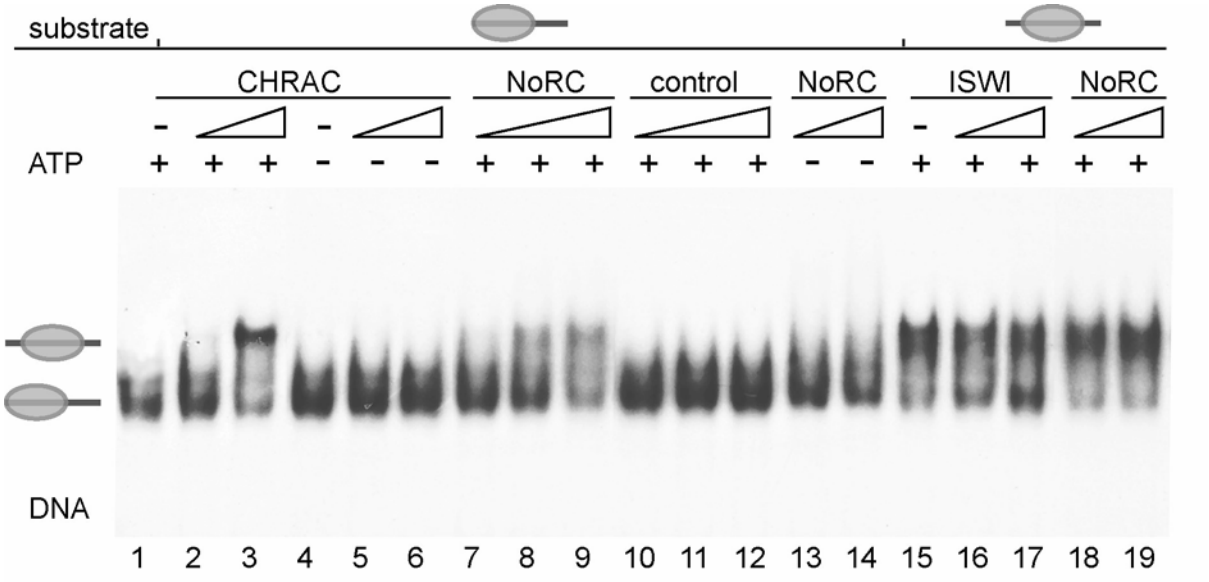

Figure 23. NoRC triggers ATP-dependent movement of mononucleosomes
Nucleosome mobilization by affinity-purified NoRC. Increasing amounts of CHRAC, immunoprecipitated NoRC and control beads were incubated with end-positioned nucleosomes (lanes 1 to 14) as indicated. In lanes 15 to 19, recombinant ISWI and NoRC were incubated with nucleosomes positioned at the center of the DNA fragment. The nucleosome remodeling reaction was supplemented with ATP as indicated, incubated at 26°C for 75 min, stopped by the addition of competitor DNA, subjected to native gel electrophoresis and visualized by autoradiography. The positions of nucleosomes are indicated at the left.

8.2.3 The N-terminus of histone H4 is required for NoRC dependent remodeling

The ISWI-containing chromatin remodeling complexes require nucleosomal substrates with intact histone H4 N-termini ('tails') for both, stimulation of the ATPase activity, and nucleosome remodeling (Clapier et al., 2001). To examine whether NoRC-mediated nucleosome movement requires the N-terminal part of histones, the remodeling assays were performed with recombinant nucleosomes lacking individual histone tails.

In the experiment shown in Figure 24, recombinant nucleosomes positioned at the end of the DNA fragment were used as substrate for both CHRAC and NoRC in the nucleosome mobility assay. Consistent with previous results, CHRAC dependent mobilization of nucleosomes did not require the tails of histone H2A, H2B and H3 (Figure 24, lanes 2, 7 and 12). If however the N-terminal tail of histone H4 was deleted, CHRAC failed to mobilize the nucleosome towards the center of the DNA fragment (lane 17). If NoRC was assayed on nucleosomal substrates lacking individual histone tails, a similar result was obtained. Like CHRAC, NoRC was able to mobilize nucleosomes lacking the N-termini of histone H2A, H2B and H3 (lanes 3 to 5, 8 to 10 and 13 to 15, respectively). However, deletion of the tail of histone H4 abolished nucleosome sliding, leaving the nucleosomal position unchanged (lanes 18 to 20).

These experiments confirmed that the NoRC complex, composed of Tip5 and Snf2h, is a novel member of the ACF / CHRAC like nucleosome remodeling machines.

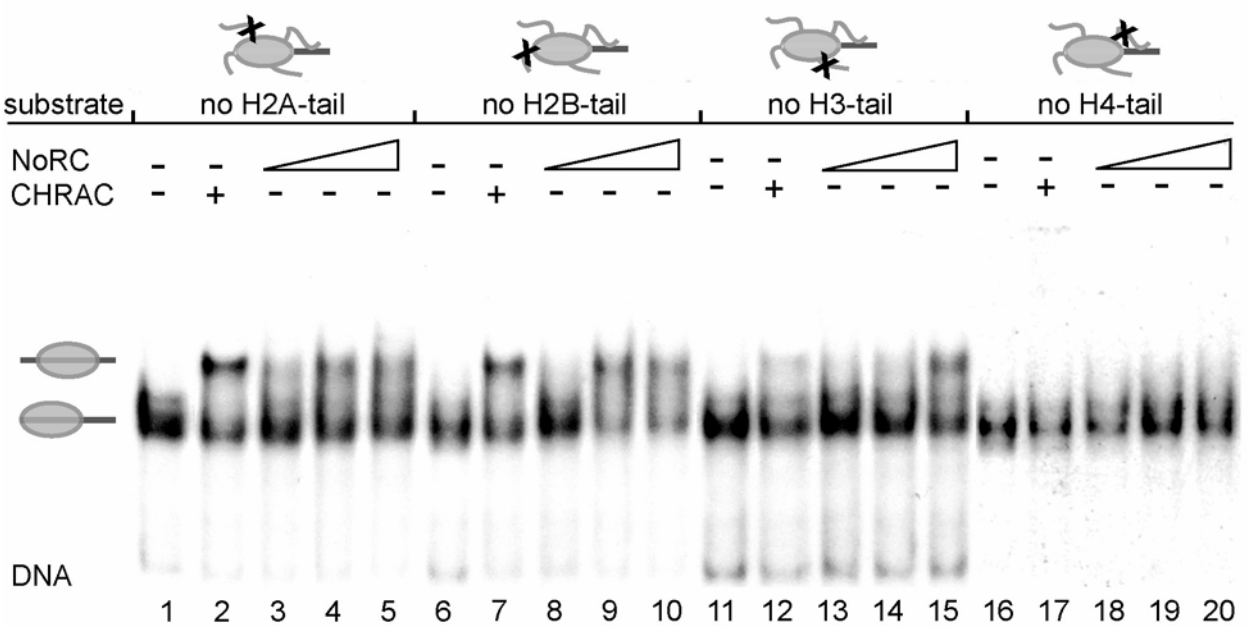

Figure 24. The N-terminus of histone H4 is required for NoRC-dependent remodeling
Increasing amounts of immunoprecipitated NoRC and purified CHRAC were incubated with end-positioned nucleosomes reconstituted from octamers containing three recombinant full-length histones and one tail-less histone, as indicated in the panels above. All reactions contained ATP. Nucleosome positions were analyzed as described in Figure 22.

8.3 Reconstitution of NoRC

A pre-requisite for further investigation of NoRC was a sufficient amount of homogenous NoRC for use in functional studies. In order to obtain high quantities, NoRC was reconstituted from its recombinant subunits. The baculo virus / Sf9 cell expression system was chosen, since this eukaryotic expression system has the advantage of high expression levels together with the ability to express large, correctly folded proteins.

8.3.1 Expression and purification of recombinant Tip5 and Snf2h

Recombinant baculovirus with Myc-epitope tagged Tip5 and Flag-epitope tagged Snf2h were constructed using the Bac-to-Bac baculovirus expression system (Life Technologies). Proteins were expressed in Sf9 cells, purified to apparent homogeneity using their affinity tags and analyzed on SDS-PAGE followed by Coomasie blue staining (Figure 25A).

8.3.2 Reconstitution of recombinant NoRC

Myc-Tip5 and Flag-Snf2h were coexpressed in Sf9 cells and purified with two different methods to obtain a highly purified, recombinant NoRC complex. In the first step, free Tip5 was separated from Snf2h and NoRC by Flag-tag affinity purification (data not shown). In the second step, free Snf2h was separated from recombinant NoRC by Biorex 70 chromatography. Fractions from the Biorex 70 chromatography were resolved by SDS-PAGE and stained with Coomassie Blue (Figure 25B). At a concentration of 300 mM KCl free Snf2h did not bind to the column (see wash 300 mM, lanes 2 and 3) while NoRC was retained. At high salt (700 mM KCl) the recombinant Tip5-Snf2h complex was eluted in a stoichometric manner (lanes 4 to 6). Recombinant NoRC containing fractions were pooled, dialyzed against storage-buffer (EX-200) and analyzed again on Coomassie Blue stained protein gels (Figure 25C).

To evaluate the molecular mass of recombinant NoRC, Flag-affinity purified recombinant proteins were subjected to size exclusion chromatography. Figure 25D shows a Coomassie blue stained protein gel showing the individual fractions of a Superose 6 gelfiltration chromatography. Like native NoRC, the recombinant complex elutes at an apparent molecular mass of approximately 800 kDa. Minor contaminations of recombinant Snf2h eluted in earlier fractions corresponding to a molecular weight of approximately 200 to 300 kDa. Recombinant Tip5 protein eluted at about 300 to 400 kDa (data not shown).

The purification behavior of recombinant NoRC and the high molecular mass indicates that also recombinant Tip5 and Snf2h form a multiprotein complex and suggests that the subunit composition of this macromolecular complex corresponds to the purified cellular NoRC.

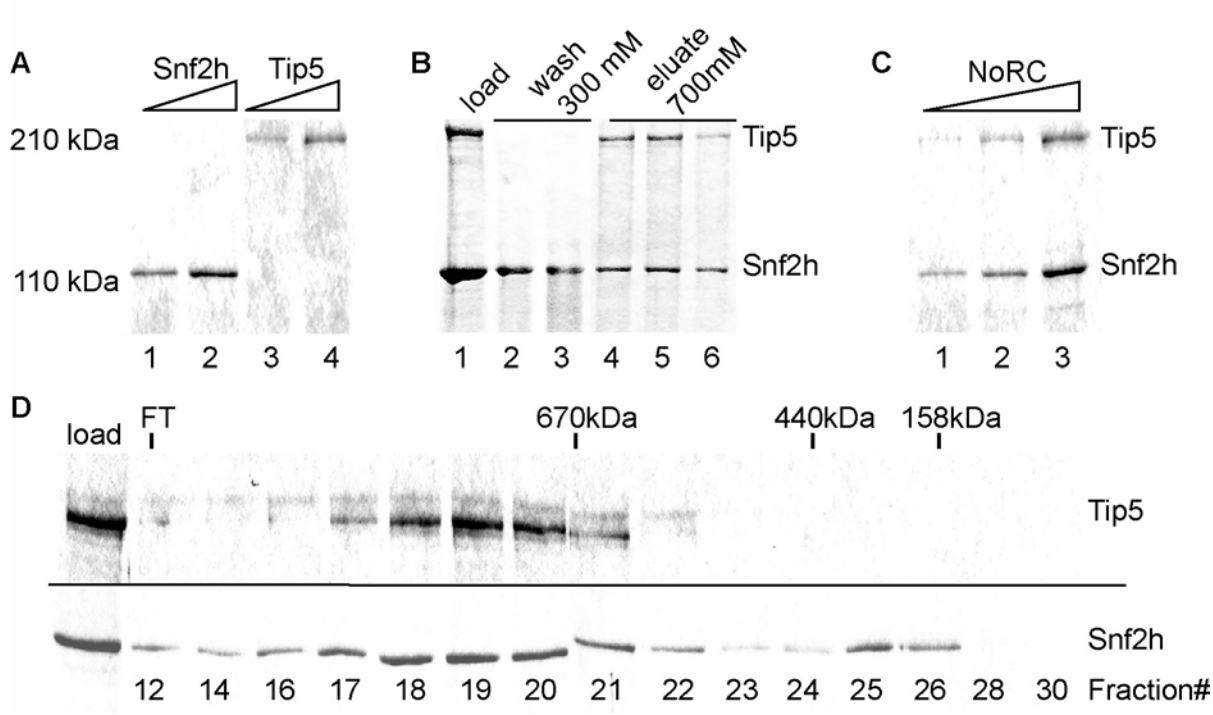

Figure 25. Reconstitution of the NoRC complex by its recombinant subunits
(**A**) Purified recombinant Flag-Snf2h and Myc-Tip5. The proteins were expressed in Sf9 cells, purified, resolved by 6% SDS-PAGE and stained with Coomassie blue. The protein sizes are indicated at the left. (**B**) Reconstitution of recombinant NoRC. Coexpressed Myc-Tip5 / Flag-Snf2h were immunopurified via the Flag-tag and subsequent subjected to a Biorex 70 column. The load (Flag-epitope eluate from Flag purification, lane 1), the wash at 300 mM KCl (lanes 2 and 3) and the 700 mM KCl eluates (lanes 4 to 6) of a Biorex 70 chromatography were analyzed by SDS PAGE and stained with Coomassie Blue. Stoichometric amounts of NoRC elute at high salt concentrations (700 mM KCl). (**C**) Purified recombinant NoRC (Myc-Tip5 / Flag-Snf2h). The proteins were coexpressed in Sf9 cells, purified in two steps as described, dialysed, resolved by 6% SDS-PAGE and stained with Coomassie blue. (**D**) Size exclusion chromatography of recombinant NoRC. Flag-affinity purified NoRC was separated on a Superose 6 column (Pharmacia). Proteins of the individual fractions were resolved by 6% SDS-PAGE and stained with Coomassie blue. NoRC eluted with a molecular weight of ~800 kDa, free Snf2h around 300 kDa. The positions of molecular weight standards (Thyroglobulin, 670 kDa; Apoferritin, 440 kDa; Aldolase, 158 kDa) are indicated.

8.3.3 Recombinant Snf2h and NoRC are nucleosome stimulated ATPases

Previous studies have established that chromatin remodeling complexes exhibit substrate-dependent ATPase activity. For instance, ISWI-containing chromatin complexes possess ATPase activity that is partially stimulated by DNA, but fully activated in the presence of nucleosomal substrate (Corona et al., 1999). The activity of other remodeling enzymes is either similar with DNA and chromatin (e.g. Swi/Snf, (Boyer et al., 2000; Richmond and Peterson, 1996)) or only stimulated by nucleosomes (e.g. Mi-2, (Brehm et al., 2000)). Similarly, the properties of recombinant NoRC in ATPase assays were evaluated.

Purified recombinant ACF, NoRC, Snf2h and Tip5 were incubated with DNA or nucleosomal substrate in the presence of radioactive γ-^{32}P ATP for 45 minutes. The reactions were stopped and hydrolyzed radioactively labeled phosphate was separated from unreacted ATP by thin layer chromatography (Figure 26A, quantification of the result in Figure 26B). As expected, the presence of ACF in the reaction resulted in a weak increase of free γ-^{32}P phosphate in the absence of substrate (B: Buffer) and ATP hydrolysis was only slightly enhanced with DNA (D). High levels of ATP hydrolysis

occurred in the presence of nucleosomal substrate (N). NoRC and Snf2h displayed similar ATPase activities; full activation of the ATPase required the presence of nucleosomal DNA. In contrast, Tip5 did not display ATPase activity. Thus, Snf2h and NoRC, but not Tip5, are nucleosome stimulated ATPases like ACF.

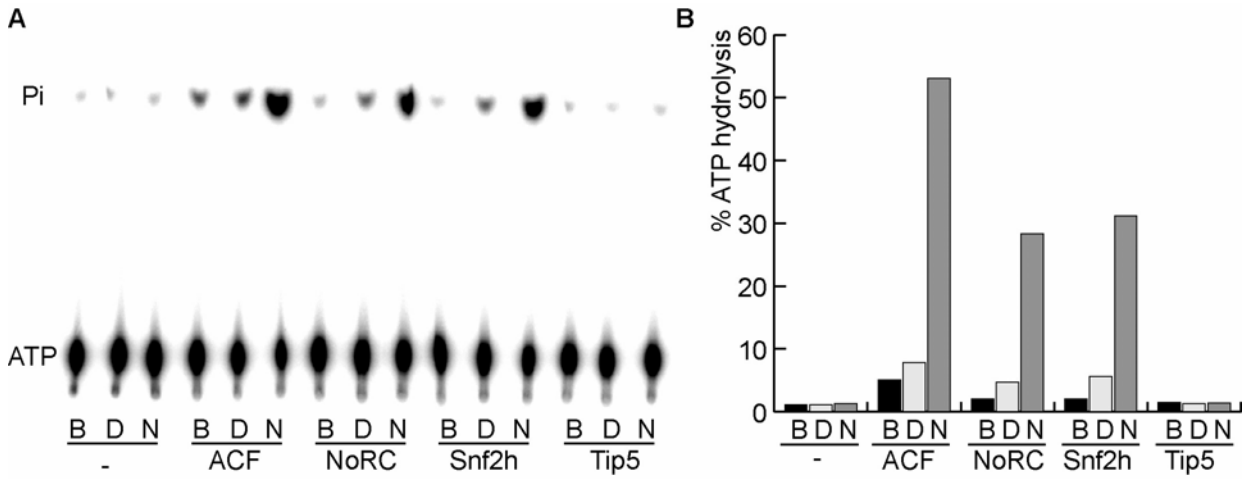

Figure 26. Recombinant Snf2h and NoRC contain nucleosomal stimulated ATPase activity
(**A**) ATPase assay. 0.2 pmol of ACF, NoRC, Snf2h, Tip5 and no protein (-) were incubated with γ-^{32}P ATP in the absence of substrate (B: Buffer), in the presence of 300 ng DNA (D) or 300 ng of nucleosomal array (N). After 45 min incubation, 1μl of the reaction was spotted on PEI-cellulose (Merck) and free phosphate (Pi) was separated from ATP by thin layer chromatography. Proteins and substrates are indicated at the bottom. (**B**) Quantification of the result shown in A. Thin layer chromatography results were quantified with a Phospho Imager (Fuji). The percentage of hydrolyzed ATP is displayed in the presence of the indicated proteins and different substrates. Abbreviations as in A.

8.3.4 The ATPase activity of NoRC depends on the N-terminus of histone H4

Similar to other ISWI-containing complexes the chromatin remodeling activity of cellular NoRC was dependent on the histone H4 N-terminus (see Figure 24). To confirm this requirement for recombinant NoRC, ATPase assays were performed using different nucleosomal substrates.

The ATPase activity of ACF, Mi-2 and NoRC were analyzed with chromatin substrates reconstituted from intact histones containing all tails (N), only the H4 tail (i4) or lacking the N-terminus of histone H4 (g4). The quantification of this experiment is shown in Figure 27. Again, NoRC, ACF and Mi-2 hydrolysed high amounts of ATP in the presence of the intact nucleosomal substrate (compare B, D and N). However, differences were observed using substrates lacking individual histone tails. The Mi-2 ATPase, known to be tail independent (Brehm et al., 2000), was similarly activated with either chromatin substrate used. In contrast, ACF and NoRC were highly active in the presence of chromatin substrate containing only the H4 tail (i4), but were inactive with nucleosomes lacking the histone H4 N-terminus (g4).

Accordingly, the enzymatic activity of recombinant NoRC depends on the histone H4 tail like the cellular NoRC complex.

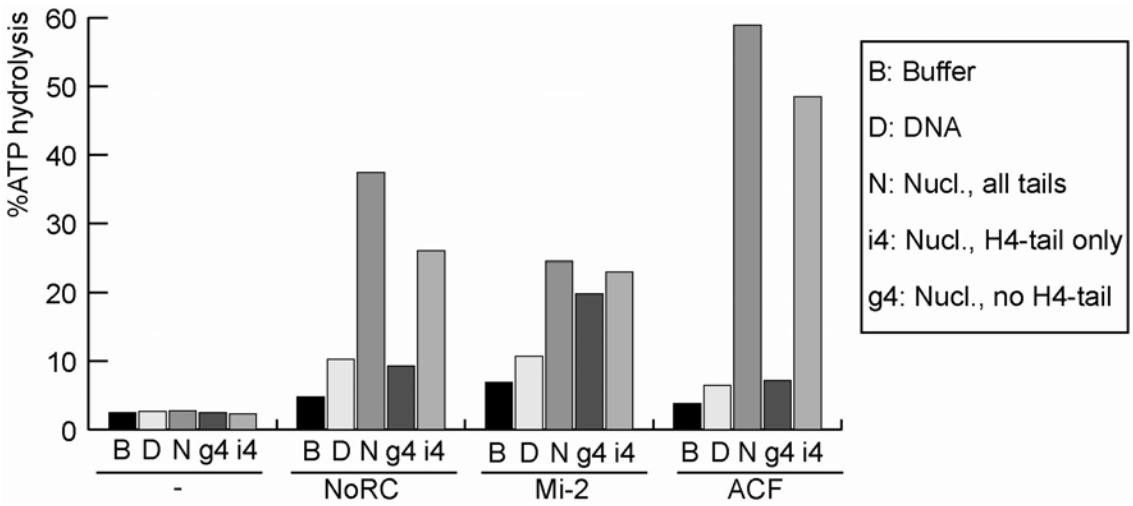

Figure 27. The ATPase activity of NoRC depends on the N-terminus of histone H4
ATPase assay with different nucleosomal substrates. ATPase activity of recombinant NoRC, Mi-2 and ACF (each 0.3 pmol) was analyzed in the absence of substrate (B: Buffer), or in the presence of 300 ng DNA (D) or 300 ng of the indicated recombinant nucleosomal arrays as described in Figure 26. N: nucleosomes reconstituted from full-length histones; g4: Histone H4 lacks the N-terminal tail, the other histones are full-length proteins; i4: Histone H4 is a full-length protein, the other histones lack the N-terminal tail. Proteins and substrates are indicated at the bottom. The bar shows the percentage of hydrolyzed ATP.

8.3.5 Recombinant NoRC exhibits ATP-dependent remodeling activity

The nucleosome mobility assay was used to characterize the nucleosome remodeling activity of reconstituted recombinant NoRC further (for details see 8.2.1).

Recombinant NoRC was incubated with nucleosomes positioned at the ends of the 247 bp DNA fragment in the presence or absence of ATP (Figure 28A). Similar to purified cellular NoRC (see Figure 23) and ACF (Figure 28, lane 4) recombinant NoRC induced ATP-dependent nucleosome movement from the border to the center of the DNA fragment, which was seen as a shift from fast migrating to slow migrating nucleosomes (Figure 28, lanes 2 and 3). This result indicates functional identity between cellular and recombinant NoRC.

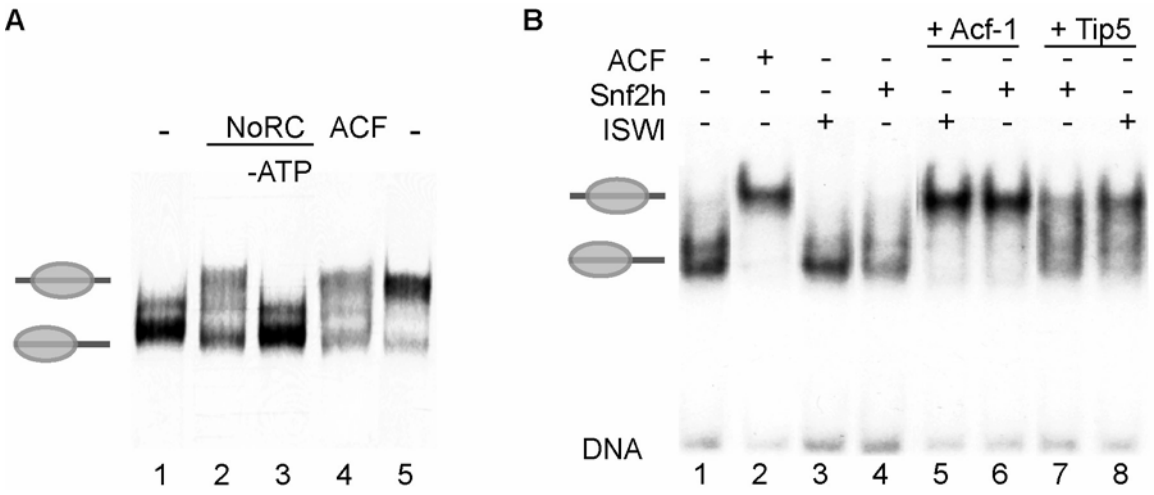

Figure 28. Recombinant NoRC exhibits ATP-dependent nucleosome remodeling activity
(**A**) Nucleosome mobility assay. Purified nucleosomes (0.1 pmoles), positioned at the end (lanes 1 to 4) or center (lane 5) of the 247 bp DNA fragment, were incubated for 90 min with 0.1 pmol recombinant NoRC in the presence (lane 2) or absence of ATP (lane 3) or with ACF (0.1 pmoles, lane 4). Nucleosome positions were analyzed by native gel electrophoresis. The positions of end-positioned and center-positioned nucleosomes are indicated on the left. (**B**) Influence of Tip5 and Snf2h on nucleosome mobility. Nucleosomes positioned at the end of the DNA fragment (lane 1) were incubated with ISWI or Snf2h alone (lanes 3 and 4), or in the presence of Acf-1 (lanes 5 and 6), or Tip5 (lanes 7 and 8). A control reaction with ACF (lane 2) indicates the position of the central nucleosome. Nucleosome positions were analyzed as in A.

8.3.6 Tip5 determines the directionality of the remodeling reaction

In order to characterize the nucleosome remodeling mechanism by NoRC and its individual subunits, the nucleosome mobility assay was used for a more detailed analysis of the remodeling activities. This assay allows differentiation between the effects of individual subunits on the activity of a remodeling machine, as shown previously (Eberharter et al., 2001).

In the experiment shown in Figure 28B, endpositioned nucleosomes were incubated with either recombinant ISWI and Snf2h alone or in the presence of Acf1 and Tip5. The reactions were supplemented with ATP, incubated at 26°C and stopped after 75 min by the addition of competitor DNA. Nucleosome positions were resolved by native gel electrophoresis. As shown before, only ACF but neither ISWI nor Snf2h, moved the end-positioned nucleosomes to the central position (Figure 28B, lanes 1 to 4). Strikingly, nucleosome movement was observed if Tip5 or Acf1 were added to the ATPases ISWI and Snf2h (lanes 5 to 8). Neither Acf1 nor Tip5 alone showed chromatin remodeling activity (data not shown).

In conclusion, similar to Acf1 within the ACF chromatin remodeling complex, the large subunit Tip5 determines the directionality of nucleosome movement catalyzed by NoRC.

8.4 DNA and nucleosome binding properties of NoRC and its subunits

Chromatin remodeling complexes react with a complex substrate of DNA packaged into chromatin. In order to understand how NoRC interacts with its substrate, the DNA and nucleosome binding properties of NoRC and its subunits Tip5 and Snf2h were studied in various assays. Both proteins contain a number of possible DNA binding domains, e.g. AT-hooks, TAM / MBD domain, DDT motif within Tip5 or the SANT / SLIDE domains within Snf2h, which might be involved in this activity.

8.4.1 Tip5 exhibits DNA binding activity

First, the DNA binding properties of Tip5 were studied in electromobility shift assays (EMSA). This method allows the visualization of protein / DNA complexes migrating as distinct bands in native polyacrylamide gels.

Increasing amounts of purified recombinant Tip5, and in comparison Acf1, the large subunit of ACF / CHRAC, were incubated with a 90 bp ^{32}P-labeled oligonucleotide for 15 min. The reactions were separated by native gel electrophoresis and visualized by autoradiography (Figure 29A). The addition of increasing amounts of Tip5 to the DNA resulted in the progressive appearance of separate retarded DNA (lanes 1 to 4), indicating one distinct Tip5 / DNA complex. Higher protein concentrations led to the accumulation of the DNA in the well of the gel, indicating the aggregation of protein / DNA complexes. Like Tip5, Acf1 shifted DNA in a concentration dependent manner (lanes 5 to 8), notably with a distinct migration behavior in the gel. These results indicate that Tip5 and Acf1 bind to DNA.

The DNA binding activity of Tip5 was verified by the addition of specific antibodies to the reaction. Purified Myc-tagged Tip5 was incubated with a 247 bp DNA fragment in the presence or absence of different antibodies and processed as in Figure 29A. Incubation of Tip5 with the DNA fragment resulted in the appearance of a specifically retarded Tip5 / DNA complex (Figure 29B, lanes 2 and 8). The presence of Tip5 specific antibodies (α-Tip5) disrupted this complex (lanes 4 and 6). Furthermore, antibodies against the Myc-tag of the protein (α-Myc) resulted in additional retardation of the DNA, a 'supershift' (lanes 10 and 12), implying a complex of Tip5, DNA and antibody. A control antibody (α-Flag) did not affect the protein / DNA complexes (lanes 13 and 14). Furthermore, the used antibodies did not alter the migration of the free DNA (lanes 3, 5, 9 and 11). The specificity of the α-Myc and α-Flag antibodies was confirmed in electromobility shift experiments with Flag-tagged ISWI (Figure 29C). Incubation with the α-Myc antibody did not alter the DNA / ISWI complexes (lanes 1 and 2), whereas the specific antibody (α-Flag) resulted in a further shift ('supershift') of the ISWI / DNA complex (lane 3).

Collectively, these experiments clearly demonstrate the DNA binding activity of Tip5.

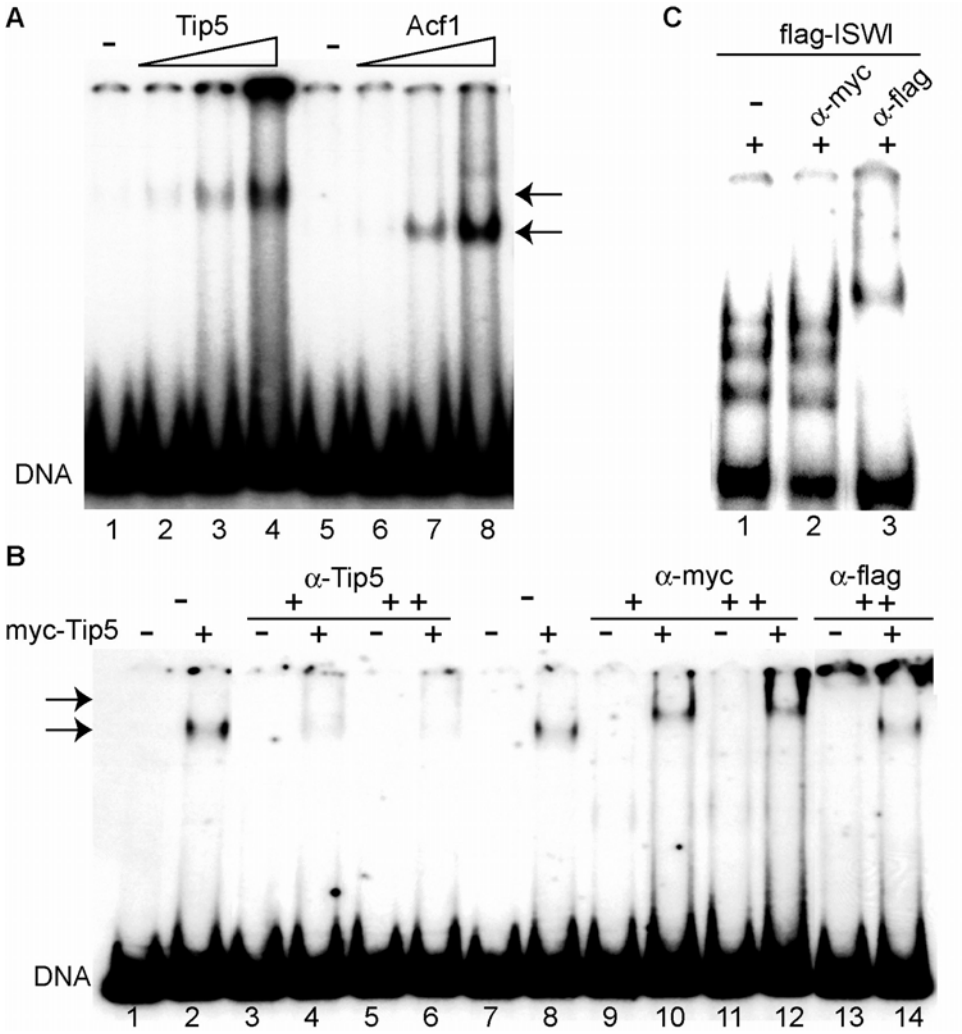

Figure 29. Tip5 exhibits DNA binding activity
(**A**) Increasing amounts of recombinant Tip5 or Acf1 were incubated with a 90 bp ^{32}P-labeled DNA fragment and analyzed for DNA binding activity in electrophoretic mobility shift assays (EMSA). Free DNA is shown in lanes 1 and 5. Increasing amounts of Tip5 (lanes 2 to 4: 20, 40 and 80 fmol) and increasing amounts of Acf1 (lanes 6 to 8: 100, 200 and 400 fmol) were added. Different protein-DNA complexes (indicated by arrows) were separated from free DNA on 4% native polyacrylamide gels and visualized by autoradiography. (**B**) Tip5 DNA binding activity is altered in the presence of specific antibodies ('supershift'). Purified Myc-tagged Tip5 (50 fmol) was incubated with a radioactive labeled 247 bp rDNA promoter fragment in the absence (lanes 2 and 8) or presence of different antibodies. Tip5 specific antibodies (α-Tip5 / N1-18) were added in lanes 3 to 6, antibodies against the Myc-epitope (α–Myc) in lanes 9 to 12 and α-Flag control antibodies in lanes 13 and 14. Analysis was performed as described in A. (**C**) Similar experiment as in B with Flag-ISWI to demonstrate the specificity of the used antibodies. Recombinant Flag-ISWI (50 fmol) was incubated with a positioned nucleosome either in the absence of antibody (lane 1), in the presence of α-Myc antibodies (lane 2) or α-Flag antibodies (lane 3).

8.4.2 Tip5 and Snf2h cooperate in DNA binding

Next, the DNA binding ability of recombinant Snf2h, Tip5 and NoRC were compared in electromobility shift assays.

A 146 bp DNA probe was incubated with similar amounts of purified Snf2h, Tip5 and NoRC and the reactions were analyzed by native gel electrophoresis as described above. Incubation of Snf2h with the DNA fragment (lane 1) generated several retarded DNA bands (Figure 30, lanes 2 to 4), suggesting that

more than one Snf2h molecule can associate with the DNA. As shown before, incubation of the DNA with limited amounts of Tip5 resulted in the formation of a specific protein / DNA complex, visible as a distinct band (lanes 5 to 7). Likewise, addition of NoRC to the reaction caused the formation a specific protein / DNA complex, migrating slower than the Tip5 / DNA complex (lanes 8 to 10). Comparison of the quantity of used protein amounts and the efficiency of protein / complex formation revealed that NoRC exhibited the strongest DNA binding activity. NoRC bound DNA approximately fourfold better than the individual subunits.

The experiment indicates that both subunits of NoRC (Tip5 and Snf2h) bind individually to DNA, but within the NoRC complex, they promote binding in a synergistic manner.

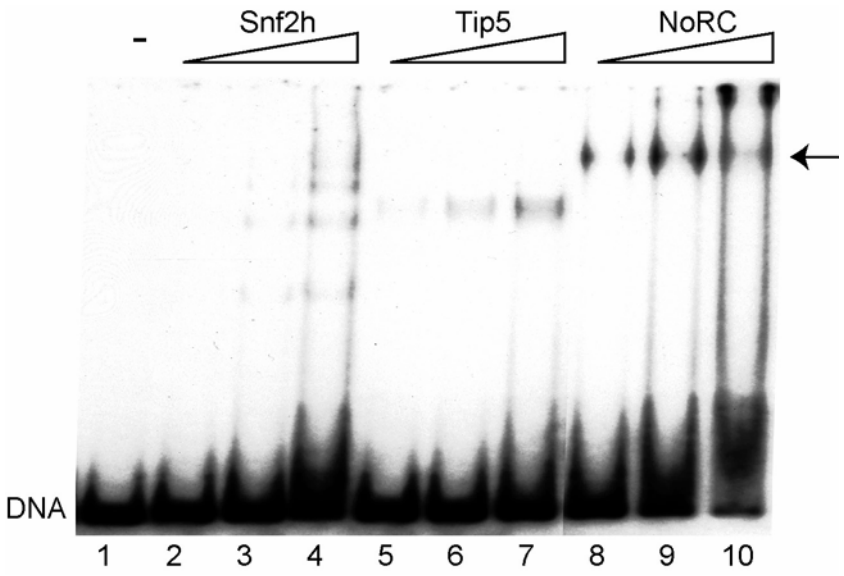

Figure 30. DNA binding properties of recombinant NoRC and its subunits
Snf2h and Tip5 cooperate in DNA binding. Electromobility shifts using free DNA (146 bp) and increasing amounts of Snf2h (lanes 2 to 4; 50 to 200 fmol), Tip5 (lanes 5 to 7; 25 to 100 fmol) and NoRC (lanes 8 to 10; 25 to 100 fmol). Different protein-DNA complexes were separated from free DNA on 4.5% native polyacrylamide gels and visualized by autoradiography. NoRC / DNA complexes are indicated with an arrow.

8.4.3 Binding of NoRC to nucleosomes needs protruding DNA

The presented electromobility shift assays demonstrate cooperative binding of Snf2h and Tip5 within NoRC to DNA. Next, the interactions of the chromatin remodeling complex with nucleosomal substrates were analyzed. Electromobility shift assays were performed with NoRC on reconstituted mononucleosomes varying in the length of the protruding linker DNA.

Nucleosomes were assembled by the histone / polyglutamic acid method (Stein et al., 1979) on radioactive labeled rDNA promoter fragments of 146, 171 and 247 bp in length. Nucleosome assemblies on the 146 bp and 171 bp DNA resulted predominantly in one visible band in EMSA. As described above (section 8.2.1), nucleosomes assembled on the 247 bp rDNA promoter fragment led to nucleosomes positioned either at the center or at the end of the DNA fragment (Längst et al., 1999). Different nucleoprotein complexes were gel purified, giving rise to mononucleosomes with DNA overhangs of 0

(146 bp), ~25 (171 bp), ~50 (247 bp, central positioned) and ~100 bp (247 bp, end-positioned nucleosome).

The different nucleosomal substrates and the free DNA were incubated with increasing amounts of NoRC and analyzed in gel retardation assays (Figure 31A). Incubation of NoRC with the individual DNA molecules resulted in NoRC / DNA complexes, independent of the DNA length (Figure 31A, panel 1, 3 and 5), suggesting similar DNA binding affinities of NoRC to the substrate. In contrast, NoRC failed to stably interact with nucleosomes assembled on the 146 bp and 171 bp DNA, but efficiently bound to positioned nucleosomes assembled on the 247 bp DNA fragment (compare panel 2, 4, 6 and 7). Nucleosomes with ~50 bp or ~100 bp of DNA overhang were bound with nearly similar affinity as free DNA. Even at elevated concentrations of NoRC, no stable NoRC / nucleosome complexes with the 146 and 171 bp nucleosomes could be resolved (data not shown).

This suggests that NoRC like ISWI (Brehm et al., 2000) preferentially recognizes and stably associate with free DNA but not nucleosomal DNA.

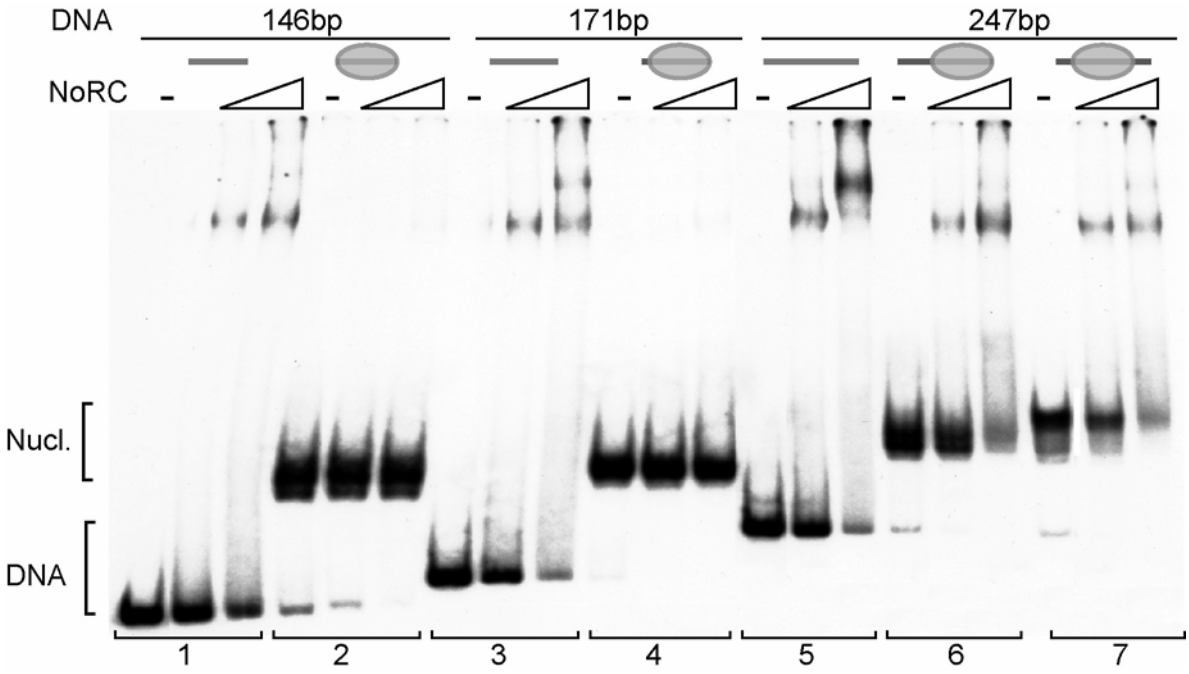

Figure 31. Binding of NoRC to nucleosomes needs accessible linker DNA
Electromobility shifts using different nucleosomal probes and NoRC. Recombinant NoRC (12, 30 and 75 pmol) was incubated with free DNA of 146 bp (panel 1), 171 bp (panel 3) and 247 bp length (panel 5), or purified nucleosomes assembled on these DNA fragments (panels 2, 4, 6 and 7). Purified nucleosomes, assembled on the 247 bp DNA fragment, were positioned either at the border (panel 6) or the center of the DNA fragment (panel 7). The assembled nucleosomes contained either no extruding DNA (146 bp fragment), ~25 bp extruding DNA (171 bp fragment), approximately ~50 bp of free DNA (247 bp, middle position) or ~100 bp linker DNA (end-positioned nucleosome). A scheme of the nucleosomes is shown on the top. Nucleoprotein complexes were separated by 3.5% native PAA gel electrophoresis. The positions of the DNA and the nucleosomes are indicated.

8.4.4 NoRC, Snf2h and ACF ATPase activity is stimulated by core nucleosomes

The experiment shown in Figure 31 illustrated that the lack of free DNA dramatically reduced the capability of NoRC to stably interact with the nucleosome. ATPase assays were used to monitor whether protruding linker DNA is also required for the activity of these enzymes.

Experiments were performed with nucleosomes assembled on DNAs with 146 bp, 247 bp and 4kb length in the presence of NoRC, Snf2h or ACF. In addition, the effects of histones and a mixture of histones and DNA on the ATPase activity of these factors were determined (Figure 32). In agreement with previous results, the ATPase activity of NoRC, Snf2h or ACF was only partially stimulated by free DNA, but the addition of polynucleosomes (4 kb) resulted in strongly stimulated ATP hydrolysis. Comparable high ATPase activity was measured with mononucleosomes having protruding DNA ends (247 bp). Despite the inability of NoRC, Snf2h and ACF to stably interact with the core nucleosome (data not shown for Snf2h and ACF), the enzymes were also activated by core nucleosomes (assembled on 146 bp DNA). Still, the rate of nucleosome-stimulated ATP hydrolysis was reproducibly weaker with core nucleosomes compared to nucleosomes assembled on longer DNA. Histones and a mixture of DNA and histones were not suitable substrates in this assay and did not stimulate ATP hydrolysis of any factor.

The experiments suggest that recognition of nucleoprotein structures in the core nucleosome by NoRC, Snf2h and ACF are sufficient to activate their ATPase activity. Apparently, the enzyme / core nucleosome interactions are rather weak, since they could not be displayed by electromobility shift assays.

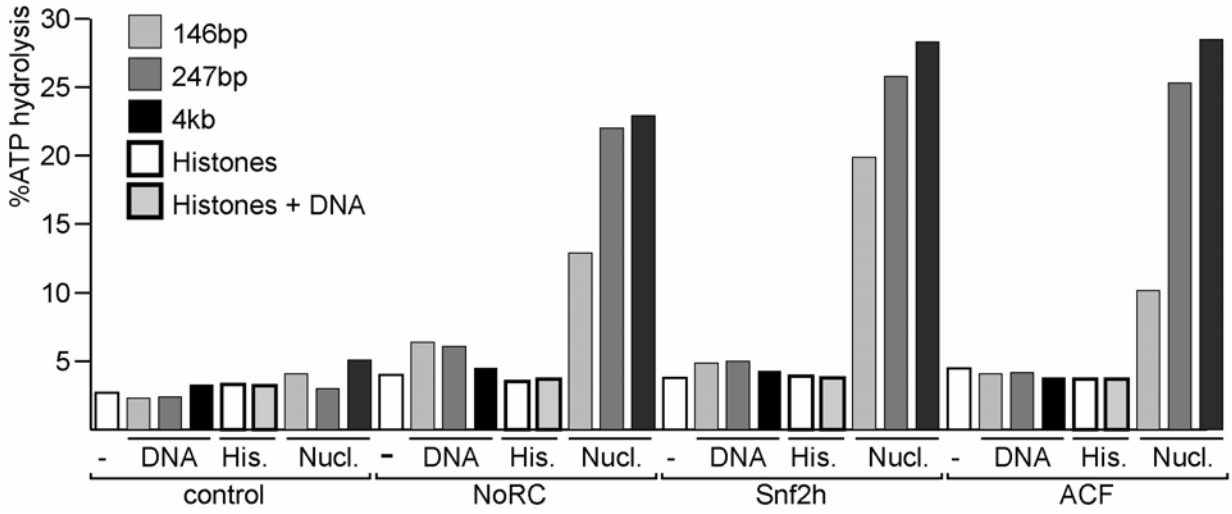

Figure 32. Substrate requirements of NoRC, Snf2h and ACF in ATPase assays
Core nucleosomes activate the ATPase activity of NoRC, Snf2h and ACF. ATPase assays of NoRC, Snf2h and ACF (each 0.3 pmol) in the presence of DNA, histones, a mixture of DNA and histones and various nucleosomal substrates (each approximately 200 ng) as indicated. Nucleosomes were salt assembled on DNA molecules with different lengths (146 bp, 247 bp and 4 kb). Reactions were performed as described before (Figure 26). Used proteins and substrates are indicated at the bottom. The bar shows the percentage of hydrolyzed ATP.

8.4.5 NoRC / nucleosome interaction: NoRC binds to nucleosomal DNA

Clearly, stable binding of NoRC to nucleosomes needs protruding DNA in electromobility shift assays. Nevertheless, whether binding occurs exclusively via contacts to free DNA or requires additional nucleosomal contacts is not determined. Supposedly, NoRC might interact with nucleosomal DNA in solution since core nucleosomes are sufficient to stimulate its ATPase activity. This is supported by ISWI / nucleosome interaction studies that revealed binding of ISWI to the linker DNA and the nucleosome, where the DNA enters / exits the nucleosome (Längst and Becker, 2001a).

DNase I protection experiments (DNase I footprint) were performed to map NoRC / nucleosome interactions in solution. Radioactively labeled 247 bp DNA was generated by PCR and assembled into nucleosomes. Positioned nucleosomes were isolated and the central nucleosomes served as substrate for the interaction mapping. DNA and the central positioned nucleosomes were mildly treated with DNase I, and the reaction was stopped after several time points. The digestion products were purified and analyzed on a denaturing sequencing gel (Figure 33A). Partial DNase I digestion of the nucleosomal DNA resulted in a characteristic cleavage pattern that differs from free DNA (compare panels 1and 2 with 3 and 4). Most prominent was a protected region, which denotes histone octamer bound DNA (indicated by the schematic picture of the central-positioned nucleosome). Since the 'central nucleosome' actually corresponds to a series of translational positions between -20 and -190 on the DNA fragment (Längst et al., 1999), the DNase I footprint lacked some precisions at the borders of the central nucleosome.

Next, NoRC / nucleosome interactions were characterized in the DNAse I footprint assay. The optimal ratio of NoRC to nucleosome was determined in initial EMSA experiments (data not shown). These reactions were scaled up and proceeded as shown in the scheme in Figure 33B. Briefly, the nucleosomal substrate was incubated with NoRC to allow complex formation and partially digested with DNase I. The reaction was stopped at different time points and directly loaded on polyacrylamide gels. The free nucleosomes and NoRC / nucleosome complexes were separated immediately by native gel electrophoresis (Figure 33D shows the result of one time point) and the individual complexes were excised. DNA was purified from the gel slices and analyzed on 7% sequencing gels (Figure 33C). While NoRC protected free DNA non-specifically from DNase I digestion (data not shown), the cleavage pattern of nucleosomal DNA was specifically altered in the presence of NoRC. NoRC interaction changed the DNase I digestion pattern predominantly at the entry and exit sites of the nucleosome (Figure 33C, panel 5 to 8). Yet, additional changes in the cleavage pattern are also seen within the nucleosome. Binding of NoRC seems to be bilateral, since interactions are visible at both ends of the nucleosomes.

All together, the electrophoretic shift assay experiments showed that extruding DNA is necessary for a stable interaction of NoRC with nucleosomes. However, NoRC´s ATPase activity is stimulated by core nucleosomes and NoRC binds to separate locations at the borders and within the nucleosome, as revealed by DNase I footprint experiments. These results imply that NoRC interacts with both nucleosomal and protruding linker DNA.

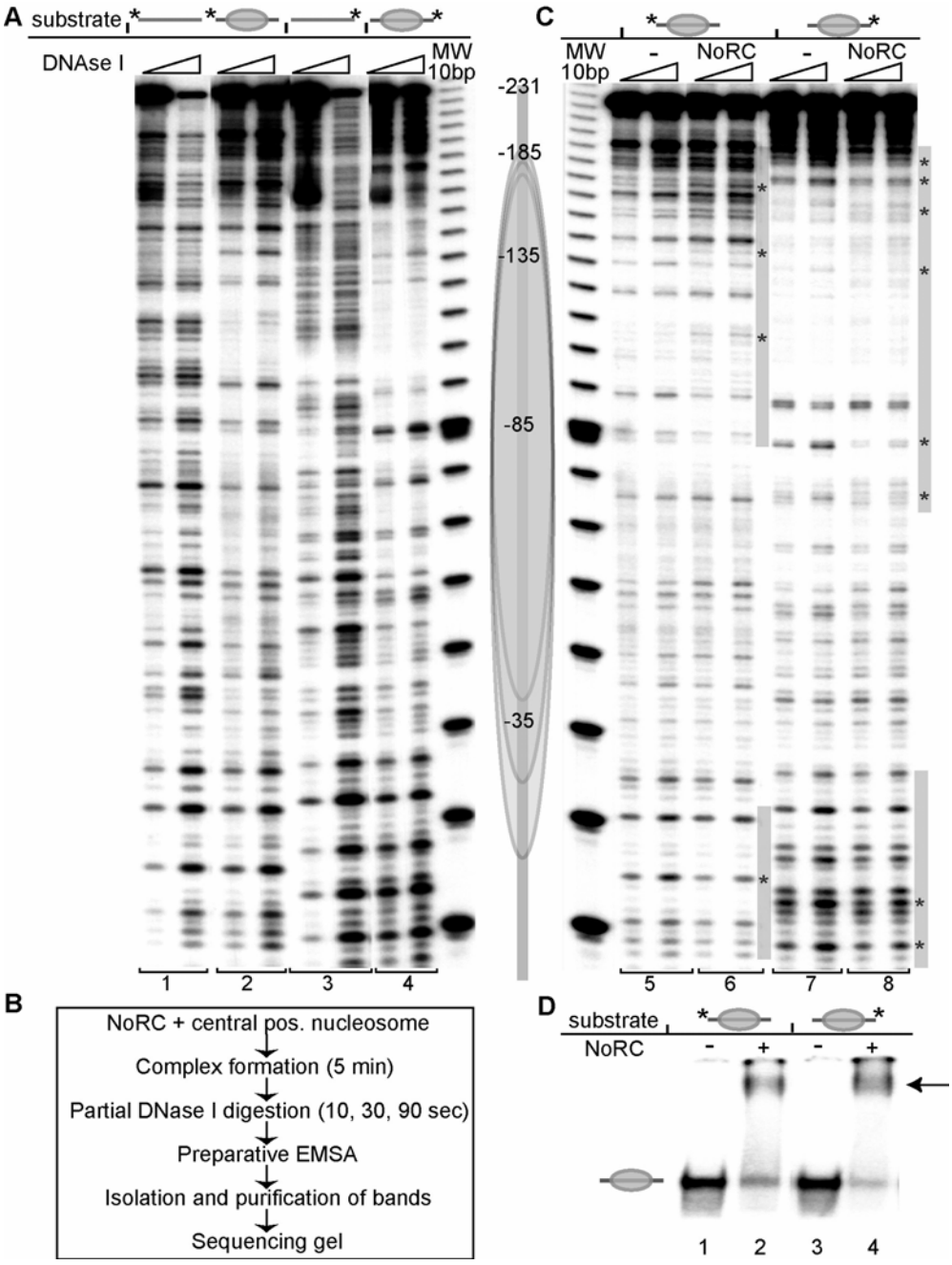

Figure 33. NoRC / nucleosome interaction studies by DNase I protection
(**A**) Partial DNase I digestion of DNA and central positioned nucleosomes. A 247 bp rDNA promoter fragment (–231 to +16 respective to the start site) was radioactively labeled either on the -231 end (panel 1 and 2) or on the +16 end (panel 3 and 4). DNA was assembled into nucleosomes and positioned nucleosomes were isolated. The free DNA (bar) and the central positioned nucleosome (gray ellipse) were treated with DNase I for different time points (10, 30 and 90 sec). Purified DNA was subsequently analyzed on a 7% sequencing gel. A scheme of the central positioned nucleosome is shown on the right. A labeled 10 bp ladder (MW) was run in parallel. (**B**) Diagram of the NoRC / nucleosome interaction mapping assay by DNase I footprint. (**C**) DNase I footprint assay. Recombinant NoRC was incubated with a purified nucleosome positioned at the center of the DNA fragment and partially digested with DNase I (10, 30 and 90 sec). The reaction was stopped by the addition of 5 mM EDTA to inactivate the DNase I and the nucleoprotein complexes were separated by gel electrophoresis. Complexes were isolated, DNA purified and analyzed on 7% sequencing gels. Nucleosomes were assembled on DNA, which was radioactively labeled either on the -231 end (panel 5 and 6) or on the +16 end (panel 7 and 8). The nucleosome position (gray ellipse) and the labeling (star) are indicated. Changes in the digestion pattern upon NoRC treatment are marked with a gray bar, strong changes are highlighted with stars. (**D**) Analytical EMSA from DNase I footprint reaction. 5% of the reaction in B, C was separated on a 4.5% PAA gel. The positions of the nucleosomes (gray ellipse) and the NoRC / nucleosome complexes (arrow) are indicated.

8.5 TTF-I: Expression and analysis of its DNA binding activity

The transcription termination factor (TTF-I) is a sequence specific DNA binding factor involved in several processes related to rDNA transcription (Grummt, 1999). Most, if not all functions of TTF-I depend on its binding to a cognate binding site, the terminator element (Grummt et al., 1986b), located in several copies at the 3´-end of the rDNA gene (T_1-T_8) and upstream of the transcription start (T_0). Binding of TTF-I to T_0 together with specific nucleosome remodeling is a prerequisite for transcriptional activation on chromatin templates (Längst et al., 1998; Längst et al., 1997a).

As Tip5 was identified as a novel interaction partner for TTF-I, it was likely that Tip5 and therefore NoRC are involved in the regulation of transcription on chromatin. To investigate this possible role of NoRC and TTF-I in transcriptional regulation further, TTF-I was expressed and analyzed.

8.5.1 Expression and purification of full-length TTF-I and TTFΔN185

The recombinant full-length protein (TTF-I) and an N-terminal deletion mutant (TTFΔN185), lacking the proposed interaction domain with Tip5 (Németh, 2002), were expressed. Full-length TTF-I cDNA was inserted into the pFastBAC HtB vector (Life Technologies) and the recombinant baculovirus was generated as described in chapter 7.2.4. Histidine tagged TTF-I and TTFΔN185 were expressed in Sf9 cells, purified over a heparin column (BioRad) followed by Ni-NTA Agarose (Qiagen) purification. Figure 34A shows the purified recombinant proteins, separated by SDS-PAGE and stained with Coomassie blue. Both, TTFΔN185 (lanes 1 and 2) and TTF-I (lanes 3 and 4) could be purified to apparent homogeneity.

8.5.2 DNA binding activity of recombinant full-length TTF-I and TTFΔN185

First, the DNA binding activity of recombinant full-length and TTFΔN185 was determined. It was shown before, that the N-terminus of full-length TTF-I masks its DNA binding activity *in vitro*. Mild protease treatment recovered the DNA binding activity of full-length TTF-I (Smid et al., 1992). Increasing amounts of TTF-I or TTFΔN185 were incubated with radioactively labeled 247 bp rDNA promoter fragments containing the TTF-I binding site T_0. In addition, TTF-I and TTFΔN185 were incubated with their substrate in the presence of V8 protease, resulting in partial proteolysis of the proteins.

Experiments were performed in the presence of high access of unspecific competitor DNA and DNA binding was monitored by EMSA. Consistent with previous results, TTFΔN185 bound with high affinity to its binding site (Figure 34B, lanes 2 to 4) whereas full-length TTF-I did not bind efficiently (lanes 8 to 10). After V8-protease treatment, both TTF-I and TTFΔN185 exhibited comparable DNA binding activity (compare lanes 5 to 7 with 11 to 13) showing that similar protein amounts were used in this assay. Thus, full-length TTF-I did not form a stable DNA-protein complex *in vitro* unless the N-terminal domain was deleted, or the protein was partially digested with V8 protease, generating a protease resistant protein core.

This suggests that the N-terminal region of TTF-I inhibits DNA binding by acting as a negative regulatory domain, which masks the DNA binding domain of TTF-I (Németh, 2002).

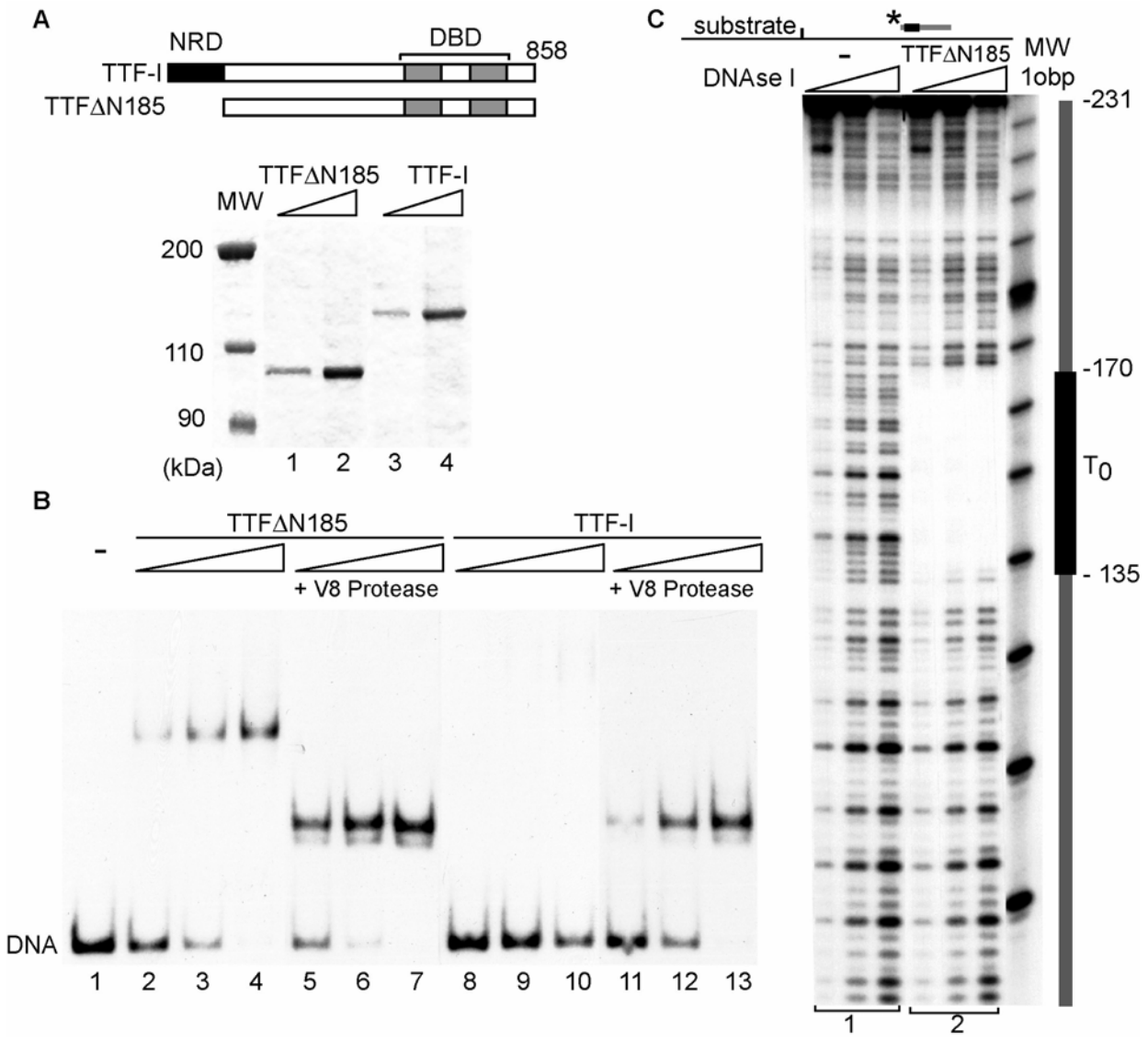

Figure 34. Expression of recombinant TTF-I and analysis of its DNA binding activity
(**A**) Expression and purification of recombinant full-length TTF-I (lanes 3 and 4) and N-terminal truncated TTFΔN185 (lanes 1 and 2). Histidine-tagged proteins were expressed in Sf9 cells, purified via Heparin chromatography and Ni-agarose columns, separated by 7.5% SDS-PAGE and stained with Coomassie blue. A protein size marker (MW) is shown at the left side. A scheme of the TTF-I domain structure is presented above (NRD: negative regulatory domain, DB: DNA binding domain). (**B**) DNA binding properties of full-length TTF-I and TTFΔN185 in electrophoretic mobility shift assays. A ^{32}P-bodylabeled 247 bp rDNA promoter fragment (lane 1), that contains the TTF-I binding site T_0 was incubated for 30 minutes with increasing amounts of recombinant TTFΔN185 (lanes 2 to 4: 2.5, 5 and 10 fmoles) or full-length TTF-I (lanes 8to 10: 2.5, 5 and 10 fmol) as indicated at the top of the figure. Parallel reactions were performed in the presence (lanes 5 to 7 and 11 to 13) of 100 ng V8 protease (added after 15 min). The protein-DNA complexes were resolved on a native 4.5% polyacrylamide gel and analyzed by autoradiography. (**C**) DNase I protection assay ('footprint') of TTFΔN185. Recombinant TTFΔN185 (40 fmol) was incubated with a 146 bp rDNA promoter fragment containing the TTF-I binding site T_0. The reaction was treated with DNase I and stopped with EDTA at different time points (10, 30, 90 sec). The DNA was purified and analyzed on 7% sequencing gels (panel 2). Free DNA is shown in panel 1. A size marker (labeled 10 bp ladder) was run in parallel. The relative positions of the DNA respective to the transcription start site, and TTF-I binding site T_0 (gray bar) are indicated on the right.

8.5.3 DNase I footprint analysis of TTFΔN185 DNA binding

As shown above, the N-terminal deletion mutant TTFΔN185 forms stable complexes with the rDNA promoter *in vitro*.

To confirm the DNA binding specificity to the terminator element T_0, TTF-DNA interactions were studied by DNase I footprint experiments. Radioactively labeled 146 bp rDNA promoter fragments (-231 to -86, respective to the rDNA transcription start site) containing the TTF-I binding site T_0, were incubated with or without TTFΔN185 for 15 min to allow complex formation. After incubation, the reactions were mildly treated with DNase I and stopped at different time points. DNA was purified and analyzed on denaturing sequencing gels. TTF-I dramatically changed the cleavage pattern of the rDNA promoter by DNase I (Figure 34C). A strong protected site ('footprint') appeared between position -170 and -140, which corresponds to the T_0 promoter element (panel 2). The result shows that TTFΔN185 is stably associated with its binding site and protects this site from DNase I digestion.

Finally, both full-length TTF-I and TTFΔN185 were expressed, purified and shown to be active, but only the N-terminal deletion mutant TTFΔN185 forms stable, high affinity complexes with DNA at its binding site.

8.6 Functional consequences of the Tip5 / TTF-I interactions

Initially, the Tip5 / TTF-I interaction was determined in a yeast-two-hybrid system (Jansa et al., 1998). Protein-protein interaction studies indicated that this interaction was mediated through the N-terminus of TTF-I (Németh, 2002). Furthermore, the N-terminus of TTF-I acts as a negative regulatory domain, inhibiting its DNA binding activity *in vitro* (see section 8.5.2 and (Evers et al., 1995)). Below, the functional consequences of the Tip5 / TTF-I interaction for DNA binding and nucleosome remodeling at the rDNA promoter were addressed.

8.6.1 Tip5 interacts with the N-terminus of TTF-I

To confirm the Tip5 / TTF-I interaction and to test whether this interaction was also present within the NoRC complex, protein-protein interaction experiments ('pulldown assay') were performed (Figure 35A).

Similar amounts of purified recombinant full-length and N-terminal truncated TTFΔN185 were incubated with immobilized NoRC, Tip5, Snf2h or beads alone, respectively. TTFΔN185 served as an internal control for the specificity of the protein interactions. After incubation and washing, unbound and bound TTF-I and TTFΔN185 were visualized by western blotting. Indeed, only full-length TTF-I but not TTFΔN185 was retained by both Tip5 and NoRC. Neither Snf2h nor control beads retained significant amounts of either TTF proteins.

Thus, the N-terminus of TTF-I interacts directly with Tip5, and this interaction exists within the NoRC complex.

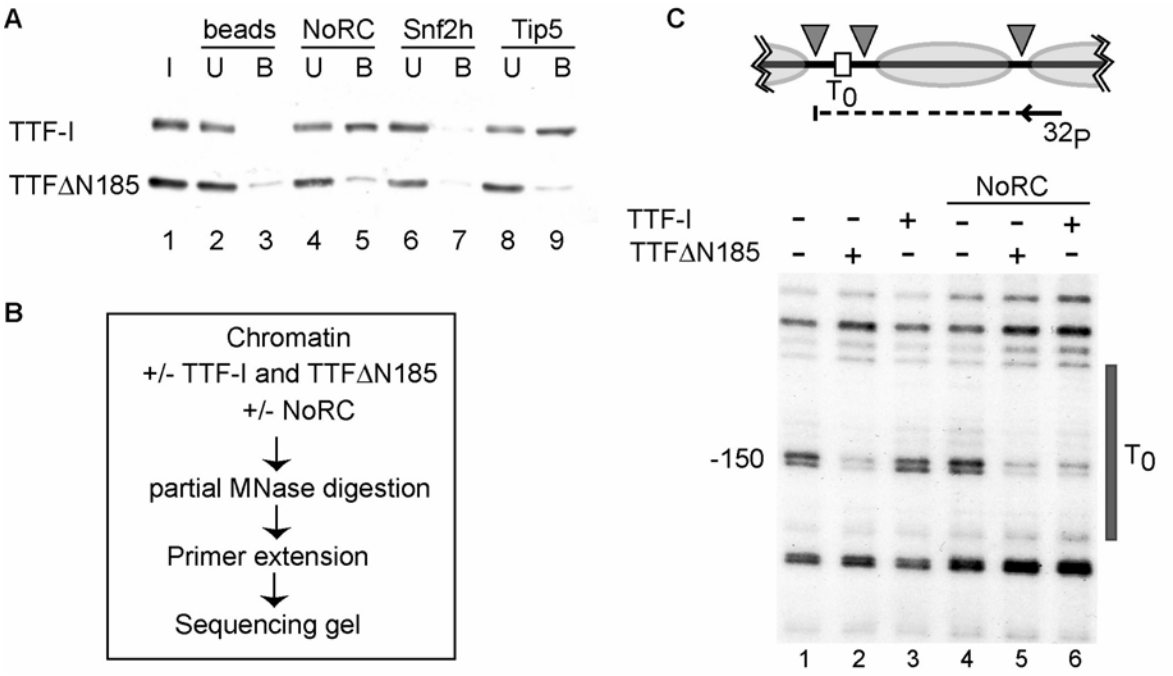

Figure 35. NoRC / TTF-I interactions mediate binding of full-length TTF-I to chromatin
(**A**) The N-terminal part of TTF-I interacts with full-length Tip5 *in vitro*. Recombinant NoRC, Snf2h and Tip5 were immobilized on beads and were incubated with a mixture of purified, recombinant full-length TTF-I and N-terminally truncated TTF-I (TTFΔN185). After washing with 10 volumes of EX-300 buffer, 10% of the input (I), unbound (U) and 50% of bead-bound proteins (B) were analyzed by 7.5% SDS-PAGE followed by immunoblot using TTF-I specific antibodies. (**B**) and (**C**) NoRC-TTF-I interaction enables DNA binding of TTF-I. Chromatin, containing the rDNA promoter fragment with the TTF-I binding site T_0, was incubated with TTFΔN185 (lanes 2 and 5) and TTF-I (lanes 3 and 6) in the absence or presence of NoRC (lanes 4 to 6). TTF-I binding to T_0 was detected by partial MNase digestion of the DNA and primer extension footprinting (A diagram of the reaction is indicated in B, a scheme of the primer extension reaction is presented above). The gray box indicates the TTF-I binding site. The diagnostic cleavage site (-150) is shown on the left.

8.6.2 The Tip5 / TTF-I interaction restores the DNA binding activity of TTF-I

The pull-down experiments revealed that the N-terminus of TTF-I interacts with Tip5 within the NoRC complex. This part of TTF-I acts as a negative regulatory domain (NRD), inhibiting DNA binding of the full-length protein (see 8.5.2). To investigate, whether Tip5 / TTF-I interactions would restore the DNA binding activity of TTF-I in chromatin, MNase footprint experiments were performed in collaboration with Gernot Längst. The assembly of DNA into nucleosomes usually reduces the accessibility of the DNA by specific DNA-binding factors due to altering DNA structure, flexibility and masking of the DNA recognition sequences on the histone-octamer surface. TTF-I is an unusual DNA binding factor, since TTFΔN185 is able to recognize its DNA binding site, even on the surface of a nucleosome (Längst et al., 1998). Sequence-specific binding of TTF-I to the nucleosomal rDNA promoter with the upstream TTF-I binding site T_0 was analyzed.

Nucleosomal arrays were incubated with TTF-I in the presence or absence of NoRC, and TTF-I binding was assayed by MNase footprinting (see scheme Figure 35B). Consistent with previous results, TTFΔN185 recognized the cognate binding site T_0 even when it was packaged into chromatin. Upon binding, TTFΔN185 protected at diagnostic cleavage site at position -150 (Figure 35C, lane 2). Full-

length TTF-I failed to bind, as the DNA-binding domain is masked by the N-terminus of the protein (lane 3). NoRC alone did not alter the chromatin structure, but TTF-I in the presence of NoRC was able to recognize T_0 and formed a stable TTF-I-chromatin complex (lanes 4 to 6). These experiments indicate that the interaction of full-length TTF-I with NoRC unmasks its DNA binding domain and allows TTF-I binding to its cognate binding site T_0 in chromatin.

8.6.3 TTF-I targeting of NoRC leads to remodeling at the rDNA promoter

The NoRC / TTF-I interaction mediates sequence-specific binding of full-length TTF-I in chromatin. These results imply that TTF-I binding to DNA results in the recruitment of NoRC to the rDNA gene promoter. The functional consequences of the TTF-I / NoRC interaction on the rDNA chromatin structure was examined in further experiments together with Gernot Längst. It was shown before, that TTF-I mediated activation of rDNA transcription on chromatin templates correlates with a remodeling reaction that positions nucleosomes upstream and downstream of the TTF-I binding site (Längst et al., 1998; Längst et al., 1997a). To address the role of the NoRC / TTF-I interaction the rDNA chromatin structure was investigated *in vitro* as described (Längst et al., 1998).

Salt assembled chromatin was incubated with TTF-I and TTFΔN185 in the presence of either NoRC or ACF, partially digested with MNase and the chromatin structure of the rDNA promoter analyzed by indirect end labeling experiments (Figure 36A). Incubation of rDNA-chromatin with TTF-I, TTFΔN185, ACF or NoRC did not significantly alter the MNase digestion pattern at the rDNA promoter (Figure 36A, lanes 1 to 5). In the presence of both ACF / NoRC and TTFΔN185, nucleosome rearrangement occurred (lanes 6 and 7). Consistent with previous results, TTFΔN185 bound to its binding site T_0, creating two MNase sensitive sites flanking the TTF-I binding site and adjacent MNase sensitive sites were protected by positioned nucleosomes (Längst et al., 1998; Längst et al., 1997a). When full-length TTF-I was used, ACF did not alter chromatin structure (lane 8), indicating that TTF-I was not able to bind to T_0 in chromatin. In the presence of NoRC however, TTF-I was able to bind to T_0 and NoRC did remodel the chromatin structure; triggering nucleosome rearrangement at the rDNA promoter (lane 9). The result indicates that recruitment of the NoRC / TTF-I complex to the rDNA promoter enables TTF-I specific rearrangement of the promoter bound nucleosomes.

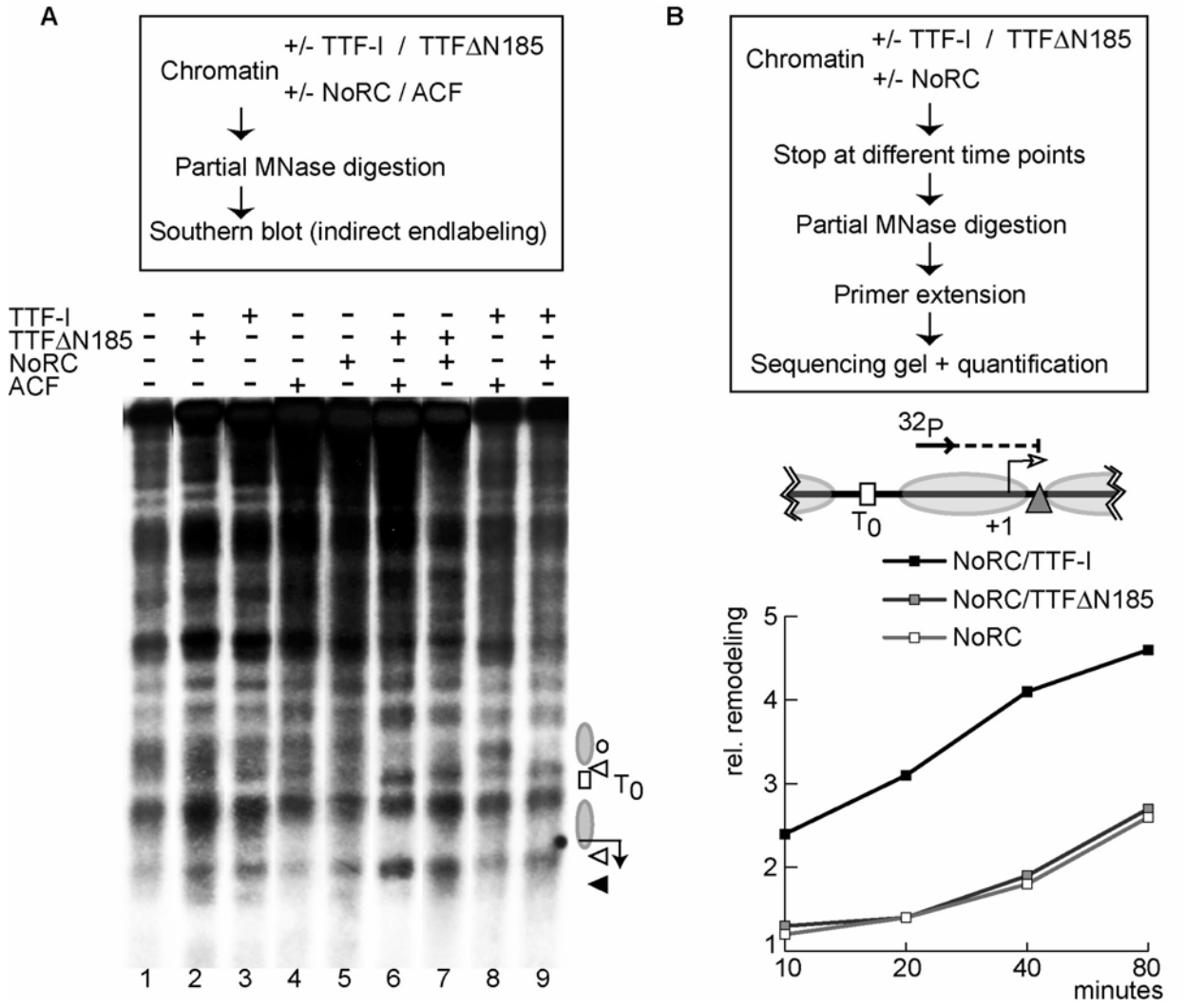

Figure 36. Recruitment of NoRC leads to nucleosome remodeling at the rDNA promoter
(**A**) NoRC-TTF-I interaction triggers chromatin remodeling at the rDNA promoter. Mapping of MNase cleavage sites by indirect end labeling, on pMrSP DNA (mouse rDNA promoter fragment (-292 to +155) fused to a 3´-terminal rDNA fragment) reconstituted into chromatin. Chromatin was incubated with TTFΔN185, TTF-I, ACF or NoRC (lanes 2 to 5), TTFΔN185 in the presence of ACF or NORC (lanes 6 and 7) and TTF-I in the presence of ACF or NoRC (lanes 8 and 9). Each reaction was digested with 1 U MNase for 30 s, purified DNA was digested with Nde I and analyzed by southern blotting. A diagram of the reaction is indicated at the top. The positions of rearranged nucleosomes (gray ellipse), the TTF-I binding site and the transcription start site (arrow) are indicated. Protected (circles) or enhanced MNase cleavage (white and black triangles) sites are indicated on the right. Position +22, the 3'-boundary of the nucleosome positioned at the rDNA promoter is marked by the black triangle. (**B**) Quantitative analysis of NoRC dependent nucleosome remodeling. Nucleosome remodeling with limiting amounts of NoRC was performed as described in A, but in contrast nucleosome positions were mapped by partial MNase digestion and primer extension of the purified DNA after 10, 20, 40 and 80 minutes of remodeling. A diagram of the reaction is indicated at the top. Primer extension products were resolved on 8% sequencing gels and quantified with a Phospho Imager (Fuji) (see also (Strohner et al., 2004)). The appearance of the MNase cleavage site at the 3'-boundary of the remodeled nucleosome (position +22) was quantified relative to the input material (chromatin in the absence of factors). Relative intensities of the MNase cleavage site at position +22, correlating with overall nucleosome remodeling, were plotted in a graph over the reaction time. The relative position of the oligonucleotide used for primer extension and the MNase sensitive sites on the rDNA promoter relative to the transcription start site are indicated.

8.6.4 Recruitment of NoRC leads to increased remodeling at the rDNA promoter

To examine whether NoRC and full-length TTF-I would lead to similar nucleosome remodeling at the rDNA promoter as observed with TTFΔN185, the positions of nucleosome boundaries in reconstituted rDNA chromatin were mapped by partial MNase digestion and analyzed by primer extension in collaboration with Gernot Längst.

A radioactively labeled oligonucleotide positioned within the realm of the promoter nucleosome was used to monitor alterations in nucleosome remodeling in high resolution. The intensity of individual MNase sensitive sites directly reflects the relative amounts of nucleosome boundaries at this position. After incubation with saturating concentrations of NoRC and TTF-I, the pattern of MNase cleavage sites was significantly altered. The most pronounced feature was the enhancement of an MNase sensitive site at nucleotide +22, which corresponds to the 3'-boundary of the positioned nucleosome at the rDNA promoter. Nucleosome remodeling reactions by TTFΔN185 were indistinguishable from remodeling reactions triggered by full-length TTF-I (data not shown, see (Strohner et al., 2004)).

In order to monitor the remodeling efficiency, chromatin was incubated with non-saturating amounts of NoRC in the presence or absence of TTFΔN185 or TTF-I (Figure 36B). Remodeling reactions were stopped after different time points and nucleosome positions were analyzed by MNase footprinting. In order to measure the remodeling efficiency, changes of the MNase sensitive sites at position +22 were quantified and compared to the non-remodeled starting material. A quantitative analysis of the nucleosomes moved to position +22 is shown in Figure 36B. Neither NoRC, nor TTFΔN185 with NoRC, significantly changed the MNase pattern during the time course. However, in the presence of full-length TTF-I and NoRC, chromatin structure was efficiently altered. Nucleosome remodeling efficiency at the rDNA promoter was augmented 2-3 fold, in the presence of the TTF-I / NoRC interaction domain.

These experiments imply that TTF-I specifically recruits NoRC to the rDNA promoter and this leads to NoRC-mediated nucleosome remodeling.

8.7 The role of NoRC in RNA polymerase I transcription regulation

Tip5 localizes in the nucleolus, the site of ribosomal RNA synthesis, and interacts with TTF-I, a factor involved in rDNA transcription regulation. Furthermore, Tip5 is part of the remodeling complex NoRC, which enables TTF-I binding to the rDNA promoter in chromatin. Therefore, NoRC is recruited to the rDNA promoter followed by a nucleosome remodeling event. These experiments suggest that NoRC influences transcription of the rRNA genes.

8.7.1 A TTF-I dependent *in vitro* transcription system on chromatin

In order to study the function of NoRC on rDNA transcription, a TTF-I dependent RNA pol I *in vitro* transcription system was established (see scheme Figure 37A). This system was recently used to show that TTF-I is required for the activation of rDNA transcription on chromatin templates. Furthermore, TTF-mediated activation of rDNA transcription on chromatin templates required an ATP-dependent remodeling reaction (Längst et al., 1998; Längst et al., 1997a). An artificial rDNA minigene (WTT), that contains the mouse rDNA promoter region spanning from -170 to +135, coupled to the full terminator region, was used as a template in the *in vitro* transcription experiments. The rDNA minigene was either used directly or reconstituted into chromatin. A partially purified nuclear extract from mouse cells

(DEAE280 fraction) was the source for RNA polymerase I and other factors required for transcription (Schnapp and Grummt, 1996). Markedly, this extract contains all the factors needed for RNA pol I transcription but lacks the termination factor TTF-I. Therefore, transcriptions carried out on 'naked' DNA result in long read-through transcripts. In contrast, the addition of exogenous TTF-I lead to short, terminated transcripts (see scheme Figure 37A, panel DNA). A different result is seen in transcription reactions performed with chromatin templates, where the presence of TTF-I is required for both termination and activation of transcription (panel chromatin).

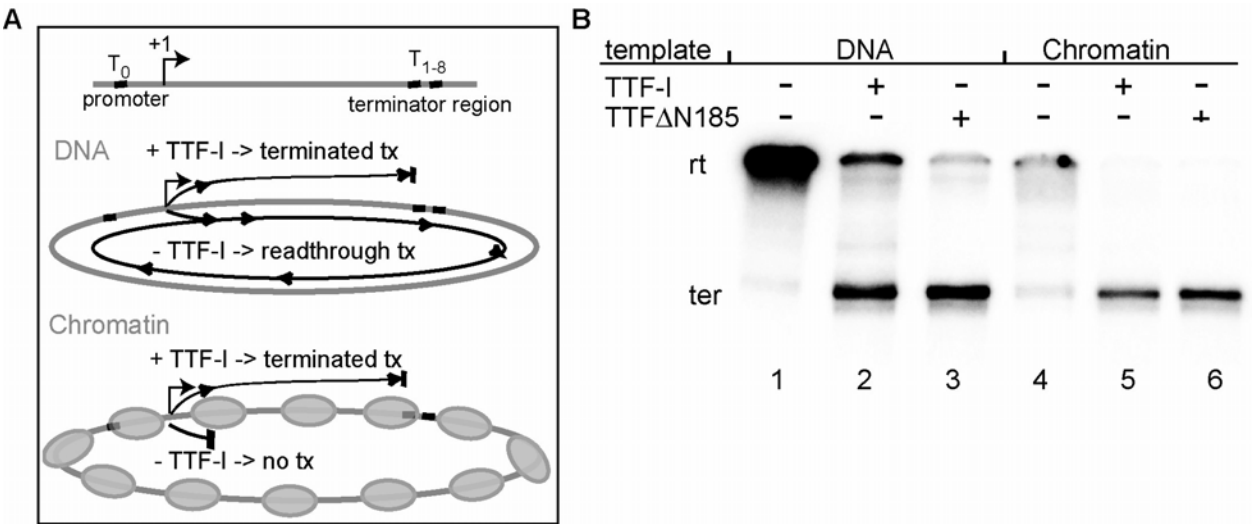

Figure 37. TTF-I dependent RNA Pol I *in vitro* transcription system
(**A**) Schematic representation of the TTF-I dependent Pol I *in vitro* transcription system. The presence of TTF-I is required for transcriptional termination but also for activation in chromatin (see text). The rDNA minigene (template) is shown at the top. The results of the *in vitro* transcription (tx) in the absence or presence of TTF-I are shown inside or outside of the DNA or chromatin template, respectively. (**B**) RNA pol I *in vitro* transcription in the context of chromatin and TTF-I. A minigene (pMrWT-T) containing the rDNA promoter and the terminator region was used for *in vitro* transcription. DNA was incubated with the transcription extract (DEAE280) in the absence or the presence of TTFΔN185 or TTF-I (lanes 1 to 3). Transcription on the minigene assembled into chromatin is shown in the lanes 4 to 6. The template was reconstituted into chromatin by salt gradient dialysis using purified histone octamers. Nucleosomal templates were purified in a sucrose gradient and the nucleosome density was monitored by DNA supercoiling and MNase digestion (Strohner et al., 2004). Transcription reactions were performed for 60 min at 30°C. The reaction contained all nucleotides necessary for transcription and traces of ^{32}P α-CTP to visualize the transcripts. The radioactive labeled products were purified and separated on 4.5% polyacrylamide gels and analyzed by autoradiography. Read-through (rt) transcripts in the absence and terminated transcripts (ter) in the presence of TTF-I are indicated on the left.

8.7.2 Full-length TTF-I terminates and activates rDNA transcription on chromatin

Previous experiments were performed with recombinant TTF-I protein lacking the N-terminal part. As discussed before, the N-terminus of TTF-I acts as a negative regulatory domain inhibiting DNA binding, but also interacts with Tip5. This interaction recovers the DNA binding ability of the full-length protein.

In the first step, the effect of full-length TTF-I on rDNA transcription on DNA and chromatin templates was compared to that of TTFΔN185 (Figure 37B). Long read through transcripts were

synthesized from naked DNA templates (lane 1). Addition of TTFΔN185 and full-length TTF-I caused transcription termination 686 nt downstream of the initiation site (lanes 2 and 3). Consistent with previous data, transcription on chromatin templates was repressed (lane 4) but in the presence of TTFΔN185 activated (lane 6). Notably, chromatin-mediated transcriptional repression was also relieved by TTF-I (lane 5).

In conclusion, these results indicate that full-length TTF-I is able to terminate and activate rDNA transcription, and suggests that the transcription extract contains activities that enable binding of TTF-I to the terminator elements.

Additionally, TTF-I dependent transcription reactions were performed with chromatin templates reconstituted from different histones as a source.

The rDNA minigene was assembled into chromatin using either purified or recombinant histone octamers. Recombinant histones contained either all tails or only the histone H4 N-terminus. Transcription assays were performed as above in the presence or absence of exogenous TTF-I (Figure 38). Long read through transcripts were synthesized from naked DNA templates (lane 1), addition of TTFΔN185 caused transcription termination (lane 2). Again, transcription on preassembled chromatin templates was repressed (lanes 3, 5 and 7). The presence of TTFΔN185 activated transcription similarly (lanes 4, 6 and 8), although in the case of recombinant histones, transcription was not completely repressed.

This experiment indicates that TTF-I dependent transcriptional activation in chromatin is possible form chromatin templates reconstituted from either purified *Drosophila* or recombinant *Xenopus* histones as a source.

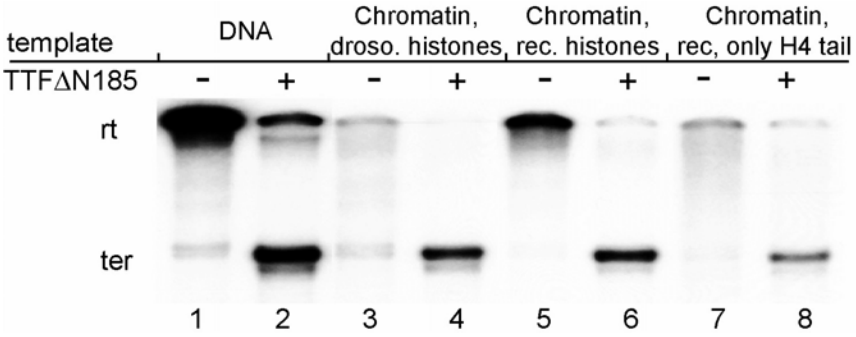

Figure 38. Transcription assay with different chromatin templates
Salt assembled Chromatin with different histones was incubated with the transcription extract in the presence or absence of TTFΔN185. Lanes 1 and 2 show the transcription from free DNA, lanes 3 to 8 with chromatin templates. Chromatin was either assembled using *Drosophila* histones (droso: lanes 3 and 4) or with recombinant histones (lanes 5 to 8). Recombinant histones contained either all tails (lanes 5 and 6) or only histone H4 tail (lanes 7 and 8). Analysis as described in Figure 37. Read-through (rt) and terminated (ter) transcripts are indicated on the left.

8.7.3 NoRC represses rDNA transcription specifically on chromatin templates

In the following section the effect of NoRC on RNA pol I transcription in the context of DNA and chromatin was analyzed.

In vitro transcription experiments were performed with DNA or nucleosomal templates, partially purified nuclear extract (DEAE-280 fraction) and recombinant TTF-I and TTFΔN185. In addition, the reaction was supplemented with increasing amounts of recombinant NoRC. After 60 min incubation, the radioactive labeled RNA transcripts were purified and analysed on 4.5% polyacrylamide gels. As seen before, termination of transcription on DNA (Figure 39A, lanes 2 and 5) and activation in chromatin (lanes 9 and 13) was dependent on TTF-I. Addition of NoRC did not affect the DNA transcription, independent of the recombinant TTF-I used (lanes 3 and 4 or 6 and 7, respectively). A different result was obtained in transcription experiments performed with chromatin templates. Increasing amounts of NoRC resulted in a concentration dependent decrease of the transcript signals (lanes 10 to 12 and 14 to 16), indicating transcription repression. NoRC repressed the transcription on chromatin templates with either TTF-I proteins. However, comparison of the reactions performed in the presence of TTF-I and TTFΔN185 revealed a roughly twofold stronger effect of NoRC with full-length TTF-I. These results indicate that NoRC abolishes rDNA transcription particularly on chromatin templates *in vitro*, and this repression is partially mediated via interaction by TTF-I.

Next, control experiments were performed. Chromatin transcriptions with TTF-I were carried out as before, adding either purified NoRC, purified NoRC heat denatured for 5 min at 95°C (heat inactivated, see ATPase assay Figure 39B) or mock-purified protein. The result is shown in Figure 39C. Once again, NoRC repressed rDNA transcription on chromatin templates in a concentration dependent manner (lanes 6 to 8). Neither the mock-purified protein (lanes 9 to 11) nor the heat inactivated NoRC (NoRC/95°C, lanes 3 to 5) showed any influence on the transcription reactions.

Taken collectively, these results show that the chromatin remodeling complex NoRC specifically represses rDNA transcription on chromatin templates *in vitro*.

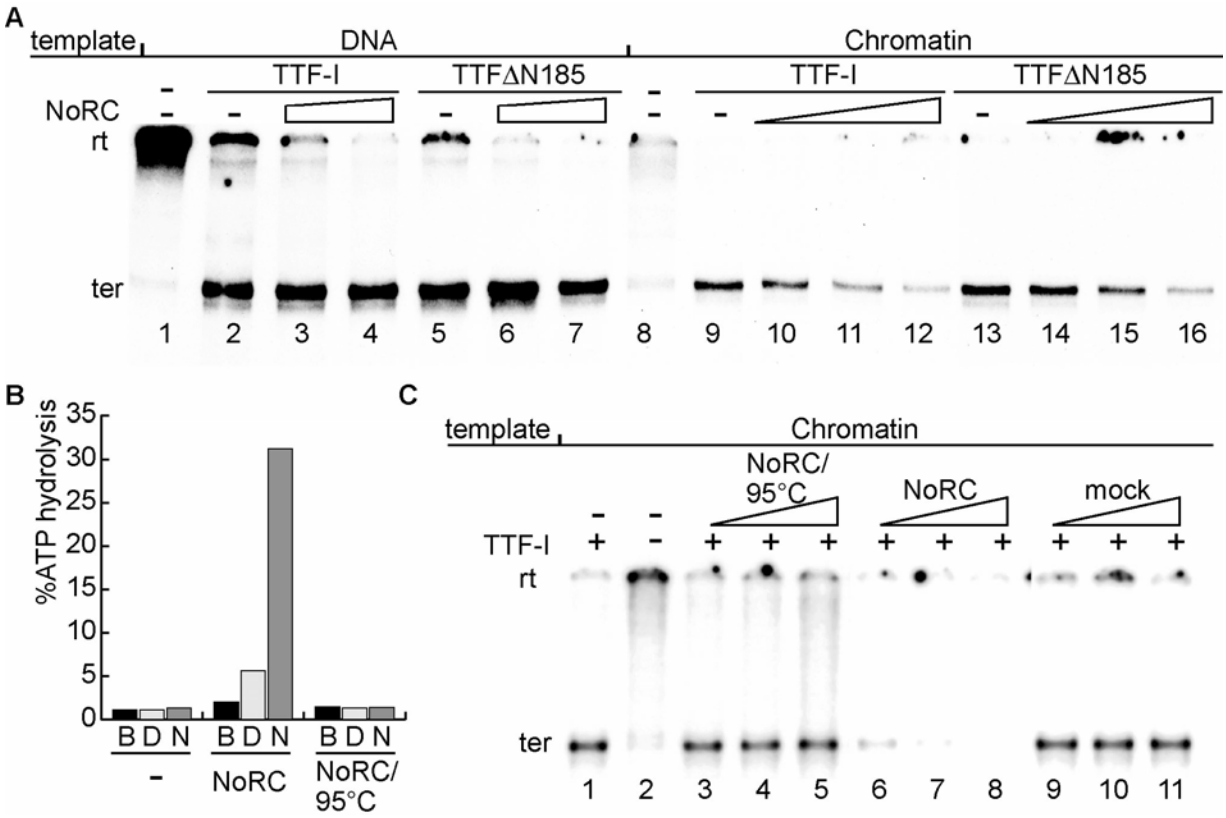

Figure 39. NoRC represses rDNA transcription only on chromatin templates
(**A**) *In vitro* transcription assay in the presence of NoRC. Increasing amounts of NoRC (lanes 3 and 4, 6 and 7: 50, 100 fmoles; lanes 10 to 12 and 14 to 16: 25, 50, 100 fmoles) were incubated with the transcription extract (DEAE280) in the presence of TTFΔN185 and TTF-I as indicated. Transcription was performed on free DNA (lanes 1 to 7) and on chromatin templates (lanes 8 to 16) for 60 min, transcripts purified and analyzed on 4.5% polyacrylamide gels. Read-through (rt) and terminated (ter) transcripts are indicated on the left. (**B**) Heat denatured NoRC has no ATPase activity. 0.2 pmol of NoRC and heat denatured NoRC (NoRC/95°C) were investigated for activity in ATPase assay in the absence (B) or in the presence of DNA (D) and nucleosomal substrate (N). Samples were analyzed as described before. (**C**) Control reactions. Neither heat denatured NoRC, nor mock purified protein show transcriptional repression effects. TTF-I dependent *in vitro* transcription reactions were performed on chromatin templates and the following proteins were added to the reactions: NoRC, heat inactivated NoRC (NoRC/95°C) and mock-purified protein, gained through a similar purification scheme. Transcripts were analyzed as described before.

8.7.4 rDNA transcription repression is specially exerted by NoRC

Next, it was investigated if repression of rDNA transcription on chromatin is mediated specifically by NoRC. NoRC and two other chromatin remodeling factors, e.g. the motor protein Snf2h and ACF were compared in parallel in rDNA transcription experiments.

Figure 40A shows a Coomassie Blue stained SDS-PAGE gel with the analyzed recombinant proteins (NoRC, Snf2h and ACF). Similar activities were judged in ATPase assays (Figure 40B) and further used in the *in vitro* transcription assay. TTF-I dependent transcription experiments were performed with DNA or chromatin templates in the presence of increasing amounts of Snf2h, NoRC and ACF. On the DNA template, exogenous Snf2h, NoRC and ACF did not affect transcription at all (Figure 40C, DNA panel). On chromatin templates, however, a concentration-dependent repression of transcription by NoRC (lanes 6 to 8), but not by Snf2h or ACF, was observed (Fig. 27C, chromatin panel).

This result demonstrates that only the chromatin remodeling complex NoRC specifically represses rDNA transcription on chromatin templates *in vitro*.

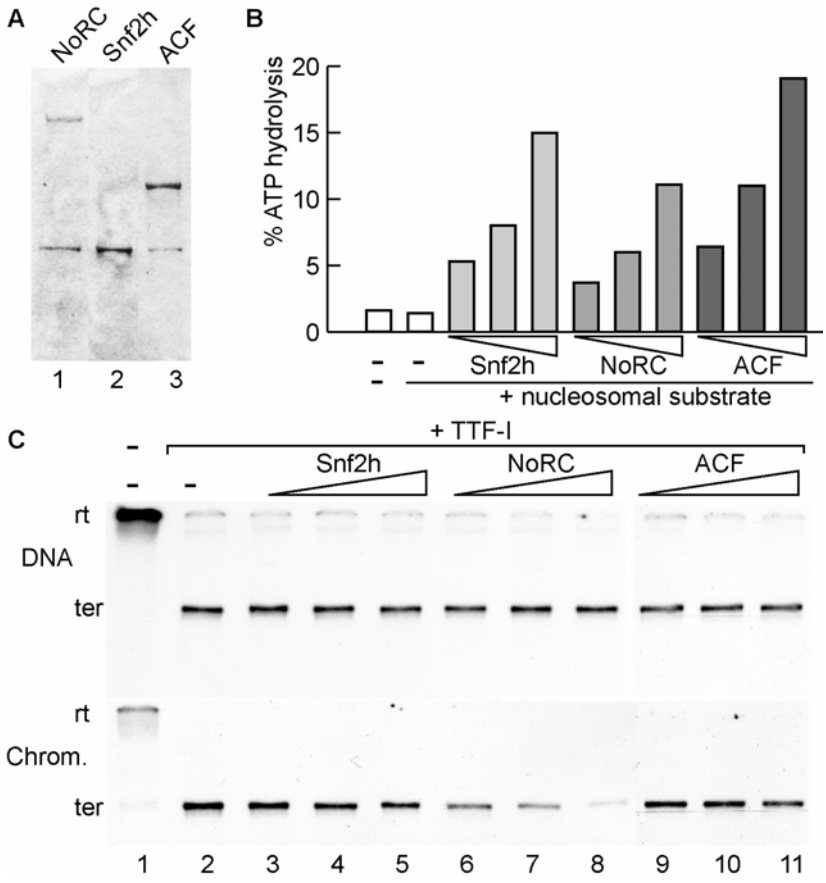

Figure 40. NoRC, but not Snf2h or ACF, represses rDNA transcription
(**A**) A Coomassie blue stained protein gel of the purified recombinant proteins used in this assay is shown. (**B**) ATPase assay. Similar amounts of Snf2h, NoRC and ACF (0.05 to 0.2 pmol) are compared for ATPase activity. Proteins were incubated for 60 min at 26°C with nucleosomal substrate and ^{32}P γATP. The hydrolysed ATP was resolved by thin layer chromatography and the result quantified with a Phospho Imager (Fuji). (**C**) Only NoRC represses rDNA transcription on chromatin templates. Increasing amounts of Snf2h (lanes 3 to 5; 25, 50, 100 fmoles), NoRC (lanes 6 to 8; 25, 50, 100 fmoles) and ACF (9 to 11; 25, 50, 100 fmoles) were added to the TTF-I dependent (lanes 1 and 2) *in vitro* transcription system. Read-through (rt) and terminated (ter) transcripts in the presence of TTF-I are indicated. Transcription reactions were performed on naked DNA and on chromatin templates as shown on the left.

8.8 Does NoRC-mediated rDNA repression depend upon chromatin remodeling?

The results above indicate a specific role of NoRC in the repression of rDNA transcription in chromatin. To reveal whether nucleosome remodeling is involved in this process, two different approaches were undertaken: rDNA transcription experiments were performed either with a chromatin remodeling inactive, ATPase mutant of NoRC, or with chromatin templates lacking the histone H4 tail, which was shown to be necessary for nucleosome remodeling.

8.8.1 Reconstitution of a chromatin remodeling deficient NoRC

A chromatin remodeling deficient NoRC complex was reconstituted from Tip5 together with an ATPase mutant of Snf2h. This ATPase mutant has a single amino acid exchange in the ATP-binding pocket domain (lysine to arginine exchange at position 211), which abolishes its ATPase and remodeling activity (Corona et al., 1999; Hakimi et al., 2002).

Recombinant NoRC, containing the wild type or the mutant Snf2h, respectively, was produced by coexpression of Snf2h with Tip5 in Sf9 cells followed by a two-step purification. Comparable amounts of the complexes were obtained (Figure 41A lanes 1 and 2). Both complexes were tested in ATPase assays and in nucleosome remodeling assays. As expected, wild type NoRC displayed nucleosomal stimulated ATPase activity and was able to mobilize the octamer along the DNA, but NoRC containing the Snf2h mutant was inactive in both assays (Figure 41B and C). Electrophoretic mobility shift assays were performed to assay the DNA binding ability of each complex. Both showed identical DNA / nucleosome binding behavior (data not shown). Similar results were obtained, when the activities of the Snf2h proteins alone were examined (data not shown).

It can be concluded, that mutation of the ATPase domain in Snf2h abolishes its ATP-dependent remodeling activities, but does not affect its ability to bind Tip5 or DNA.

8.8.2 Effect of the NoRC mutant on rDNA transcription

The chromatin remodeling deficient mutant and the wild type NoRC were compared in *in vitro* transcription assays. Similar amounts of wild type and mutant NoRC were added to a TTF-I dependent *in vitro* transcription assay on DNA or chromatin and proceeded as before (Figure 41D, DNA: lanes 1 to 6 and chromatin: lanes 7 to 14, respectively). Unexpectedly, a similar repression was observed on chromatin templates with either recombinant wild type NoRC or with NoRC reconstituted with the ATPase-deficient Snf2h mutant. This lead to the conclusion that the remodeling activity of NoRC might not be necessary for transcription repression, but Tip5 alone might be sufficient.

In a second experiment, the influence of recombinant Tip5 on rDNA transcription on DNA and chromatin templates was assessed (data not shown). Again, neither NoRC nor Tip5 affected transcription on free DNA. On chromatin templates, however, Tip5 repressed rDNA transcription, although to a lower extent then NoRC (see also (Strohner et al., 2004)).

This suggests that Tip5 alone might bind to nucleosomal rDNA and inhibit Pol I transcription. However, other remodeling activities present in the transcription extract could be associated with the template and provide nucleosome repositioning within the reaction. *In vitro* transcription assays with a highly purified system, lacking additional remodeling activities, are necessary to answer this question.

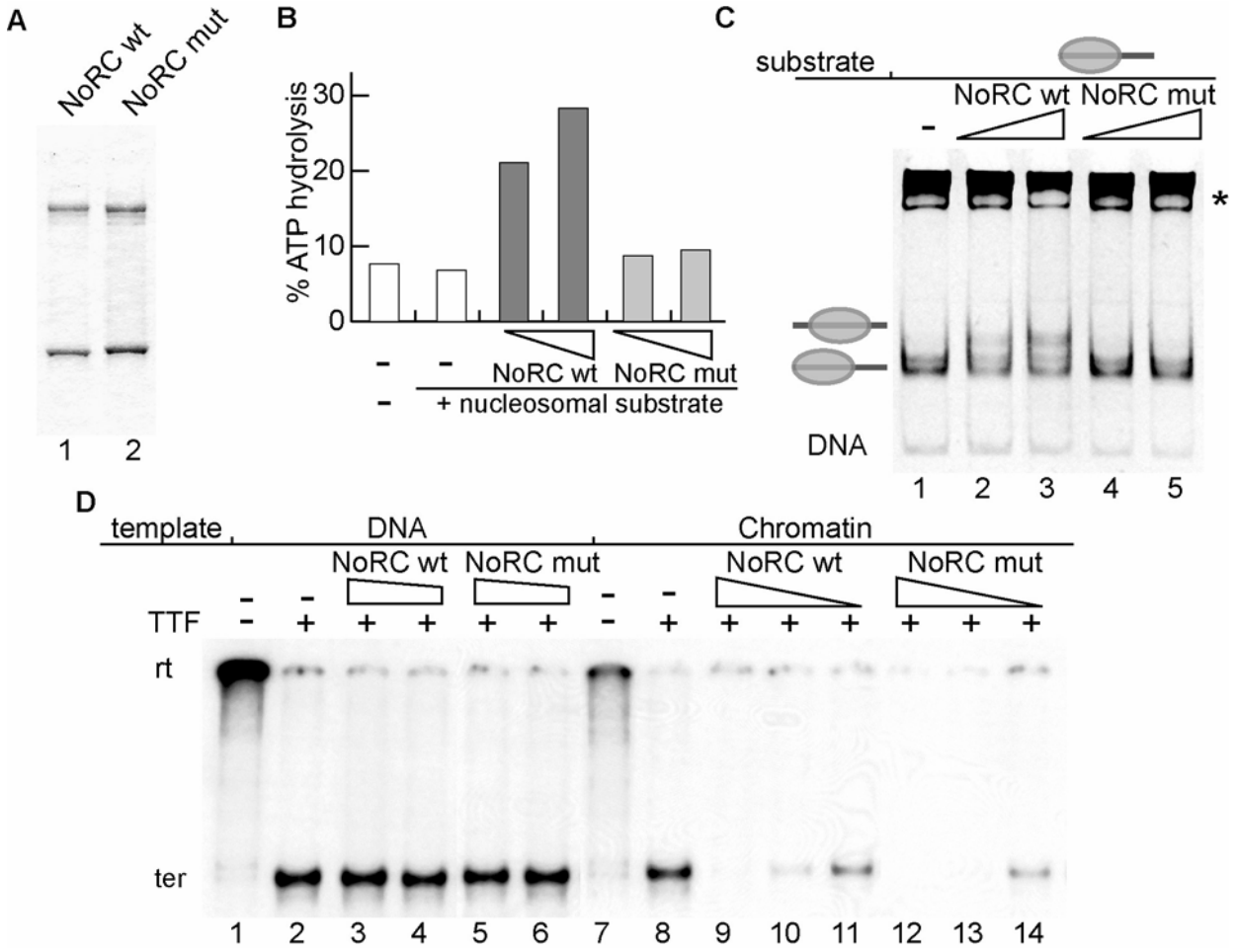

Figure 41. Reconstitution and analysis of a chromatin remodeling deficient NoRC complex
(**A**) Reconstitution of recombinant NoRC containing wild type (lane 1) or an ATPase deficient Snf2h mutant (Snf2h KR211, lane 2). Tip5 and the individual Snf2h constructs were coexpressed in Sf9 cells, purified in two steps as described, resolved by 6% SDS-PAGE and stained with Coomassie blue. (**B**) ATPase assay with wild type (wt) NoRC and mutant (mut) NoRC in the presence of 300 ng of nucleosomal arrays. The bar shows the percentage of hydrolyzed ATP. (**C**) Nucleosome mobility assay. Non-labeled mononucleosomes (1 pmol), positioned at the end of the DNA fragment (lane 1), were incubated for 90 min with increasing amounts of wild type (wt) NoRC (lanes 2 and 3: 50 and 100 fmoles) or with mutant NoRC (lanes 4 and 5: 50 and 100 fmoles) in the presence of ATP. Nucleosome positions were analyzed by native gel electrophoresis and were visualized using SYBR green (Roche). The positions of end-positioned and center-positioned nucleosomes and free DNA are indicated on the left. Competitor DNA, that was added to stop the reaction, is marked with a star. (**D**) *In vitro* transcription assay: The chromatin remodeling inactive NoRC mutant abolishes transcription similar to the wild type. Increasing amounts of NoRC containing wild type Snf2h (NoRC wt) (lanes 3 and 4, 9 to 11: 25, 50 and 100 fmoles), and NoRC, reconstituted with the Snf2h KR211 mutant (NoRC mut) (lanes 5 and 6, 12 to 14: 25, 50 and 100 fmoles) were added to an *in vitro* transcription reaction on DNA (lanes 1 to 6) or chromatin (lanes 7 to 14). Reactions were supplemented with TTF-I and the transcription extract, incubated for 60 min at 30°C and transcripts analyzed as described. Read-through transcription and terminated transcription on naked DNA and on chromatin templates are indicated.

8.8.3 The histone H4 tail is a prerequisite for NoRC-mediated rDNA repression

Another way to uncouple chromatin remodeling from NoRC-mediated rDNA repression, is to perform *in vitro* transcriptions on nucleosomal templates that either lack or contain only the N-terminus of histone H4. As nucleosome remodeling by NoRC is known to require the tail of histone H4 (see previous results 8.2.3 and 8.3.4), this experimental approach should reveal whether binding and interaction or in addition chromatin remodeling causes transcriptional repression.

Chromatin was reconstituted from recombinant histones, that either lacked (g4) or contained only the N-terminus of histone H4 (i4), and analysed in ATPase assays with NoRC. These results confirmed the requirement of the histone H4 tail for NoRC's activity (Figure 42A).

The same chromatin templates were used in a TTF-I dependent transcription system. As shown before (Figure 38), TTF-I was able to activate transcription from these chromatin templates. In accordance with the results of the ATPase assay, NoRC affected the chromatin transcription in an H4 tail dependent manner. NoRC did not repress Pol I transcription on tail-less nucleosomes (Figure 42B, lanes 1 to 4), but repressed transcription on templates lacking all histone tails except the N-terminus of histone H4 (lanes 5 to 8). Furthermore, the enhanced transcription signals on the H4 tail lacking templates are consistent with the histone H4 tail being a prerequisite for rDNA repression.

These results demonstrate that the N-terminus of histone H4 is required for rDNA repression and suggest that the ATPase activity of NoRC might be necessary for repression of transcription.

8.8.4 Binding of NoRC to nucleosomes does not depend on the histone H4 tail

One explanation for the histone H4 tail dependence of NoRC-mediated rDNA repression could be a preferred chromatin binding to the different nucleosomal arrays. Therefore, nucleosome interaction experiments were performed with mononucleosomal substrates lacking or containing the histone H4 tail.

Increasing amounts of Tip5, Snf2h and NoRC were incubated with end-positioned nucleosomes, containing or lacking the N-terminus of histone H4 and analysed by EMSA on 1.3% agarose gels. As shown before, all three proteins bound to the intact nucleosomes (Figure 42C). Only slight differences are visible with nucleosomes lacking the histone H4 tail (g4, lanes 1 to 7) or containing all tails (N, lanes 8 to 14). While Tip5 and NoRC bind to some extent better to the histone H4 tail-less substrate, Snf2h alone seems to have a minor preference to the H4 tail and binds better to the intact nucleosome. Markedly, NoRC binding to the H4 tailless substrate is not abolished.

These results indicate that the histone H4 tail is not required for NoRC binding to the chromatin template during repression of transcription. Instead, they suggest, that the ATPase activity and thus chromatin remodeling is actively involved in rDNA repression.

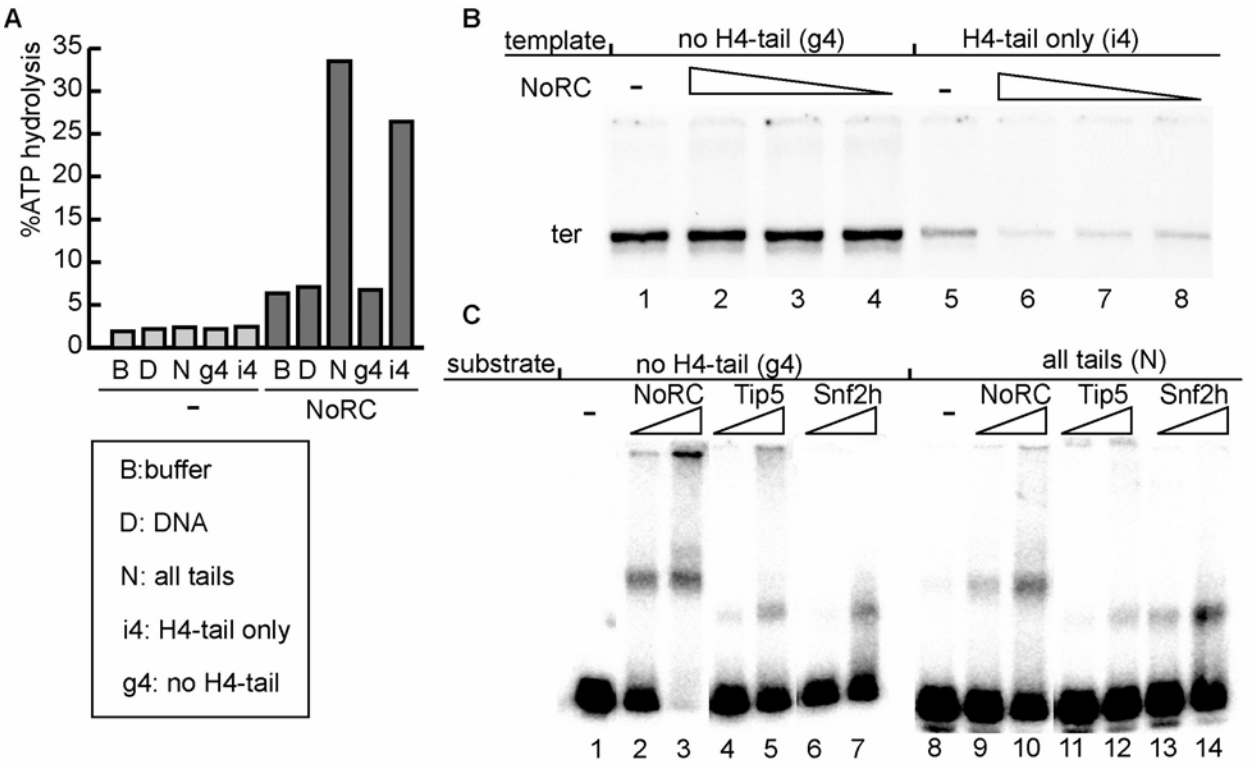

Figure 42. The histone H4 tail is required for NoRC-mediated transcriptional repression
(**A**) ATPase assay with different nucleosomal substrates. The ATPase activity of recombinant NoRC in the absence of substrate (B: Buffer), in the presence of DNA (D) or the indicated recombinant octamers reconstituted into nucleosomal arrays was analyzed. (N: nucleosomes reconstituted from full-length histones; g4: histone H4 lacks the N-terminal tail, the other histones are full-length proteins; i4: histone H4 is a full-length protein, the other histones lack the N-terminal tail). The bar shows the percentage of hydrolyzed ATP. (**B**) Transcription assay. NoRC repression of rDNA transcription on chromatin templates is histone H4 tail dependent. Transcription was assayed with the nucleosomal arrays either lacking only the histone H4 N-terminus (lanes 1 to 4) or containing only the histone H4 N-terminus and lacking the N-termini of H3, H2A and H2B (lanes 5 to 8) in the presence of TTF-I and decreasing amounts of NoRC (100, 50, 25 fmoles; lanes 2 to 4; 6 to 8). (**C**) EMSA. Binding of NoRC, Tip5, Snf2h is not influenced by the lack of histone H4 tail. Mononucleosomes positioned at the end of the DNA fragment were reconstituted from recombinant full-length histones (N) and from histones, where H4 lacks the N-terminal tail but the other histones are full-length proteins (g4). Recombinant NoRC, Tip5 and Snf2h were incubated for 15 min with the different nucleosomal substrates at 30°C and analyzed on 1.3% agarose gels.

8.9 Characteristics of NoRC-mediated transcriptional repression

The molecular basis of NoRC-mediated transcriptional repression on chromatin might be versatile. NoRC binding and chromatin remodeling could sterically interfere with transcriptional activators. Alternatively, the remodeling activity of NoRC may inhibit transcription elongation and / or termination on chromatin templates, thereby decreasing the amount of transcripts. NoRC might establish repressed genes by a 'hit-and-run' mechanism leaving a transcriptionally incompetent chromatin structure behind. The complex could act as a landing platform for other transcriptional repressors, e.g. histone modifying enzymes. To distinguish between these possibilities the following experiments were performed.

8.9.1 Time course of a transcription reaction

First, the transcription kinetics of the TTF-I dependent transcription system on DNA and nucleosomal templates were estimated.

Transcription reaction were performed on DNA and chromatin templates in the presence of TTF-I as before, but stopped at different time points (10, 20, 40, 60 min) and analyzed by gel electrophoresis (Figure 43A). Transcripts continuously accumulated throughout the experiment with both templates (lanes 1 to 4). A delayed appearance of signals in the case of the nucleosomal template (chromatin panel) might be due to a more complex initiation process or the generation of less transcripts. The time course experiment indicated that the transcription reactions are performed in a non-saturating time scale. Further transcription assays were conducted for 60 min, ensuring that the transcription did not reach a plateau.

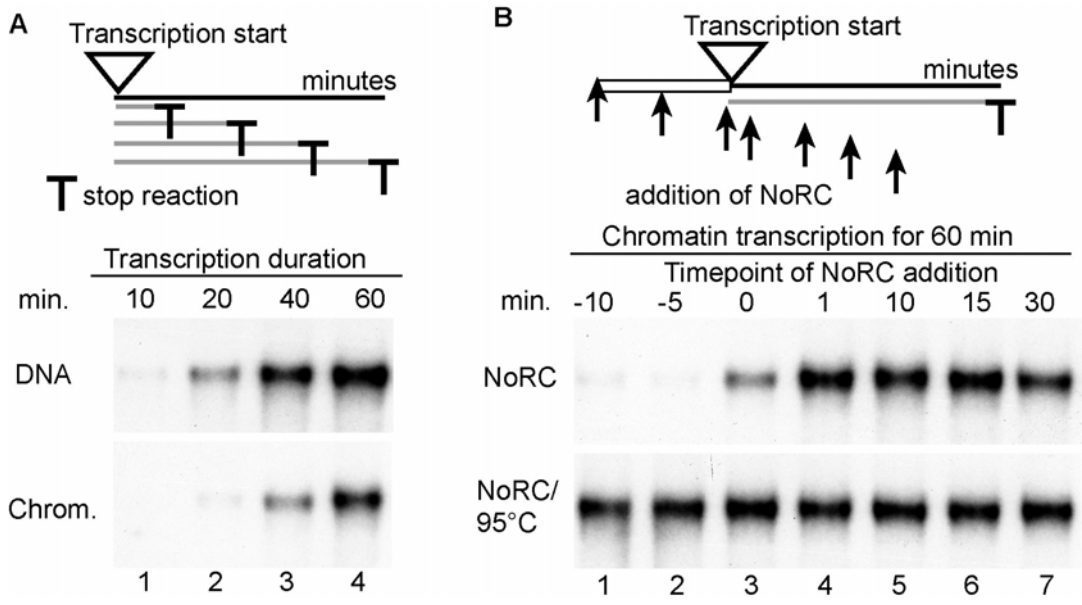

Figure 43. NoRC-mediated repression occurs prior preinitiation complex formation
(**A**) Time course of an *in vitro* Pol I transcription. Transcription reactions were performed with DNA or chromatin templates as indicated, in the presence of TTF-I for 10, 20, 40 and 60 minutes (lanes 1 to 4), stopped and analyzed as described before. The scheme of the reaction is indicated at the top. (**B**) Transcription repression requires NoRC prior to preinitiation complex formation. NoRC (50 fmol) or heat denatured NoRC (NoRC/95°C, 50 fmol) were pre-incubated with the chromatin template (-10, -5 min; lanes 1 and 2) or added at different time points to the transcription reaction (0, 1, 10, 15, 30 min; lanes 3 to 7) as indicated above. The transcription reactions were incubated for 60 min in the presence of TTF-I and the transcripts were analyzed on 4.5% polyacrylamide gels.

8.9.2 NoRC affects transcription initiation but no post-initiation events

Subsequently, the role of NoRC in pre- or post initiation processes was investigated. If NoRC exerts a specific effect on rDNA transcription initiation, NoRC should only repress RNA polymerase I transcription prior to initiation complex formation and should not affect transcription elongation. Therefore time-of-addition transcription experiments were performed.

NoRC was added at different time points either to the chromatin template or directly to the transcription reaction (see scheme Figure 43B). In addition, reactions with heat-inactivated NoRC were undertaken. Preincubation of the template and addition of NoRC together with the transcription extract repressed rDNA transcription (Figure 43B, lanes 1 to 3), whereas addition of NoRC after incubation of the template with the transcription factors had no effect (lanes 4 to 7). Control reactions, using heat-inactivated NoRC did not affect transcription at all (lanes 1 to 7; NoRC/95°C).

This experiment shows that NoRC acts prior to transcription initiation and implies that the complex establishes a repressive promoter structure without affecting transcription elongation.

8.9.3 NoRC-mediated repression is independent of histone deacetylation *in vitro*

Active genes are generally associated with hyperacetylated histones, while non-acetylated (deacetylated) histones are found at silenced / repressed genes (Brown et al., 2000; Iizuka and Smith, 2003; Kurdistani and Grunstein, 2003). NoRC might recruit histone deacetylases (HDAC) to the rDNA promoter to generate a transcription incompetent chromatin structure. To test, whether posttranslational histone modifications influence NoRC-mediated repression of transcription, histone deacetylation was inhibited by adding Trichostatin A (TSA), a potent inhibitor of most HDACs, excluding the Sir2 group (Finnin et al., 1999).

First, the transcription extract was tested for histone deacetylase activity. ^{3}H-marked chicken histones were incubated with the DEAE280 fraction (Schnapp and Grummt, 1996) in the absence or presence of 500 nM TSA. The transcription extract contained significant quantity of histone deacetylase activities as measured by the release of labeled acetate (Figure 44A). These enzymatic activities could be inhibited by the addition TSA to the reaction. The clear loss of deacetylation activity, comparable to the background, suggests that the DEAE fraction contains mainly TSA sensitive HDACs. Consequently, the transcriptional repression mediated by NoRC was studied in the absence or presence of TSA (Figure 44B). As shown before, addition of NoRC resulted in a concentration dependent repression of rDNA transcription (upper panel, lanes 1 to 7). The addition of TSA to the reaction had no influence on the outcome of the experiment. Neither chromatin transcription, nor NoRC-mediated transcriptional repression, was changed by TSA (lower panel).

In conclusion, NoRC-mediated rDNA transcriptional repression is not influenced by changes of the histone acetylation status *in vitro*.

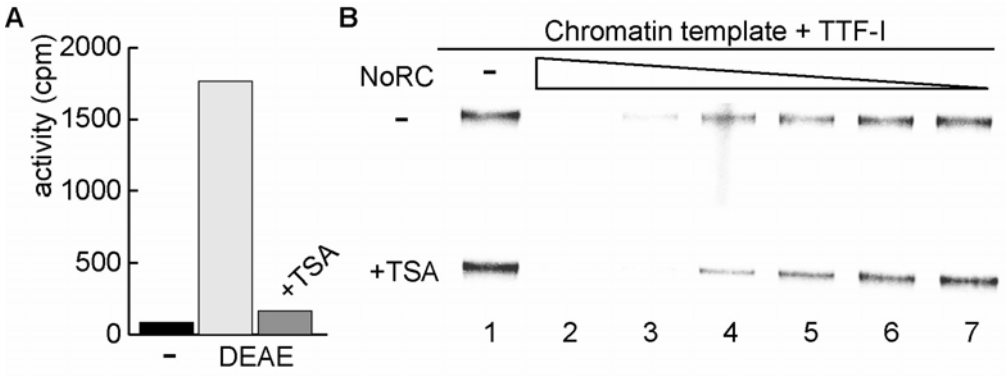

Figure 44. NoRC-mediated repression is independent of histone deacetylation
(**A**) TSA treatment inhibited the histone deacetylase activities of the transcription extract. Acetylated histones, labeled with radioactive ^{3}H-Acetyl-CoA were incubated with the partially purified transcription extract (DEAE 280). After incubation for 60 min under transcription conditions, the reaction was spotted onto filter and free H^{3}-acetate measured by scintillation counting. The addition of 500 nM TSA to the extract blocked all histone deacetylase activities. (**B**) TSA treatment did not influence NoRC-mediated transcriptional repression. Transcription assay on chromatin templates in the presence of TTF-I were conducted in the absence (upper panel) or presence of 500 nM TSA (+TSA panel) with decreasing amounts of NoRC (lanes 2 to 7).

8.9.4 NoRC stably associates with the repressed rDNA template

Next, it was asked, whether NoRC exerts its effect according to a hit and run model or if NoRC is physically associated with the rDNA promoter.

In vitro transcription assays were performed with two different templates, varying in transcript length. One template encodes a 690-nucleotide transcript (SP-), while the other generates a prolonged transcript of 860 nucleotides (SP+BH). Both templates were assembled into chromatin and used in the *in vitro* transcription assay either alone, together, or were mixed at different time points to the reaction. NoRC and TTF-I were preincubated with the first template, and the transcription started subsequent to the addition of the second template with the transcription extract (Figure 45A). Control transcriptions were performed with single templates and with varying the addition order of the different templates, as depicted in the Figure 45A.

As shown before, NoRC did not affect transcription on DNA templates (Figure 45B, lanes 3 to 5), but repressed transcription on chromatin templates. Indeed, NoRC repressed transcription on either, individual provided nucleosomal templates (for SP-, lanes 6-9; for SP+BH, lanes 10 to 13). Furthermore, repression occurred upon mixing both templates with NoRC at the same time before transcription was started (SP-& SP+BH, lanes 14 to 17). However, a different result was obtained, if the second template was added at a later time point. Transcriptional repression was only observed on the first template that was preincubated with NoRC. In more detail, preincubation of either template SP- (lanes 18 to 20) or SP+BH (lanes 21 to 23) for 15 min with NoRC led to inhibition of transcription. In contrast, no significant change of the transcription rate was detected for the second template (SP+ and SP vice versa).

These results indicate that NoRC is stably associated with the rDNA promoter on chromatin templates, thereby maintaining rDNA transcription in a repressed state. This suggests that the association of NoRC with the rDNA prevents preinitiation complex formation.

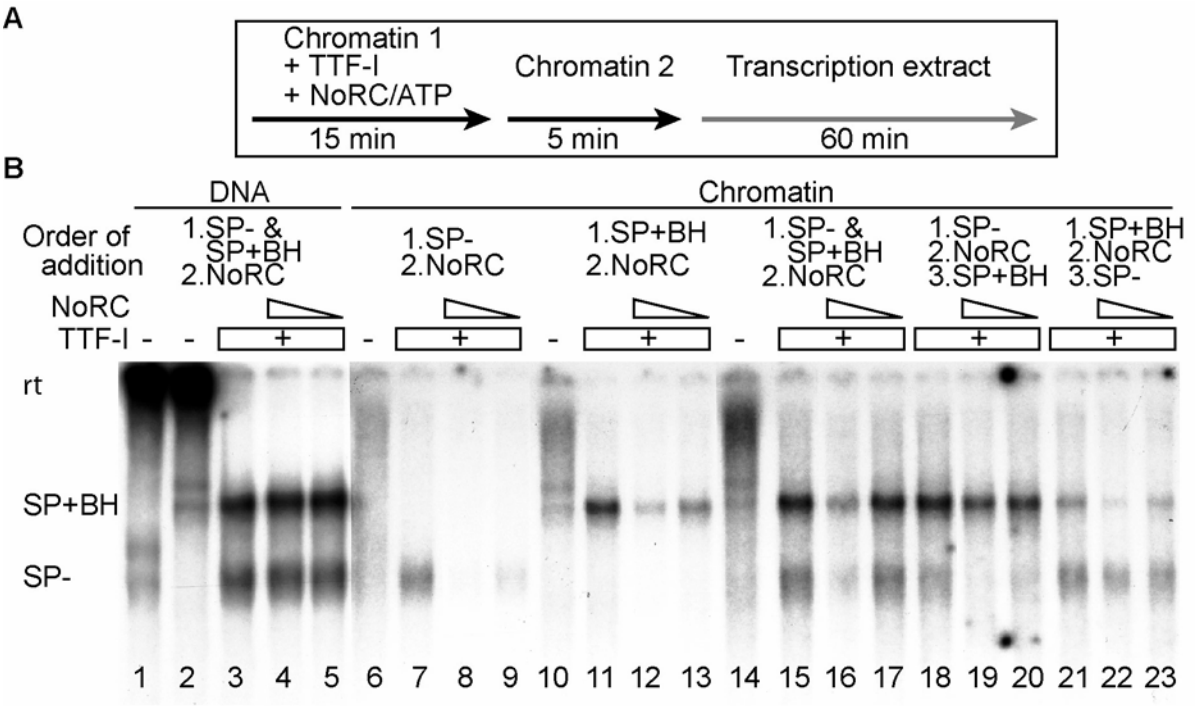

Figure 45. NoRC stably associates with the repressed chromatin template
(**A**) Diagram of the 'template commitment' transcription reaction. Transcriptional repression mediated by NoRC was analyzed by providing two templates at different time points with limiting amounts of NoRC. (**B**) NoRC stably associates with the rDNA promoter to prevent transcription. Increasing amounts of NoRC were added to a two-template transcription reaction. These templates differ in the resulting transcript's length (SP-: 690 and SP+BH: 860 nucleotides). Templates were assembled into chromatin and added solitary (lanes 6 to 13), together (1 to 5 and 14 to 17) or at different time points (lanes 18 to 23). The reaction was provided with TTF-I, NoRC and transcription extract as shown in A. For further details: see text. The transcription reactions were incubated for 60 min and the transcripts were analyzed on 4.5% polyacrylamide gels.

8.10 NoRC binds preferred to a structured (bent) region within the rDNA promoter

The experiments presented here indicate a physical association between NoRC and the rDNA promoter, during ribosomal RNA gene repression. In the following section, the binding specificity of Tip5 and NoRC to the rDNA promoter is investigated.

8.10.1 Tip5 binds preferentially to structured (bent) DNA

Initial experiments did not display any sequence specific DNA binding activity of Tip5 and NoRC. The presence of high amounts of unspecific competitor DNA (either salmon sperm DNA, poly dI/dC or plasmid DNA) abolished the DNA binding ability of NoRC (data not shown).

Nevertheless, NoRC may recognize structured DNA. A common feature of ribosomal gene promoters is that they lack sequence homology but retain structural similarity and contain intrinsically distorted regions (Schroth et al., 1992). Indeed, also the mouse rDNA promoter contains defined regions with specific local curvature (Längst et al., 1997b). In addition, substrate binding and the activity of chromatin remodeling factors is influenced by structured or distorted DNA (Bonaldi et al., 2002; Längst and Becker, 2001a). Proteins, that bind preferentially to structured DNA are known to bind with high affinity to cruciform (4-way junction) DNA. The archetypical example of these proteins is HMGB1 (Bianchi et al., 1989), a small abundant nuclear protein, involved in different nuclear processes (Agresti and Bianchi, 2003). Remarkably, HMGB1 acts as well as a DNA chaperone during nucleosome remodeling (Bonaldi et al., 2002). Furthermore, recent functional studies on the ATPase ISWI showed binding to cruciform DNA (Grüne et al., 2003).

In initial experiments, binding of Tip5 to cruciform DNA was analyzed. Cruciform DNA and two linear, double stranded, DNA fragments ('DNA sequence controls') were prepared as described (Bianchi et al., 1989). Increasing amounts of Tip5 were incubated with either the cruciform DNA or the linear DNA and analyzed in electromobility shift assays. Weak binding of Tip5 to either of the linear DNA fragments was visible in the conditions used (Figure 46A, panel 1 and 3). Even at highest concentrations, Tip5 barely bound to DNA. In contrast, the presence of low amounts of Tip5 led to the formation of protein / cruciform DNA complexes, increasing with the applied Tip5 concentration (see panel 2). These differences in Tip5 - DNA affinity indicates its preferential binding to structured DNA.

A similar approach was undertaken to test Tip5 binding to rDNA promoter fragments, which greatly differ in their predicted DNA curvature. The relative DNA curvature of the mouse rDNA promoter was calculated with the Bolshoy algorithm according to Schatz (Schatz and Langowski, 1997), using the described 'bandit' program (Figure 46B). One oligonucleotide contains limited curvature (Oligo 101, rDNA promoter -192 to -153) while the other oligonucleotide is greatly curved (Oligo 102, rDNA promoter -137 to -98). Electromobility shift assays were carried out in the presence of Tip5 and Acf1 (Figure 46C). Again, Tip5 showed preferred binding to the structured (bent) DNA fragment (compare lanes 2 and 3 with 7 and 8, respectively), whereas Acf1 exhibits similar affinities for both oligonucleotides (lanes 4 and 5; 9 and 10).

Taken together, these experiments suggest a preferred binding of Tip5 to structured (bent) DNA. In addition, they indicate a putative NoRC - binding region within the mouse rDNA promoter.

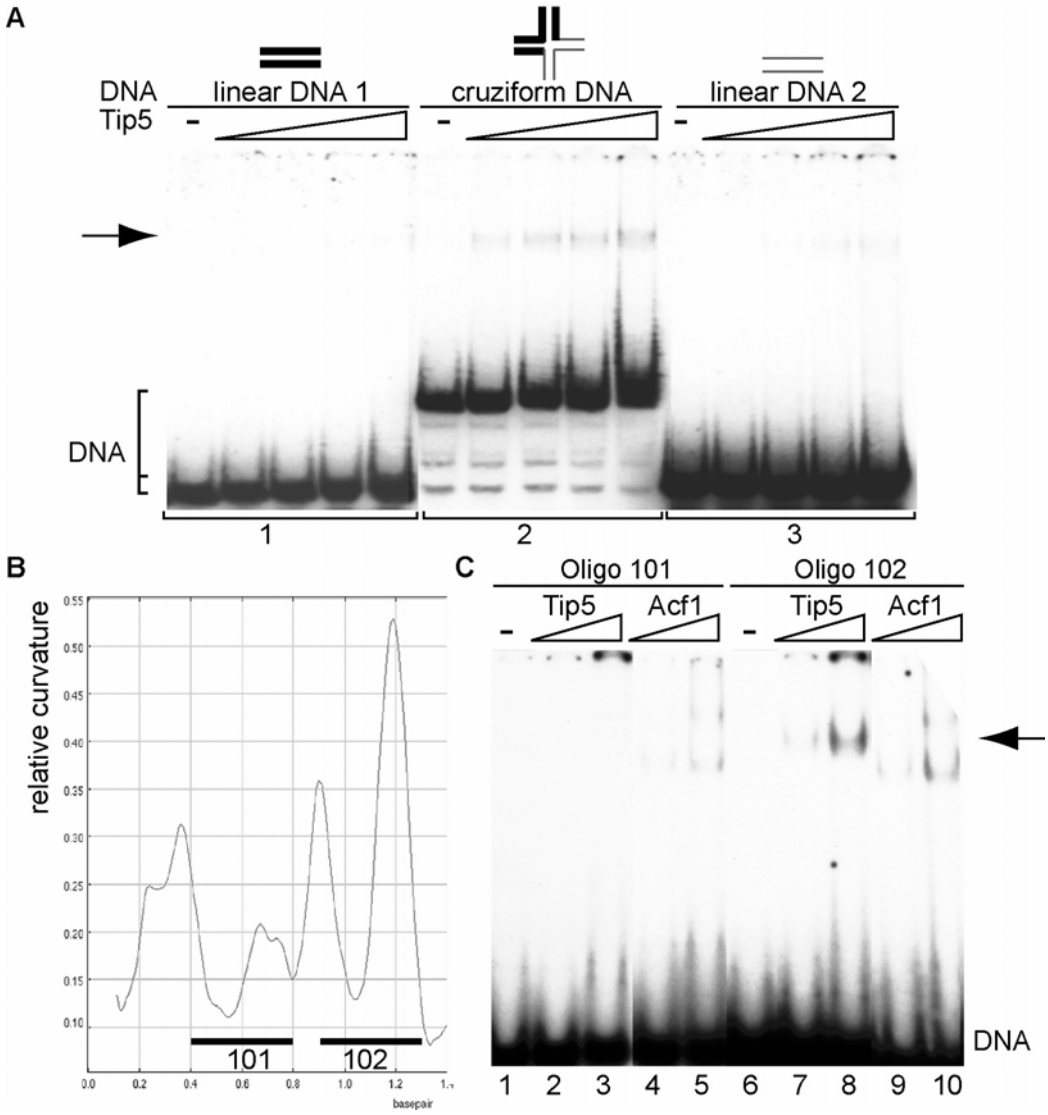

Figure 46. Tip5 binds preferred to cruciform and structured (bent) DNA
(**A**) EMSA. ^{32}P-labeled cruciform DNA (panel 2), and the two linear controls (panel 1 and 3), which cover the used nucleotide sequence, were incubated with purified Tip5 and analyzed for DNA binding in electrophoretic mobility shift assays. DNA without addition of protein is shown in the first lane of each panel, followed by adding increasing amounts of Tip5 (2 to 16 fmol). Different protein-DNA complexes were separated from free DNA on 5% native polyacrylamide gels. The structure of the used oligonucleotides is illustrated above. (**B**) DNA curvature prediction of a part of the murine rDNA promoter (-231 to -95, relative to transcription start site). The relative DNA curvature of the rDNA promoter sequence was calculated using a DNA curvature prediction program (Bolshoy algorithm / bandit program (Schatz and Langowski, 1997)). The further used 40bp oligonucleotides are shown; while oligo 101 (-192 to -153) contains nearly no curvature, oligo 102 (-137 to -98) is strongly bended. (**C**) EMSA of Tip5 and Acf1 with the two different structured rDNA promoter fragments shown in B. Increasing amounts of Tip5 (lanes 2 and 3, 7 and 8) and Acf1 (lanes 4 and 5, 9 and 10) were incubated with the radioactively labeled oligonucleotides 101 and 102 and analyzed for DNA binding in electrophoretic mobility shift assays.

8.10.2 NoRC favours a structured (bent) region within the rDNA promoter

The DNA binding specificity of NoRC to the 'structured' rDNA promoter region compared to the 'unstructured' neighboring DNA was analyzed further.

In initial experiments, the optimal ratio of NoRC to DNA for binding to DNA was estimated. A 40 bp rDNA promoter fragment with a great predicted curvature (see before 8.10.1), was radioactively labeled and incubated with increasing amounts of NoRC. Reactions were analysed by EMSA on agarose gels. Consistent with previous results, a concentration dependent formation of NoRC / DNA complexes was observed (Figure 47A).

Next, the radioactively labeled rDNA promoter fragment was incubated with limiting amounts of NoRC in the presence of decreasing amounts of competitor DNA. The competitor DNA contained either the same 'structured' DNA fragment or a neighboring rDNA promoter fragment, with only a weak predicted curvature ('unstructured'). The resulting DNA / protein complexes were resolved by native gel electrophoresis on 1.3% agarose gels. Figure 47B revealed, that approximately 10 times greater amounts of the 'unstructured' fragment (lanes 2 to 5) were necessary to compete the binding of NoRC, compared to the corresponding rDNA promoter fragment (lanes 7 to 11). Thus, NoRC displayed preferred binding to the structured (bent) rDNA promoter fragment.

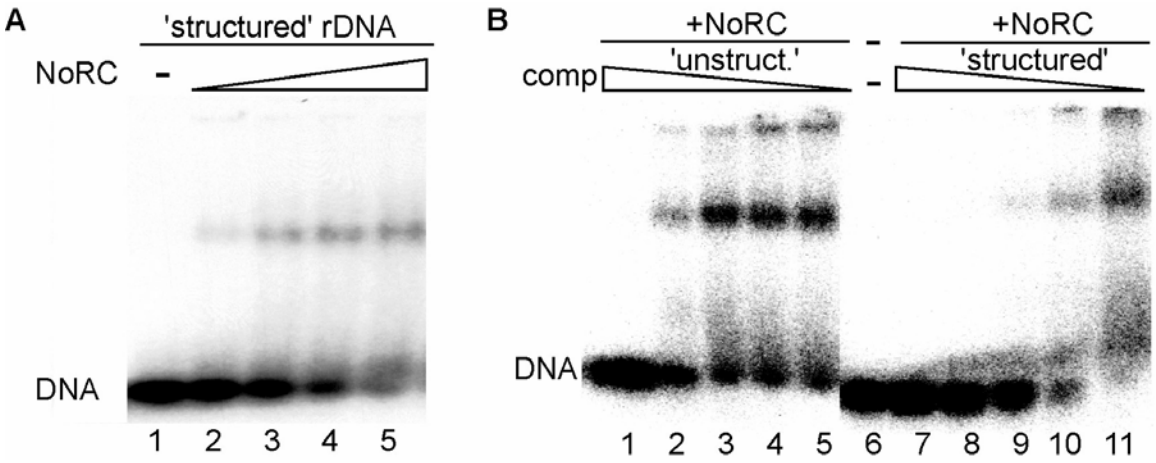

Figure 47. NoRC binds preferred to a structured region within the mouse rDNA promoter
(**A**) EMSA, NoRC binds with high affinity to a structured region within the rDNA promoter. Increasing amounts of recombinant NoRC (2.5, 5, 10, 20 fmol, lanes 2 to 5) were incubated with a 40 bp endlabeled rDNA promoter fragment (5 fmol, lane 1) spanning the region from -150 to -111 respective to the rDNA promoter. Protein-DNA complexes were separated from free DNA on 1.3% native agarose gels in electrophoretic mobility shift assays and visualized by autoradiography. (**B**) DNA competition experiment: NoRC binding to the structured 40 bp rDNA promoter fragment (-150 to -111) was competed by adding decreasing amounts of the same fragment ('structured', lanes 7 to 11) or a neighboring 'unstructured' 40 bp fragment (-192 to -153, lanes 2 to 5). Approximately 20 fmol of NoRC were incubated with 5 fmol of labeled DNA and decreasing quantity of competitor DNA (9, 27, 81, 243, 841 fmol). Analysis was performed on 1.3% agarose gels.

8.10.3 Binding to the rDNA promoter does not depend on DNA methylation

Methylation of the mouse rDNA promoter region is known to repress rDNA transcription both in transfection experiments and in *in vitro* assays using chromatin templates (Santoro and Grummt, 2001). Furthermore, DNA methylation of a single cytosine within a CpG repeat at position -133 abolishes rDNA transcription. Recently it was shown by chromatin immunoprecipitation experiments, that NoRC associates with methylated rDNA genes (Santoro et al., 2002).

Therefore, binding of Tip5 to the methylated rDNA promoter was investigated *in vitro*. The structured 40 bp rDNA promoter fragment (-150 to -111) was chosen, as it contains the functionally important CpG site at -133 (Santoro and Grummt, 2001). The DNA fragment was methylated with Sss-I methylase. Mock methylation was performed in the absence of the cosubstrate SAM. DNA methylation of the rDNA promoter fragment was confirmed by digestion with a methylation sensitive restriction enzyme (data not shown). The binding efficiency of Tip5 was compared to Acf1 in electromobility shift assays. No significant difference between the methylated and unmethylated DNA fragment was detectable. In the presence of Tip5, similar amounts of both oligonucleotides were retarded (Figure 48 lanes 2 and 3; lanes 7 and 8), indicating equal binding affinities for both DNA fragments. Analogous results were obtained with Acf-1 (lanes 4 and 5; lanes 9 and 10). Further experiments revealed either no preferred binding of NoRC to the methylated rDNA promoter fragment (data not shown).

These results indicate, that methylation of the rDNA promoter (specially at cytosine -133) does not influence the DNA binding affinity of Tip5 and NoRC *in vitro*.

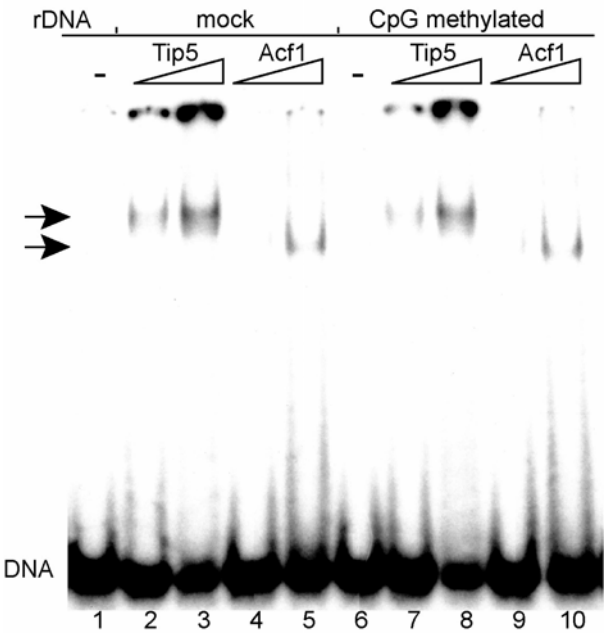

Figure 48. Tip5 binding to the rDNA promoter is independent of CpG methylation
Binding of Tip5 and Acf1 to methylated DNA. Recombinant Myc-Tip5 and Flag-Acf1 were assayed for binding to ^{32}P-labeled, unmethylated (lanes 1 to 5) or methylated (lanes 6 to 10) rDNA promoter fragment (-150 to -110). Methylation was performed by Sss-I methylase and efficiency was controlled by HpaII digestion (not shown). 25 or 50 fmoles of purified Tip5 (lanes 2, 3, 7 and 8) and 200 and 400 fmol of Acf1 (lanes 4, 5, 9 and 10) were incubated with the oligonucleotides. No protein was added in lanes 1 and 6. Protein-DNA complexes were separated from free DNA on 4.5% native polyacrylamide gels in electrophoretic mobility shift assays. Protein / DNA complexes (arrows) and free DNA (DNA) are indicated on the left side.

8.11 DNA methylation in the context of chromatin

DNA methylation plays an important role in gene silencing (Bird and Wolffe, 1999) and silenced rDNA genes have been shown to be methylated (Grummt and Pikaard, 2003). The mechanism of DNA methylation within a chromatin environment is ill-defined. Recent data provide the first insights. DNMT1 methylation is possible but restricted within nucleosomal DNA and the ability of DNMT1 to methylate nucleosomal sites depends on the nature of the DNA substrate (Okuwaki and Verreault, 2004). Similar experiments were performed to study DNA methylation in the context of chromatin.

Initial experiments were performed with a commercial bacterial CpG methylase (Sss-I, NEB), which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence (5'...CG...3'). Methylation efficiency was determined by subsequent digestion with a methylation sensitive endonuclease (Hpa II). Since DNA methylation blocks restriction enzyme cleavage, efficient DNA methylation is inversely correlated to restriction enzyme accessibility.

8.11.1 Chromatin interferes with DNA methylation by Sss-I methylase

The methylation efficiency of Sss-I was tested on two different chromatin substrates, a 146 bp core nucleosome (Figure 49A) and a chromatin array (Figure 49B) and in parallel their respective free DNA. The 146 bp DNA contains three defined cleavage sites for Hpa II, the circular plasmid DNA, contains multiple Hpa II sites.

The methylation reaction was performed for 60 minutes at 30°C, DNA was purified, and digested with the methylation sensitive restriction enzyme Hpa II. Cleavage products were resolved by gel electrophoresis. DNA methylation inhibited restriction enzyme cleavage of the 146 bp DNA (Figure 49A, lanes 2 and 3). In contrast, restriction enzyme cleavage of nucleosomal DNA is only partially blocked after the methylation reaction (lanes 5 and 6), showing that only a minor fraction of nucleosomal DNA was methylated. Notably, the restriction enzyme pattern indicated that the nucleosomal DNA was not uniformly less accessible to Sss-I methylase. Sites within the nucleosome were worse substrate for DNA methylation than sequences located at the border of nucleosome (e.g. compare diagnostic sites a position 14 and 53).

A similar approach conducted with a circular plasmid DNA revealed analogous results (Figure 49B). Free DNA was quantitatively methylated, and therefore not cleaved by the restriction enzyme Hpa II (lanes 2 and 3). In contrast, DNA packaged into chromatin was only partially protected from digestion (lanes 5 and 6), indicating that the DNA was only weakly methylated. Again, a different cleavage pattern occurred after the methylation reaction. Compared to unmethylated DNA, restriction enzyme digestion resulted in larger DNA fragments, suggesting that not all sites are similarly protected for the Sss-I methylase.

In conclusion, these experiments clearly show that the ability of Sss-I to methylate DNA is greatly interfered in the context of chromatin.

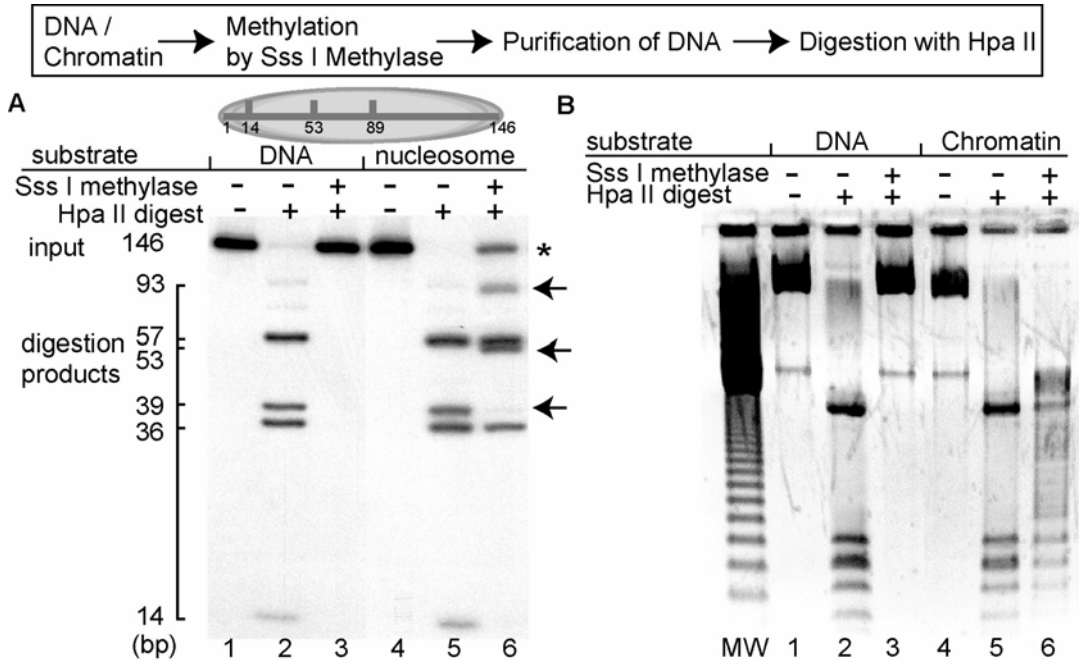

Figure 49. DNA methylation is interfered in a chromatin environment
(**A**) A 146 bp DNA fragment (lanes 1 to 3) and a mononucleosome (lanes 4 to 6), assembled on this fragment, were incubated with Sss-I methylase for 60 min. The methylation reaction was stopped and deproteinized. DNA was purified, digested with the methylation sensitive endonuclease Hpa II as indicated and analyzed on 7% PAA gels. The DNA contained three distinct Hpa II cleavage sites (at position 14, 53 and 89 respectively, see picture). A scheme of the reaction is depicted on the top. Minor contaminations of free DNA in the nucleosome reactions are complete methylated and indicated (star). Diagnostic methylation / cleavage sites are indicated (arrows). (**B**) Circular plasmid DNA (8 kbp) (lanes 1 to 3) and a chromatin array, salt assembled on this DNA (lanes 4 to 6) were analyzed for DNA methylation competence by Sss-I as described in A. Cleavage products were resolved on 1.3% agarose gels. A 123 bp size marker (MW) was run in parallel.

8.11.2 Chromatin remodeling enhances DNA methylation within a nucleosome

DNA methylation is restricted in the context of chromatin. Does chromatin remodeling restore the methylation efficiency and allow access to the substrate? DNA methylation experiments were performed with a 247 bp nucleosome positioned at the center of the DNA. The DNA fragment contained two analytical Hpa II cleavage sites at the border and within the nucleosome. Reaction was supplemented with ISWI and ATP to study the effect of nucleosome remodeling. Methylation efficiency was analyzed by restriction enzyme digestion with the two isoschizomers Hpa II and Msp I (Msp I is not sensitive to DNA methylation).

A scheme of the methylation reaction and the resulting cleavage products are shown in Figure 50A. Free DNA was fully methylated (Figure 50B, lanes 1 to 5) as analyzed by Msp I (not methylation sensitive, lanes 3 and 5) and Hpa II (methylation sensitive, lanes 2 and 4) cleavage. The nucleosomal DNA is partially protected from Sss-I modification, therefore Hpa II digestion occurs to some extend (lanes 6 to 10). Presence of ISWI and ATP in the reaction led to complete DNA methylation and cleavage was restricted (lanes 11 and 12). In contrast, in the absence of ATP, the nucleosomes displayed the same restriction enzyme cleavage pattern (lanes 13 and 14) as nucleosomal DNA alone (lanes 9 and 10).

Thus, DNA methylation on chromatin is enhanced in the presence of ISWI and ATP, suggesting that chromatin remodeling can play an important role in the process of DNA methylation within nucleosomes.

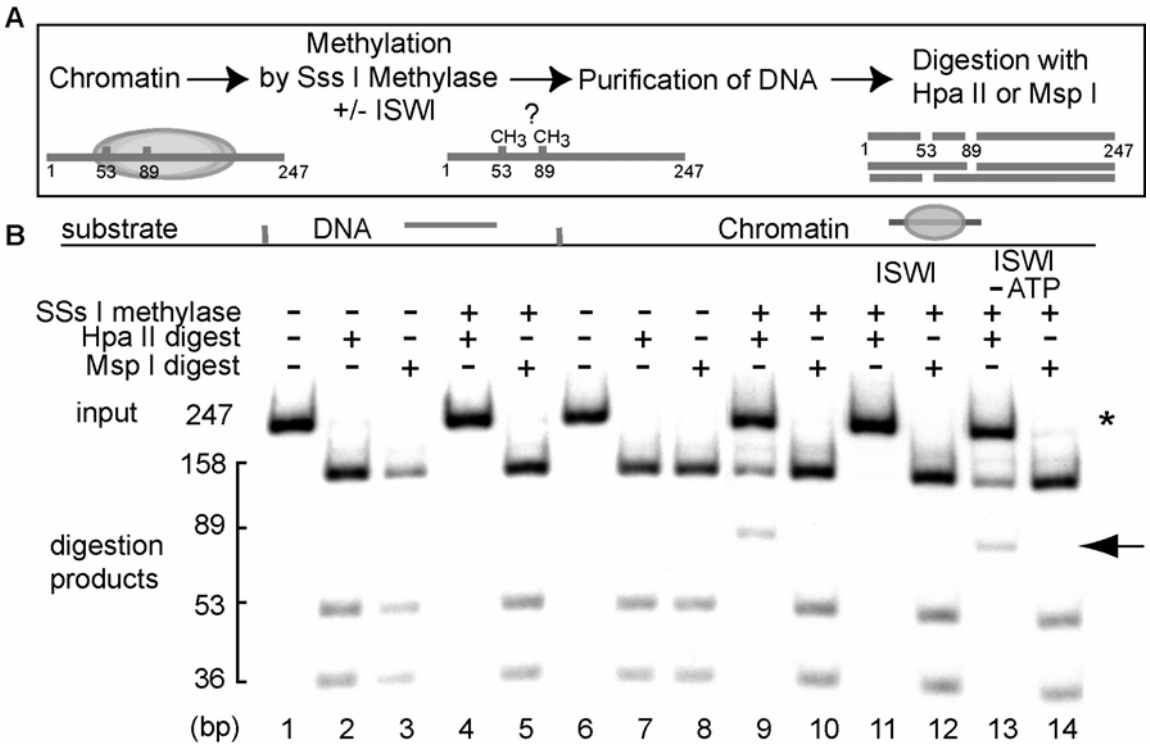

Figure 50. Chromatin remodeling by ISWI facilitates DNA methylation of Sss-I
(**A**) Diagram of the experimental setup and depiction of possible restriction enzyme cleavage products. Nucleosome position and diagnostic methylation / cleavage sites are indicated. (**B**) A mononucleosome positioned at the center of a 247 bp DNA fragment, which contained two analytical Hpa II cleavage sites at the border and within the nucleosome (A), was incubated with Sss-I methylase in the presence of ISWI as indicated (lanes 6 to 14). Control reaction on DNA was performed on lanes 1 to 5. ATP was left out of the methylation reaction in lanes 13 and 14. Reactions were incubated for 75 min at 26°C, stopped and the DNA purified. Methylation efficiency was analyzed by Hpa II and Msp I digestion. Hpa II activity is inhibited by DNA methylation. The cleavage products were resolved on 7% PAA gels. Note, that minor contaminations of free DNA and different translational nucleosome positions allow DNA methylation to some extent (star). A diagnostic methylation / cleavage site (at position 89) is indicated (arrow).

9 DISCUSSION

This doctoral thesis presents the identification and characterization of a novel chromatin remodeling complex, termed NoRC (Nucleolar Remodeling Complex). NoRC specifically represses rDNA transcription in chromatin. Several studies investigated the role of NoRC in the last years. The results, including the work presented in this thesis, reveal an active and specific role of NoRC in concert with TTF-I in rDNA gene silencing. Furthermore, they imply that TTF-I has a dual role in the regulation of the rDNA genes in chromatin, namely involvement in both activation and silencing of rDNA transcription (see Figure 51).

9.1 TTF-I recruits a chromatin remodeling complex

Within this work, I found that interaction of Tip5 in NoRC with TTF-I leads to recruitment of NoRC to the rDNA promoter followed by nucleosome remodeling. The interaction domain is within the N-terminus of TTF-I, a region known to act as a negative regulatory domain, masking the DNA binding activity of TTF-I (Evers et al., 1995; Sander et al., 1996). Indeed, the interaction with NoRC / Tip5 enables TTF-I DNA binding to chromatin. The Tip5 / TTF-I interaction was analyzed in detail in several *in vitro* and *in vivo* assays in other studies (Jansa et al., 1998; Németh et al., 2004; Strohner et al., 2001). A repeated 25 amino acid sequence within the N-terminus of TTF-I interacts with a region within the N-terminal part of Tip5 (aa ~600 to ~700), which is sufficient to facilitate DNA binding (Németh et al., 2004). Furthermore, the Tip5 / TTF-I interaction is necessary for NoRC-mediated rDNA repression *in vivo*.

I show herein, that only NoRC is able to facilitate nucleosome remodeling with full-length TTF-I, confirming the role of the Tip5 / TTF-I interaction. Co-targeting of Tip5 and TTF-I to the rDNA promoter results in binding of TTF-I and nucleosome remodeling mediated by NoRC. TTF-I is a multifunctional protein and other factors might interact with the N-terminus as well thereby restoring the DNA binding activity of TTF-I. Interacting proteins may modulate the multiple functions of TTF-I, such as transcription termination and control of rDNA gene transcription in chromatin (see also section 9.5).

Targeting of chromatin remodeling complexes to gene promoters appears to be a common theme. Several sequence specific binding factors induce rearrangements of nucleosomal arrays in respect to their binding site, e.g. binding of nuclear factor kappa B or nuclear factor E2 mediate nucleosome alteration in chromatin *in vitro* (Armstrong and Emerson, 1996; Pazin et al., 1996). Furthermore, the nucleosome remodeling factor NURF was identified in a search for a remodeling activity which act in concert with the GAGA transcription factor to rearrange the chromatin structure at the hsp70 promoter (Tsukiyama et al., 1994; Tsukiyama and Wu, 1995). In addition, studies on the *Drosophila* hsp26 promoter *in vitro* revealed that binding of GAGA factor and heat shock factor in chromatin caused ATP-dependent nucleosome rearrangements (Wall et al., 1995). Consequently, it was shown, that the large subunit NURF301 of the *Drosophila* NURF complex specifically interacts with the transcription factors GAGA and heat shock factor (Xiao et al., 2001). In mammals, the transcription factor EKLF specifically recruits the Swi/Snf complex to the ß-globin promoter, generating specific DNase I hypersensitive sites (Kadam et al., 2000).

In yeast, Ume6-dependent targeting of the ISW2 chromatin remodeling complex was shown to repress transcription of early meiotic genes (Goldmark et al., 2000).

We propose a similar mechanism, in which TTF-I recruits a chromatin remodeling activity (NoRC) to the rDNA promoter. However, if TTF-I dependent targeting of a remodeling complex to the rDNA promoter rearranges chromatin structure *in vivo* remains to be established.

9.2 A chromatin remodeling complex mediating transcription repression?

In this thesis I show, that NoRC specifically abolishes rDNA transcription on chromatin templates *in vitro*. Remarkably, only NoRC but not Snf2h or ACF inhibited rDNA transcription on chromatin templates. NoRC-mediated repression requires the histone H4 tail, indicating that nucleosome remodeling is crucial in this process.

These results were unexpected, as previous studies revealed that binding of TTF-I to its promoter-proximal target site mediates ATP-dependent nucleosome remodeling, which correlates with efficient transcription initiation on otherwise repressed nucleosomal rDNA templates (Längst et al., 1998; Längst et al., 1997a). The effect of Tip5 / NoRC on rDNA transcription was investigated in several other studies *in vivo* (Németh et al., 2004; Santoro et al., 2002; Zhou et al., 2002). All of them reported specific repression of rDNA transcription by Tip5 / NoRC. The results imply a role for NoRC in rDNA gene silencing and a dual role for TTF-I in rDNA gene regulation in chromatin (see section 9.4).

The generally repressive nature of chromatin structure has long been appreciated in transcription regulation. Initially, chromatin remodeling was expected to produce a more accessible chromatin structure, thereby activating gene expression. However, recent studies have challenged this view, suggesting that many ATP-dependent chromatin remodeling activities can also function as repressors of transcription, implying that active repression is an important regulatory mechanism.

Indeed, only some members of the ISWI-containing chromatin remodeling machines were shown to activate transcription from chromatin templates, such as NURF and RSF (LeRoy et al., 1998; Mizuguchi et al., 1997). Other ISWI containing complexes have been implicated in processes such as maintaining chromosome structure and heterochromatin formation, rather than opening the chromatin structure (Corona and Tamkun, 2004; Havas et al., 2001). Furthermore, localization studies of ISWI and Pol II on salivary gland polytene chromosome, showed a mutually exclusive staining, indicating a negative role for ISWI on the regulation of transcription (Deuring et al., 2000). The related yeast Isw2 complex, together with the Rpd3-Sin3 histone deacetylase complex, was shown to directly repress transcription of the early meiotic genes during mitotic growth (Goldmark et al., 2000). Repression requires both the Isw2 remodeling activity and the histone deacetylase activity. Further analysis revealed parallel repressive functions for these complexes for a number of genes (Fazzio et al., 2001).

Not only the ISWI remodeling complexes are involved in transcriptional repression. Recent functional studies suggest that many ATP-dependent chromatin remodeling activities can function as repressors of transcription, such as members of the Swi/Snf family, originally described as activators. Genome wide expression analysis suggest a direct role of Swi/Snf and RSC complexes in transcriptional repression in yeast (Damelin et al., 2002; Martens and Winston, 2002; Martens and Winston, 2003; Sudarsanam and Winston, 2000). In addition, many members of the CHD / Mi-2 family of chromatin remodeling complexes are described as transcriptional repressors (Tsukiyama, 2002). Indeed, Mi-2

interacts with several corepressors (Bowen et al., 2004). The combined action of histone deacetylation, DNA methylation and chromatin remodeling within the NuRD complexes is suggested to form a repressive chromatin structure.

In conclusion, transcriptional repression by chromatin remodeling factors seems to be an important mechanism to stably inhibit gene expression.

9.3 Is chromatin remodeling involved in NoRC-mediated repression?

NoRC-mediated rDNA repression is specific on chromatin templates. However, a chromatin remodeling deficient NoRC mutant abolishes rDNA transcription to the same extend as the wild type. Furthermore, also Tip5 repressed rDNA transcription, although to a lower extent then NoRC (data not shown). Somehow contradictory, the N-terminus of histone H4 plays a crucial role in transcriptional repression in chromatin and this histone tail is important for nucleosome remodeling by NoRC.

The chromatin specific transcriptional repression may result either from the passive association of NoRC / Tip5 with the rDNA promoter and / or from the active remodeling of the promoter-proximal nucleosome. In both scenarios, nucleosome remodeling and NoRC binding would establish an inaccessible chromatin structure, thereby inhibiting the binding of initiation factors. Several lines of experimental evidence suggest that NoRC-mediated transcriptional repression *in vitro* is due to active chromatin remodeling.

First, transcriptional repression was observed only for chromatin templates and not for naked DNA, but no preferred chromatin binding of NoRC was detectable in electrophoretic mobility shift assays. Second, targeting of NoRC by TTF-I to the rDNA promoter leads to active nucleosome rearrangement, confirming the functional significance of the NoRC / TTF-I interaction. Third, NoRC-dependent rDNA repression occurs prior to initiation factor binding and cannot revert to an activated state in chromatin. Fourth, repression requires the tail of histone H4. NoRC did not repress transcription for nucleosomal templates that lack the tail of histone H4 but did inhibit Pol I transcription for histone octamers containing only the tail of histone H4. The requirement of the histone H4 tail for NoRC-mediated nucleosome remodeling has been demonstrated. As repression depends on the N terminus of histone H4, the passive mechanism would require the specific interaction of Tip5 / NoRC with this histone tail. Electrophoretic mobility shift assays that were performed with intact nucleosomes and nucleosomes lacking the H4 tail did not reveal preferential binding of Tip5 or NoRC to the N-terminus of histone H4, thus arguing against a specific interaction. On the other hand, NoRC reconstituted with the Snf2h ATPase mutant did repress rDNA transcription, implying that nucleosome remodeling is not required. Still this complex enables TTF-I DNA binding. Upon TTF-I binding, the rDNA promoter chromatin structure is rearranged by chromatin remodeling activities in the transcription extract (Längst et al., 1998) and does not allow to distinguish direct between the active and passive repression mechanisms. Remodeling activities present in the transcription extract could be associated with the template, TTF-I and NoRC and provide nucleosome repositioning within the reaction. A highly purified *in vitro* transcription system lacking additional remodeling activities would be necessary to eliminate this possibility.

These results, taken together with data demonstrating that overexpression of an ATPase-deficient Snf2h mutant abolished Tip5-mediated transcriptional repression *in vivo* (R. Santoro and I. Grummt, unpublished observation), underscores the possible role of nucleosome remodeling in NoRC-mediated rDNA repression.

9.4 NoRC is involved in rDNA gene silencing

Even in actively growing cells, where rRNA synthesis accounts for most transcriptional activity, only a subset of the rRNA genes is active, reflected in an 'open chromatin' conformation. The remainder of the genes stays silent, in a 'closed' chromatin state. The level of cellular rRNA is regulated by changing the rate of transcription initiation of active rDNA genes rather than by activating silent transcription units (Conconi et al., 1989; French et al., 2003; Grummt, 2003; Grummt and Pikaard, 2003).

The work herein demonstrated that NoRC mediates transcriptional repression on chromatin templates. NoRC-mediated repression turned out to inhibit preinitiation complex formation, most likely by forming a repressive chromatin structure. Commitment experiments revealed that NoRC stays stably associated with the repressed chromatin templates. The presented results suggest that NoRC stably represses rDNA genes, and therefore imply a role in rDNA gene silencing rather than in regulating the transcription initiation rate.

Indeed, recent work shed light into the pathway establishing rDNA gene silencing and confirmed NoRC as a key player in this process. The laboratory of Ingrid Grummt showed impressively the link between histone modification, DNA methylation and silencing of the rDNA genes (Santoro and Grummt, 2001; Santoro et al., 2002; Zhou et al., 2002). Initial experiments showed that DNA methylation of a CpG site at position -133 relative to the transcription start site is associated with the silent rDNA copies in the cell. Methylation of this site inhibits promoter binding by the RNA polymerase I transcription activator UBF and was shown to directly repress rDNA transcription in chromatin but not on free DNA (Santoro et al., 2001). Transfection of rDNA minigenes together with Tip5 resulted in the repression of the reporter, which was prevented by 5-aza-2´-deoxycytosine, a chemical inhibitor of cytosine methylation (Santoro et al., 2002). The transfected rDNA reporter became methylated only in the presence of Tip5. Immunoprecipitation experiments showed that histone H3 methylated on Lys9, a hallmark of silenced genes was associated with the methylated promoter. By contrast hyperacetylated histone H4 or the large subunit of RNA polymerase I – hallmarks of active genes – were only associated with the active, non-methylated genes (Santoro et al., 2002). The probable mechanism by which this occurs is suggested by co-immunprecipitation experiments, showing an association of DNA methyltransferases (DNMT1 and DNMT3b), as well as the Sin3 co-repressor complex (including the histone deacetylases HDAC1 and HDAC2) with Tip5 (Santoro et al., 2002; Zhou et al., 2002).

Collectively, these data suggest that NoRC serves as a scaffold that coordinates the activity of complexes for the establishment and maintenance of the inactive state of rDNA genes, and link chromatin remodeling to both DNA methylation and specific histone modifications.

Initial experiments presented in this thesis demonstrate that DNA methylation is restricted if the DNA is assembled into chromatin. However, chromatin remodeling by ISWI facilitates methylation of nucleosomal DNA. It is tempting to speculate, that also NoRC could contribute directly to the establishment or maintenance of the rDNA methylation state. This seems to be an attractive model, as Tip5 interacts directly with DNMT1 and DNMT3b. Accordingly, NoRC might recruit the DNA methylases to the rDNA promoter and facilitates DNA methylation of nucleosomal DNA through chromatin remodeling. As a result, the inactive genes are stably marked and UBF binding is inhibited. Furthermore, NoRC-mediated repression does not dependent on histone deacetylation *in vitro*, suggesting

that histone deacetylation is not necessary for the establishment of a repressed gene but rather for subsequent steps. Again, NoRC recruits HDAC activities and might facilitate their action. This suggests an ordered mechanism of rDNA gene silencing, initiated by TTF-I / NoRC recruitment and remodeling as the first step followed by DNA methylation and histone deacetylation (Figure 51). Nevertheless, a detailed analysis of the onset of rDNA gene silencing, the maintenance and the precise order wait to be performed.

9.5 A dual role of TTF-I in rDNA gene regulation in chromatin

As mentioned before, two stably propagated chromatin states of the ribosomal RNA genes exist: an actively transcribed state and a silenced conformation. Active genes contain acetylated histones (Hirschler-Laszkiewicz et al., 2001; Johnson et al., 1987; Mutskov et al., 1996; Vavra et al., 1982), whereas silenced genes have heterochromatic markers such as DNA methylation and specific histone methylation patterns (Brock and Bird, 1997; Lachner and Jenuwein, 2002; Santoro et al., 2002; Stancheva et al., 1997). However, the activity status of an rDNA gene is erased by the replication machinery, generating two newly replicated coding regions that are packaged into nucleosomes (Lucchini and Sogo, 1995; Lucchini et al., 2001).

Disruption of nucleosomes at the replication fork is followed by a rapid re-assembly of nucleosomes (within seconds) after the passage of the replication machinery and the subsequent binding of histone H1 (Gasser et al., 1996; Lucchini et al., 2001). A general property of newly replicated chromatin is its increased nuclease sensitivity compared to bulk chromatin. Nuclease insensitivity is restored within 15 minutes after replication in a process termed ‘maturation’ (Klempnauer et al., 1980). This process is thought to involve the modification of histones and chromatin folding into higher order structures (Perry and Annunziato, 1991). Methylation of DNA, a mark of inactive rDNA chromatin occurs after nucleosome assembly, a few minutes after the replication fork passes or is delayed for several hours within a minor fraction of the DNA (Gruenbaum et al., 1983; Woodcock et al., 1986).

Therefore, replication may generate a ‘window of opportunity’ for transcription factors, in which they could bind to the immature chromatin and establish transcriptional active or repressed promoters. Transcriptional activity is re-established after replication by mechanisms that open chromatin structure, allowing transcription, or conversely by mechanisms that lead to stable silencing of rRNA genes.

Indeed, studies showed that the transcription termination factor TTF-I is involved in the activation of murine rDNA genes on chromatin templates (Längst et al., 1998; Längst et al., 1997a) and demonstrated that TTF-I dependent nucleosome positioning establishes a specific promoter architecture that is compatible with, if not prerequisite to pre-initiation complex formation. In a search for TTF-I interacting partners that might activate transcription, Tip5 was identified. However, the results presented here imply a specific role of Tip5 / NoRC in rDNA gene silencing. TTF-I dependent targeting of NoRC leads to a repressive chromatin environment. Furthermore, a recent study demonstrated that the recruitment of NoRC by TTF-I is necessary to silence rDNA genes *in vivo* (Németh et al., 2004) and decreased levels of NoRC resulted in higher rDNA transcription activity *in vivo* (A. Németh, unpublished observation). Clearly, alternative factors and mechanisms must exist to activate rDNA transcription in chromatin.

Accordingly, TTF-I facilitates activation and silencing of rDNA genes with different factors, respectively. Therefore, we suggest a dual role for TTF-I in the regulation of the rDNA genes in chromatin (see Figure 51).

In this scenario, TTF-I is the key player that interacts with distinct factors after replication to determine the state of the rDNA genes during the 'window of opportunity'. Distinct nucleosome remodeling events lead to activation and repression of rDNA transcription in chromatin and further mechanisms maintain the established activity state. Indeed, localization experiments revealed that TTF-I colocalizes with the active transcription machinery and with the inactive machinery present in certain mitotic nucleolar organizer regions (NORs), when rDNA transcription is repressed (Sirri et al., 1999).

In regard to this model, TTF-I is the molecular switch, which determines the activity state of an rDNA gene through interactions with distinct factors. As mentioned before, the N-terminus of TTF-I acts as negative regulatory domain (NRD), inhibiting DNA binding (Németh et al., 2004). Regulation of the DNA binding activity of TTF-I, can determine its function in rDNA gene regulation in chromatin, as shown with NoRC. Furthermore, full-length TTF-I is functionally active in transcription reactions with extracts, i.e. it binds to its target sequence, terminates and activates transcription on chromatin templates. Remarkably, no proteolytic cleavage products can be detected during the transcription reaction (data not shown). This suggests that either modifications of the NRD or interactions with other cellular proteins facilitate DNA binding of TTF-I and modulate its functions.

With regard to post-translational modifications, TTF-I is phosphorylated at multiple sites during mitosis and the phosphorylated form is easier solubilized from chromatin than the non-phosphorylated form (Sirri et al., 1999). This suggests, that phosphorylation of TTF-I may play a role in regulating the binding of this protein to chromatin. However, the precise function of TTF-I phosphorylation is not known. TTF-I is also acetylated. It interacts with the histone acetyltransferase PCAF (Muth et al., 2001) and the N-terminal part of TTF-I is acetylated *in vitro* (A. Németh, unpublished observation). It remains to be investigated whether these modifications influence the function and DNA binding activity of TTF-I. Post-translational modifications of TTF-I and interaction with other factors are not mutually exclusive in the regulation of TTF-I functions. Specific modifications of TTF-I might be important to facilitate specific interactions. However, this remains to be investigated.

Except for Tip5 in NoRC, no TTF-I N-terminal interaction proteins are characterized so far. The mechanisms and factors for activating rDNA transcription in chromatin are less investigated. A remodeling activity is expected to take part, as ATP-dependent chromatin remodeling at the rDNA promoter is required to activate RNA polymerase I transcription on chromatin (Längst et al., 1998; Längst et al., 1997a). I show that transcription is possible from chromatin templates, reconstituted from histones lacking the H4 N-terminus. This implies that a chromatin remodeling activity that does not depend on the histone H4 tail is involved in transcriptional activation. A member of the Swi/Snf or CHD / Mi-2 family could be responsible for this activity. Nevertheless, the TTF-I interaction partners for activation rDNA transcription in chromatin remain to be identified.

Finally, the TTF-I dependent activity state of the rDNA genes is stable propagated throughout the cell cycle. Maintenance of the silenced gene is facilitated by DNA methylation, histone deacetylation and histone methylation (see before). In contrast, histone acetylation is present in active rDNA gene copies (Hirschler-Laszkiewicz et al., 2001; Johnson et al., 1987; Mutskov et al., 1996; Santoro et al., 2002;

Vavra et al., 1982). Furthermore, the histone H3 variant H3.3 accumulates on highly transcribed regions like the active rDNA arrays (Ahmad and Henikoff, 2002). Therefore, histone H3.3 might be a stable mark of activated rDNA genes. Still, the relationship between histone H3.3 and the rDNA gene activity state needs to be addressed by further research.

Future experiments will elucidate more details about the onset, the precise mechanisms, the missing factors and their connections in rDNA gene regulation in chromatin. Clearly, a milestone in understanding the regulation of rDNA genes in chromatin will be the identification of an activating chromatin remodeling factor. Furthermore, analysis of the chromatin state *in vivo* under various conditions, such as different Tip5 / NoRC levels will lead to a better understanding of the silenced state. This will help to receive a more complete picture of the mechanisms regulating rDNA transcription in chromatin and to adjust our working model according to the results.

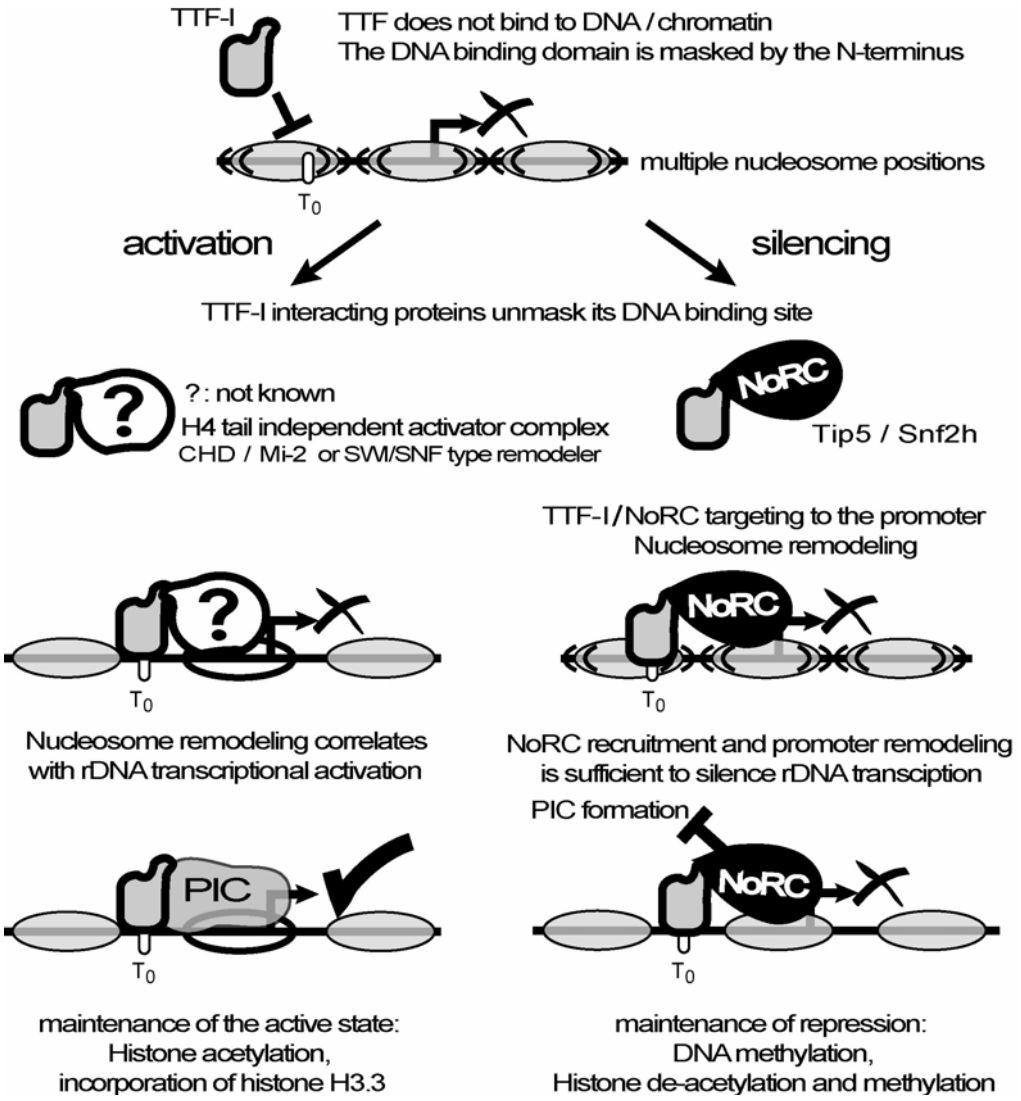

Figure 51. Working model showing the regulation of the rDNA genes in chromatin
TTF-I plays a dual role in rDNA gene regulation, in that it establishes either the active or the repressed state of the gene. The functional role of TTF-I is defined by interacting proteins, which restore the DNA binding activity. NoRC interacts via the Tip5 subunit with TTF-I and is the key player in the establishment and maintenance of the silent state of the rDNA genes. The factors activating rDNA transcription in chromatin are ill-defined.

9.6 Domains within Tip5 which specify its function

NoRC is composed of the nucleolar protein Tip5 and the ATPase Snf2h, the mammalian homolog of ISWI. Most ISWI-containing remodeling complexes contain a large subunit of the WAL / BAZ protein family (WSTF-, Acf1-like / bromodomain adjacent zinc finger, respectively) (Corona and Tamkun, 2004; Eberharter and Becker, 2004; Jones et al., 2000; Poot et al., 2000). Indeed, also Tip5 is a member of this family. All members of the WAL / BAZ family are characterized by their distinct modular organization. Intriguingly, most prominent are shared domains within the central and C-terminal region of the proteins, such as the DTT domain, the BAZ domains, the WAKZ motif, the PHD fingers and the bromodomain, while other motifs in the N-terminus are unique for the individual proteins (see Figure 13, introduction). As the ATPase subunit is common in these chromatin remodeling complexes, it is tempting to speculate that the other subunits confer to the specificity and targeting.

WSTF and Acf1, but not Tip5, contain at their N-terminal region a WAC motif, a novel protein domain, which appears to target these proteins to heterochromatin (Bozhenok et al., 2002; Collins et al., 2002; Tate et al., 1998). The WAC domain is important for DNA binding of ACF (Fyodorov and Kadonaga, 2002a) and interacts directly with the small histone fold subunits of CHRAC (Kukimoto et al., 2004)(F. Hartlepp *et al.*, submitted). In contrast, the characteristic features within the N-terminus of Tip5 are a motif termed TAM, that is related to the MBD motif present in methyl-CpG binding proteins (see below) and several AT hooks, known as auxiliary DNA binding modules. It turned out, that a specific region within the N-terminus containing two AT-hooks (aa ~600 to ~700) is necessary for the interaction with TTF-I (Németh et al., 2004). Moreover, the N-terminal part of Tip5 is sufficient to target the protein into the nucleolus (R. Santoro, unpublished observations). Hence, the results indicate that the N-terminus of Tip5 denotes its precise target.

However, transcriptional repression mediated by NoRC requires the central and C-terminal part of the protein (Németh et al., 2004; Zhou et al., 2002). Within these regions are the conserved domains of the WAL / BAZ members (see Figure 13). The ISWI interaction region is mapped to several conserved regions including the DDT domain and the BAZ domains (Eberharter, 2004; Fyodorov and Kadonaga, 2002a; Jones et al., 2000). Accordingly, Tip5 contains these regions and interacts with Snf2h, the mammalian homologuos of ISWI, forming the chromatin remodeling complex NoRC. Hence, the interaction with Snf2h might be necessary for transcriptional repression.

Tip5 contains a PHD finger bromodomain bipartite module at its C-terminus. The bromodomain has been implicated in recognition of histone modifications. The structure of the bromodomain has been resolved and shown to allow specific interactions with acetylated histone H4 tails (Dhalluin et al., 1999; Owen et al., 2000). It has been demonstrated that the bromodomain targets proteins to the promoter in an acetylation dependent manner (Hassan et al., 2002; Horn and Peterson, 2001). A recent study demonstrated that the bromodomain and PHD finger cooperate in nucleosome binding within the histone acetyltransferase p300. In this model, both the bromodomain and the PHD finger contact the nucleosome while simultaneously interacting with each other (Ragvin et al., 2004). It is possible, that also Tip5 interacts specifically with histones / nucleosomes through these domains. Tip5 might be sensitive to the acetylation state of specific lysine residues within histone tails and these interactions might be required to stably anchor NoRC to chromatin.

Another described role for these domains is the recruitment of cofactors. Characterization of the bipartite PHD finger / bromodomain unit of the KAP-1 protein revealed that these domains are collectively required for optimal transcriptional repression (Capili et al., 2001). The repression is likely due to an interaction of the PHD finger with a histone deacetylase complex (Schultz et al., 2001). It was shown that the C-terminal part of Tip5, containing the PHD finger and the bromodomain, interacts with HDAC1 and HDAC3 within the Sin3 corepressor complex (Zhou et al., 2002). This interaction was suggested to be necessary for transcriptional repression, as deletion of the C-terminus of Tip5 abrogated its repressive effect. However, these studies did not address, if the remodeling activity within the mutated NoRC complex was affected. In deletion studies on Acf1, the large subunit of ACF, it was demonstrated that the PHD fingers are not only important for histone / nucleosome interaction of ACF. In addition, deletion of the PHD fingers strongly impaired nucleosome remodeling by ACF (Eberharter, 2004).

Further studies with Tip5 and NoRC should address the influence of the domains on remodeling activity, specific factor recruitment and the influence of histone modification on NoRC function.

9.7 DNA binding activity of Tip5 and NoRC

The experiments in this study demonstrate that Tip5 and Snf2h can bind to DNA and they function cooperative within the NoRC complex. Tip5 and Snf2h contain a number of domains that have been implied in DNA binding. Such as the AT-hooks, the TAM / MBD domain, the DDT motif within Tip5 or the SANT / SLIDE domains within Snf2h (Aasland et al., 1996; Aravind and Landsman, 1998; Doerks et al., 2001; Grüne et al., 2003; Nan et al., 1993). Studies with the TAM / MBD domain of Tip5 revealed that this motif is capable of DNA binding, and this activity is furthermore stimulated by its neighboring AT-hooks (Németh, 2002; Strohner et al., 2001). Intriguingly, the TAM (Tip5, ARBP, MeCP2) domain is described in close relation to the MBD motif present in methyl-CpG binding proteins suggesting that Tip5 might bind to methylated DNA (Aravind and Landsman, 1998; Nan et al., 1993; Wade, 2001).

Methylation of a single CpG dinucleotide within the rDNA promoter represses rDNA transcription (Santoro and Grummt, 2001) and NoRC was shown to be associated with methylated rDNA copies (Santoro et al., 2002). Furthermore, the rDNA promoter fragment that is preferentially bound by NoRC contains this methylation site. Nevertheless, neither Tip5 nor the TAM / MBD domain showed methylation sensitive binding to this DNA fragment (Németh, 2002; Strohner et al., 2001). Indeed, the MBD domain within Tip5 is imperfect. Structural analysis of several methylated DNA binding proteins pinpointed to critical conserved amino acids for binding to methylated DNA which are missing in the TAM domain of Tip5 ((Wade, 2001) and references therein). Accordingly, direct binding of Tip5 / NoRC to methylated DNA appears to be unlikely.

Binding of NoRC to the rDNA promoter, but not to transcribed regions was shown *in vivo* by chromatin immunoprecipitations (Santoro et al., 2002). Notably, NoRC is targeted and recruited to the rDNA promoter by TTF-I. Furthermore, NoRC displays preferred binding *in vitro* to a structured (bent) rDNA promoter element next to the TTF-I binding site. A scenario is possible, where a combination of different signals specifically attracts and strengthens binding of NoRC at the rDNA promoter. It is tempting to speculate, that NoRC is recruited to the rDNA promoter via TTF-I and stays there stably associated through a variety of signals, such as the TTF-I interaction, preferential DNA binding and specific histone modifications, which leads to the establishment of a repressive chromatin environment.

9.8 Tip5 and NoRC homologous in other species?

Within the WAL / BAZ family exists a non-characterized member, called BAZ2B, a protein certainly closely related to Tip5 (termed also BAZ2A). BAZ2B contains the characteristic TAM / MBD domain but misses the two AT-hooks within the TTF-I interaction region of Tip5 (Jones et al., 2000). However, it would be especially interesting to characterize BAZ2B further and to compare it with Tip5.

Throughout the animal kingdom, several Tip5 or BAZ2B homologous are found by sequence homology search, but very little is known about these proteins. Toutatis, a predicted *Drosophila* homologous, was found in a genetic screen for dominant modifiers of segment identity during development. The gene product is described as novel member of the trithorax group, but its precise function is elusive (Fauvarque et al., 2001). Unexpectedly, a Tip5 / BAZ2B homologous in chicken, the F22 protein, was defined in GenBank description as an extracellular matrix protein expressed in the retina. Furthermore, the gene ZK 783.4 in *Caenorhabditis elegans* encodes for the Tip5 / BAZ2B related protein FLT-1 (FLecTin-1). This protein is a putative homologous of Flectin, an extracellular matrix protein thought to provide a microenvironment of great elasticity (www.wormbase.org). The protein is similar to the WAL / BAZ protein family but not to other extracellular matrix proteins. FLT-1 is expressed throughout larval and adult stage, with high levels in neuronal cells. In genome wide RNAi knock down experiments, a FLT-1 knock down displays wild type phenotype (www.wormbase.org). Four different ISWI-containing complexes were purified from *Xenopus laevis* (Bozhenok et al., 2002; Guschin et al., 2000). Acf1 and WSTF homologs were described and are present in two of these complexes, but the subunits of other complexes remain to be identified. Indeed, a *Xenopus* homologous of Tip5 can be found in the Genebank suggesting that a *Xenopus* NoRC exists. It will be interesting to see, if one or several of these Tip5 / BAZ2B homologous proteins have also a function in the regulation of rDNA gene transcription. This might be elucidated by future research.

9.9 Biochemical comparison of cellular and recombinant NoRC

The results presented and discussed until here indicate that Tip5 / NoRC interacts with TTF-I and represses specifically rDNA transcription both *in vitro* and *in vivo*. The purified cellular NoRC complex is composed of Tip5 and Snf2h and contains ATP-dependent chromatin remodeling activity in a histone H4 tail dependent fashion. Further comparison was performed. Reconstitution of recombinant NoRC led to a functional and equivalent complex, as revealed in a variety of biochemical assays.

The reconstitution confirmed the tight association of Tip5 and Snf2h, as it was stable under the stringent salt and detergent conditions used for the purification. The recombinant complex showed similar subunit composition as the cellular one and had an equivalent size as revealed in gel filtration experiments. This confirmed that NoRC consists of two subunits, similar to several ISWI-containing remodeling complexes. NoRC has an apparent molecular mass of 800 kDa, as revealed by size exclusion chromatography, suggesting several copies of one or both subunits within the complex. The molecular mass of NoRC matches approximately for a tetramer or pentamer protein complex, such as two Tip5 and two to three Snf2h molecules. Comparing the amount of Snf2h and Tip5 on 'Coomassie gels' suggests that more Snf2h might be present within the complex. In gel filtration experiments elute the recombinant subunits at molecular sizes, which correspond to theoretical dimers of the proteins. Indeed, the composition of recombinant Snf2h measured in MALS (multi angle light scattering) experiments fits best

with a dimer (data not shown). Furthermore, the subunit composition of the related chromatin remodeling complex ACF was determined in fluorescence cross correlations spectroscopy experiments (FCCS). The data imply an ACF complex composed of two ISWI and two Acf1 molecules (Strohner *et al.*, submitted). This suggests, that also the NoRC complex could contain two Snf2h and two Tip5 subunits. Nevertheless, additional studies are necessary to determine the exact subunit composition of NoRC, such as MALS, FCCS or analytical ultra centrifugation experiments.

The purified complex and the recombinant NoRC contain ATP-dependent chromatin remodeling activity. NoRC can induce nucleosome sliding on a short DNA fragment in an ATP-dependent manner, similar to other members of the ISWI-containing remodeling complexes, such as NURF, CHRAC and ACF (Eberharter et al., 2001; Hamiche et al., 1999; Längst et al., 1999). Remarkably, the catalytic subunit ISWI is able to induce histone octamer rearrangements without additional cofactors (Längst et al., 1999). The large subunits Acf1 and NURF301 of the ACF and the NURF remodeling complex, respectively, determine the directionality of the nucleosome movement (Eberharter et al., 2001; Xiao et al., 2001). Indeed, nucleosome positioning depends on the large subunit (G. Längst, unpublished observations). In the nucleosome mobility assay, also the large subunit Tip5 within the NoRC complex determined the nucleosome sliding direction. Therefore, it's tempting to speculate, that the outcome of ISWI remodeling is directly influenced by the additional factors also in the native chromatin environment.

Characteristic for members of the ISWI family is that the N-terminal 'tail' of histone H4 is critical for their activity (Clapier et al., 2001; Hamiche et al., 2001). The remodeling activity of recombinant ISWI is strongly impaired by disruption of a three amino acid region localized at the base of the histone H4 tail, a region which contacts nucleosomal DNA. Furthermore, ISWI is sensitive to the acetylation state within the histone H4 N-terminus (Clapier et al., 2002; Corona et al., 2002). Accordingly, also the remodeling activity of cellular and recombinant NoRC was shown to depend on the histone H4 tail. This suggests that also the activity of NoRC might be influenced by the histone acetylation state.

Like other members of the ISWI family of remodeling factors (Längst and Becker, 2001b), NoRC has a specific chromatin inducible ATPase activity. NoRC and ACF are also stimulated by mononucleosomes (146 and 247 bp DNA in length), but to a lesser extend by core nucleosomes. In contrast, a simple mixture of histones and DNA did not stimulate the activity. This indicates that NoRC recognizes specific structural features of a nucleoprotein complex. Supposedly, NoRC undergoes weak interaction with core nucleosomes. Consistent with this, discrete complexes with core nucleosome particles could not be resolved in electrophoretic mobility assays. However, NoRC forms stable complexes with nucleosomes that contain additional linker DNA. The DNA length for efficient NoRC binding was approximately 50 bp of DNA for half maximal binding as determined in a DNA length competition assay (data not shown). Nevertheless, NoRC interacts with linker DNA and nucleosomal DNA, as shown in DNase I footprint experiments. These results imply that NoRC might bind to free and nucleosomal DNA by two distinct ways. Similarly, ISWI and ACF do not form stable complexes with core nucleosomes (data not shown and (Brehm et al., 2000; Whitehouse et al., 2003)) but interact with linker DNA and the nucleosome (Längst and Becker, 2001a)(Strohner *et al.*, submitted). In addition, NURF shows binding to linker DNA and a region asymmetrically surrounding the nucleosome dyad (Schwanbeck et al., 2004). In this study the nucleosomal interaction region was mapped close to residues of the histone H4 tail that have been implicated in ISWI function. These results suggest a common way in which ISWI chromatin remodeling complexes bind to the nucleosomal substrate.

9.10 Studies on nucleosome remodeling

In this doctoral thesis, I present the identification and characterizing of NoRC and its role in rDNA silencing. Beside this, I was involved in several other studies upon the mechanisms by which ISWI-containing chromatin remodeling complexes alter nucleosome positions. These results are not further described and discussed in this thesis.

One study led to the discovery of a role for HMGB1 as a DNA chaperone during nucleosome remodeling (Bonaldi et al., 2002).

Nucleosome remodeling complexes CHRAC and ACF contribute to chromatin dynamics by converting chemical energy into sliding of histone octamers on DNA. Their shared ATPase subunit ISWI binds DNA at the sites of entry into the nucleosome. A prevalent model assumes that DNA distortions catalyzed by ISWI are converted into relocation of DNA relative to a histone octamer. HMGB1, one of the most abundant nuclear non-histone proteins, binds with preference to distorted DNA. We have now found that transient interaction of HMGB1 with nucleosomal linker DNA overlapping ISWI-binding sites enhances the ability of ACF to bind nucleosomal DNA and accelerates the sliding activity of limiting concentrations of remodeling factor. By contrast, an HMGB1 mutant with increased binding affinity was inhibitory. These observations are consistent with a role for HMGB1 as a DNA chaperone facilitating the rate-limiting DNA distortion during nucleosome remodeling.

In addition, experiments were performed providing evidence for a ‘loop recapture mechainsm’ for nucleosome remodeling by ISWI containing remodeling complexes (Strohner *et al.*, submitted).

The ATPase ISWI is the molecular motor of several nucleosome remodeling complexes including ACF. In order to unravel the mechanism of nucleosome remodeling, we analyzed ACF-nucleosome interactions and the characteristics of ACF-dependent nucleosome mobility. In contrast to the asymmetric ISWI-nucleosome interactions, ACF complex contacts the nucleosome symmetrically at both DNA entry/exit sites. By two-color fluorescence cross correlation spectroscopy measurements, it was demonstrated that ACF can bind three or four DNA duplexes simultaneously in a complex that consists of two Acf1 and ISWI molecules. Using biotinylated nucleosomal substrates coupled to magnetic beads, nucleosome movement by mechanisms involving DNA-twisting was excluded. Furthermore, an ACF-dependent local detachment of DNA from the nucleosome was demonstrated in a novel assay based on the preferred intercalation of ethidium bromide to free DNA. The experimental findings provide evidence for a loop recapture mechanism where ACF introduces a DNA loop at the nucleosomal entry site that propagates over the histone octamer surface and leads to nucleosome repositioning.

10 APPENDIX

10.1 Curriculum Vitae

Name:	Strohner
First name:	Ralf
Nationality:	German
Date of birth:	16. August 1972
Place of birth:	Weinheim, Germany
Marital status:	Single

Scientific education:

01/09/00 – present	PhD at the Adolf-Butenandt-Institut (Munich) Department of Molecularbiology; Head: Prof. Dr. P. B. Becker 'NoRC, a novel chromatin remodeling complex, involved in ribosomal RNA gene silencing'; Supervisor: PD Dr. G. Längst
06/05/00 – 31/07/00	Research assistent at the laboratory of Prof. I. Grummt, DKFZ (Heidelberg)
01/10/94 – 05/05/00	Study of biology at the Ruprecht-Karls-University (Heidelberg)
05/05/00	Master degree in biology (molecular biology, cell biology and biochemistry)
07/99 - 05/00	Diploma thesis, 'Tip5, a novel TTF-I interacting protein: cloning, biochemical purification and functional analysis', at the laboratory of Prof. I. Grummt, DKFZ (Heidelberg)
04/98 - 06/98	Work placement, "Initial investigations on channel lining segments of the NMDA–receptor" at the laboratory of PD Dr. R. Schoepfer, LMP / Department of Pharmacology (UCL, London), supported by a short time fellowship (Boehringer Ingelheim Fonds)
11/97 - 12/97	Work placement at the laboratory of Prof. I. Grummt, DKFZ (Heidelberg)
01/10/96	Bachelor degree in biology
24/06/94 – 30/09/94	Work as a technical assistant at the Paul-Ehrlich-Institut (Langen)
10/08/91 – 23/06/94	Technical assistant training at the Paul-Ehrlich-Institut (Langen) and the Co. E. Merck (Darmstadt)
28/06/91	High school qualification

Conference poster presentations:

Conference Jacques Monod
'Signaling and Control of Transcription'
Aussois (France), 09. - 13. June 2001

12th IMP SPRING CONFERENCE
'Epigenetic Programming of the Genome'
Vienna (Austria), 23. – 25. May 2002

5th EMBL Transcription Meeting
EMBL, Heidelberg (Germany), 24. – 28. August 2002

FASEB Summer Research Conferences
'Chromatin and Transcription Meeting'
Snowmass, Colorado (USA), 12. – 17. July 2003

1st Symposium of Transregio 5
'Chromatin: Assembly and Inheritance of Functional States'
Munich (Germany), 09. – 11. October 2003

10.2 List of publications

Strohner, R., Németh, A., Jansa, P., Hofmann-Rohrer, U., Santoro, R., Längst, G., and Grummt, I. (2001). NoRC--a novel member of mammalian ISWI-containing chromatin remodeling machines. Embo J *20*, 4892-4900.

Bonaldi, T., Längst, G., Strohner, R., Becker, P. B., and Bianchi, M. E. (2002). The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding. Embo J *21*, 6865-6873.

Strohner, R., Németh, A., Nightingale, K. P., Grummt, I., Becker, P. B., and Längst, G. (2004). Recruitment of the nucleolar remodeling complex NoRC establishes ribosomal DNA silencing in chromatin. Mol Cell Biol *24*, 1791-1798.

Németh, A., Strohner, R., Grummt, I., and Längst, G. (2004). The chromatin remodeling complex NoRC and TTF-I cooperate in the regulation of the mammalian rRNA genes *in vivo*. Nucleic Acids Res *32*, 4091-4099.

Strohner, R., Wachsmuth, M., Dachauer, K., Mazurkiewicz, J., Rippe, K., and Längst, G. (2004). A 'loop recapture' mechanism for nucleosome remodeling by ISWI containing complexes. *Submitted.*

Print-outs of the publications are attached at the end of this doctoral thesis.

11 REFERENCES

Aalfs, J.D., Narlikar, G.J. and Kingston, R.E. (2001) Functional differences between the human ATP-dependent nucleosome remodeling proteins BRG1 and SNF2H. *J Biol Chem*, **276**, 34270-34278.

Aasland, R., Gibson, T.J. and Stewart, A.F. (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem Sci*, **20**, 56-59.

Aasland, R., Stewart, A.F. and Gibson, T. (1996) The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB. *Trends Biochem Sci*, **21**, 87-88.

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

Agalioti, T., Chen, G. and Thanos, D. (2002) Deciphering the transcriptional histone acetylation code for a human gene. *Cell*, **111**, 381-392.

Agresti, A. and Bianchi, M.E. (2003) HMGB proteins and gene expression. *Curr Opin Genet Dev*, **13**, 170-178.

Ahmad, K. and Henikoff, S. (2001) Centromeres are specialized replication domains in heterochromatin. *J Cell Biol*, **153**, 101-110.

Ahmad, K. and Henikoff, S. (2002) The histone variant h3.3 marks active chromatin by replication- independent nucleosome assembly. *Mol Cell*, **9**, 1191-1200.

Aihara, T., Miyoshi, Y., Koyama, K., Suzuki, M., Takahashi, E., Monden, M. and Nakamura, Y. (1998) Cloning and mapping of SMARCA5 encoding hSNF2H, a novel human homologue of Drosophila ISWI. *Cytogenet Cell Genet*, **81**, 191-193.

Akey, C.W. and Luger, K. (2003) Histone chaperones and nucleosome assembly. *Curr Opin Struct Biol*, **13**, 6-14.

Albert, A.C., Denton, M., Kermekchiev, M. and Pikaard, C.S. (1999) Histone acetyltransferase and protein kinase activities copurify with a putative Xenopus RNA polymerase I holoenzyme self-sufficient for promoter-dependent transcription. *Mol Cell Biol*, **19**, 796-806.

Allfrey, V.G., Faulkner, R. and Mirsky, A.E. (1964) Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. *Proc Natl Acad Sci U S A*, **51**, 786-794.

Andersen, J.S., Lyon, C.E., Fox, A.H., Leung, A.K., Lam, Y.W., Steen, H., Mann, M. and Lamond, A.I. (2002) Directed proteomic analysis of the human nucleolus. *Curr Biol*, **12**, 1-11.

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.

Aoyagi, S., Wade, P.A. and Hayes, J.J. (2003) Nucleosome sliding catalyzed by the xMi-2 complex does not occur exclusively via a simple twist-diffusion mechanism. *J Biol Chem.*

Aravind, L. and Landsman, D. (1998) AT-hook motifs identified in a wide variety of DNA-binding proteins. *Nucleic Acids Res*, **26**, 4413-4421.

Armstrong, J.A., Bieker, J.J. and Emerson, B.M. (1998) A SWI/SNF-related chromatin remodeling complex, E-RC1, is required for tissue-specific transcriptional regulation by EKLF in vitro [In Process Citation]. *Cell*, **95**, 93-104.

Armstrong, J.A. and Emerson, B.M. (1996) NF-E2 disrupts chromatin structure at human beta-globin locus control region hypersensitive site 2 in vitro. *Mol Cell Biol*, **16**, 5634-5644.

Armstrong, J.A., Papoulas, O., Daubresse, G., Sperling, A.S., Lis, J.T., Scott, M.P. and Tamkun, J.W. (2002) The Drosophila BRM complex facilitates global transcription by RNA polymerase II. *Embo J*, **21**, 5245-5254.

Ausubel, F.M. (1999) *Short protocols in molecular biology : a compendium of methods from Current protocols in molecular biology*. Wiley, New York.

Badenhorst, P., Voas, M., Rebay, I. and Wu, C. (2002) Biological functions of the ISWI chromatin remodeling complex NURF. *Genes Dev*, **16**, 3186-3198.

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

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, **410**, 120-124.

Barak, O., Lazzaro, M.A., Lane, W.S., Speicher, D.W., Picketts, D.J. and Shiekhattar, R. (2003) Isolation of human NURF: a regulator of Engrailed gene expression. *Embo J*, **22**, 6089-6100.

Bartsch, I., Schoneberg, C. and Grummt, I. (1988) Purification and characterization of TTFI, a factor that mediates termination of mouse ribosomal DNA transcription. *Mol Cell Biol*, **8**, 3891-3897.

Baxter, J., Merkenschlager, M. and Fisher, A.G. (2002) Nuclear organisation and gene expression. *Curr Opin Cell Biol*, **14**, 372-376.

Bazett-Jones, D.P., Leblanc, B., Herfort, M. and Moss, T. (1994) Short-range DNA looping by the Xenopus HMG-box transcription factor, xUBF. *Science*, **264**, 1134-1137.

Becker, P.B. and Hörz, W. (2002) ATP-dependent nucleosome remodeling. *Annu Rev Biochem*, **71**, 247-273.

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.

Bednar, J., Horowitz, R.A., Grigoryev, S.A., Carruthers, L.M., Hansen, J.C., Koster, A.J. and Woodcock, C.L. (1998) Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin. *Proc Natl Acad Sci U S A*, **95**, 14173-14178.

Beisel, C., Imhof, A., Greene, J., Kremmer, E. and Sauer, F. (2002) Histone methylation by the Drosophila epigenetic transcriptional regulator Ash1. *Nature*, **419**, 857-862.

Bianchi, M.E. (1988) Interaction of a protein from rat liver nuclei with cruciform DNA. *Embo J*, **7**, 843-849.

Bianchi, M.E., Beltrame, M. and Paonessa, G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1. *Science*, **243**, 1056-1059.

Bird, A. (2002) DNA methylation patterns and epigenetic memory. *Genes Dev*, **16**, 6-21.

Bird, A., Taggart, M. and Macleod, D. (1981a) Loss of rDNA methylation accompanies the onset of ribosomal gene activity in early development of X. laevis. *Cell*, **26**, 381-390.

Bird, A.P., Taggart, M.H. and Gehring, C.A. (1981b) Methylated and unmethylated ribosomal RNA genes in the mouse. *J Mol Biol*, **152**, 1-17.

Bird, A.P. and Wolffe, A.P. (1999) Methylation-induced repression--belts, braces, and chromatin. *Cell*, **99**, 451-454.

Blum, H., Beier, H. and Gross, H. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **8**, 93-99.

Bochar, D.A., Savard, J., Wang, W., Lafleur, D.W., Moore, P., Cote, J. and Shiekhattar, R. (2000a) A family of chromatin remodeling factors related to Williams syndrome transcription factor. *Proc Natl Acad Sci U S A*, **97**, 1038-1043.

Bochar, D.A., Wang, L., Beniya, H., Kinev, A., Xue, Y., Lane, W.S., Wang, W., Kashanchi, F. and Shiekhattar, R. (2000b) BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell*, **102**, 257-265.

Bodem, J., Dobreva, G., Hoffmann-Rohrer, U., Iben, S., Zentgraf, H., Delius, H., Vingron, M. and Grummt, I. (2000) TIF-IA, the factor mediating growth-dependent control of ribosomal RNA synthesis, is the mammalian homolog of yeast Rrn3p. *EMBO Rep*, **1**, 171-175.

Bonaldi, T., Längst, G., Strohner, R., Becker, P.B. and Bianchi, M.E. (2002) The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding. *Embo J*, **21**, 6865-6873.

Bouazoune, K., Mitterweger, A., Längst, G., Imhof, A., Akhtar, A., Becker, P.B. and Brehm, A. (2002) The dMi-2 chromodomains are DNA binding modules important for ATP-dependent nucleosome mobilization. *Embo J*, **21**, 2430-2440.

Bowen, N.J., Fujita, N., Kajita, M. and Wade, P.A. (2004) Mi-2/NuRD: multiple complexes for many purposes. *Biochim Biophys Acta*, **1677**, 52-57.

Boyer, L.A., Langer, M.R., Crowley, K.A., Tan, S., Denu, J.M. and Peterson, C.L. (2002) Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. *Mol Cell*, **10**, 935-942.

Boyer, L.A., Logie, C., Bonte, E., Becker, P.B., Wade, P.A., Wolffe, A.P., Wu, C., Imbalzano, A.N. and Peterson, C.L. (2000) Functional delineation of three groups of the ATP-dependent family of chromatin remodeling enzymes. *J Biol Chem*, **275**, 18864-18870.

Bozhenok, L., Wade, P.A. and Varga-Weisz, P. (2002) WSTF-ISWI chromatin remodeling complex targets heterochromatic replication foci. *Embo J*, **21**, 2231-2241.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-254.

Bradsher, J., Auriol, J., Proietti de Santis, L., Iben, S., Vonesch, J.L., Grummt, I. and Egly, J.M. (2002) CSB is a component of RNA pol I transcription. *Mol Cell*, **10**, 819-829.

Brehm, A., Längst, G., Kehle, J., Clapier, C.R., Imhof, A., Eberharter, A., Muller, J. and Becker, P.B. (2000) dMi-2 and ISWI chromatin remodelling factors have distinct nucleosome binding and mobilization properties. *Embo J*, **19**, 4332-4341.

Brehm, A., Tufteland, K.R., Aasland, R. and Becker, P.B. (2004) The many colours of chromodomains. *Bioessays*, **26**, 133-140.

Briggs, S.D., Xiao, T., Sun, Z.W., Caldwell, J.A., Shabanowitz, J., Hunt, D.F., Allis, C.D. and Strahl, B.D. (2002) Gene silencing: trans-histone regulatory pathway in chromatin. *Nature*, **418**, 498.

Brock, G.J. and Bird, A. (1997) Mosaic methylation of the repeat unit of the human ribosomal RNA genes. *Hum Mol Genet*, **6**, 451-456.

Brown, C.E., Lechner, T., Howe, L. and Workman, J.L. (2000) The many HATs of transcription coactivators. *Trends Biochem Sci*, **25**, 15-19.

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y. and Allis, C.D. (1996) Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell*, **84**, 843-851.

Buttgereit, D., Pflugfelder, G. and Grummt, I. (1985) Growth-dependent regulation of rRNA synthesis is mediated by a transcription initiation factor (TIF-IA). *Nucleic Acids Res*, **13**, 8165-8180.

Byvoet, P., Shepherd, G.R., Hardin, J.M. and Noland, B.J. (1972) The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells. *Arch Biochem Biophys*, **148**, 558-567.

Cairns, B.R., Kim, Y.J., Sayre, M.H., Laurent, B.C. and Kornberg, R.D. (1994) A multisubunit complex containing the SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. *Proc Natl Acad Sci U S A*, **91**, 1950-1954.

Cairns, B.R., Lorch, Y., Li, Y., Zhang, M., 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.

Cairns, B.R., Schlichter, A., Erdjument-Bromage, H., Tempst, P., Kornberg, R.D. and Winston, F. (1999) Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. *Mol Cell*, **4**, 715-723.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S. and Zhang, Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science*, **298**, 1039-1043.

Capili, A.D., Schultz, D.C., Rauscher, I.F. and Borden, K.L. (2001) Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains. *Embo J*, **20**, 165-177.

Carlson, M. and Laurent, B.C. (1994) The SNF/SWI family of global transcriptional activators. *Curr Opin Cell Biol*, **6**, 396-402.

Carruthers, L.M., Bednar, J., Woodcock, C.L. and Hansen, J.C. (1998) Linker histones stabilize the intrinsic salt-dependent folding of nucleosomal arrays: mechanistic ramifications for higher-order chromatin folding. *Biochemistry*, **37**, 14776-14787.

Chen, T.A., Sterner, R., Cozzolino, A. and Allfrey, V.G. (1990) Reversible and irreversible changes in nucleosome structure along the c-fos and c-myc oncogenes following inhibition of transcription. *J Mol Biol*, **212**, 481-493.

Chen, Z.J. and Pikaard, C.S. (1997) Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. *Genes Dev*, **11**, 2124-2136.

Chiba, H., Muramatsu, M., Nomoto, A. and Kato, H. (1994) Two human homologues of Saccharomyces cerevisiae SWI2/SNF2 and Drosophila brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. *Nucleic Acids Res*, **22**, 1815-1820.

Chubb, J.R. and Bickmore, W.A. (2003) Considering nuclear compartmentalization in the light of nuclear dynamics. *Cell*, **112**, 403-406.

Clapier, C.R., Längst, G., Corona, D.F., Becker, P.B. and Nightingale, K.P. (2001) Critical Role for the Histone H4 N Terminus in Nucleosome Remodeling by ISWI. *Mol Cell Biol*, **21**, 875-883.

Clapier, C.R., Nightingale, K.P. and Becker, P.B. (2002) A critical epitope for substrate recognition by the nucleosome remodeling ATPase ISWI. *Nucleic Acids Res*, **30**, 649-655.

Clos, J., Buttgereit, D. and Grummt, I. (1986) A purified transcription factor (TIF-IB) binds to essential sequences of the mouse rDNA promoter. *Proc Natl Acad Sci U S A*, **83**, 604-608.

Collins, N., Poot, R.A., Kukimoto, I., Garcia-Jimenez, C., Dellaire, G. and Varga-Weisz, P.D. (2002) An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. *Nat Genet*, **32**, 627-632.

Comai, L. (1999) The nucleolus: a paradigm for cell proliferation and aging. *Braz J Med Biol Res*, **32**, 1473-1478.

Conconi, A., Sogo, J.M. and Ryan, C.A. (1992) Ribosomal gene clusters are uniquely proportioned between open and closed chromatin structures in both tomato leaf cells and exponentially growing suspension cultures. *Proc Natl Acad Sci U S A*, **89**, 5256-5260.

Conconi, A., Widmer, R.M., Koller, T. and Sogo, J.M. (1989) Two different chromatin structures coexist in ribosomal RNA genes throughout the cell cycle. *Cell*, **57**, 753-761.

Copenhaver, G.P., Putnam, C.D., Denton, M.L. and Pikaard, C.S. (1994) The RNA polymerase I transcription factor UBF is a sequence-tolerant HMG-box protein that can recognize structured nucleic acids. *Nucleic Acids Res*, **22**, 2651-2657.

Corona, D.F., Clapier, C.R., Becker, P.B. and Tamkun, J.W. (2002) Modulation of ISWI function by site-specific histone acetylation. *EMBO Rep*, **3**, 242-247.

Corona, D.F., Eberharter, A., Budde, A., Deuring, R., Ferrari, S., Varga-Weisz, P., Wilm, M., Tamkun, J. and Becker, P.B. (2000) Two histone fold proteins, CHRAC-14 and CHRAC-16, are developmentally regulated subunits of chromatin accessibility complex (CHRAC). *Embo J*, **19**, 3049-3059.

Corona, D.F., Längst, G., Clapier, C.R., Bonte, E.J., Ferrari, S., Tamkun, J.W. and Becker, P.B. (1999) ISWI is an ATP-dependent nucleosome remodeling factor. *Mol Cell*, **3**, 239-245.

Corona, D.F. and Tamkun, J.W. (2004) Multiple roles for ISWI in transcription, chromosome organization and DNA replication. *Biochim Biophys Acta*, **1677**, 113-119.

Costanzi, C. and Pehrson, J.R. (1998) Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. *Nature*, **393**, 599-601.

Cote, J., Quinn, J., Workman, J.L. and Peterson, C.L. (1994) Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science*, **265**, 53-60.

Cremer, T. and Cremer, C. (2001) Chromosome territories, nuclear architecture and gene regulation in mammalian cells. *Nat Rev Genet*, **2**, 292-301.

Culotta, V. and Sollner, W.B. (1988) Sites of topoisomerase I action on X. laevis ribosomal chromatin: transcriptionally active rDNA has an approximately 200 bp repeating structure. *Cell*, **52**, 585-597.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. and Pirrotta, V. (2002) Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell*, **111**, 185-196.

Damelin, M., Simon, I., Moy, T.I., Wilson, B., Komili, S., Tempst, P., Roth, F.P., Young, R.A., Cairns, B.R. and Silver, P.A. (2002) The genome-wide localization of Rsc9, a component of the RSC chromatin-remodeling complex, changes in response to stress. *Mol Cell*, **9**, 563-573.

Dammann, R., Lucchini, R., Koller, T. and Sogo, J.M. (1993) Chromatin structures and transcription of rDNA in yeast Saccharomyces cerevisiae. *Nucleic Acids Res*, **21**, 2331-2338.

Dammann, R., Lucchini, R., Koller, T. and Sogo, J.M. (1995) Transcription in the yeast rRNA gene locus: distribution of the active gene copies and chromatin structure of their flanking regulatory sequences. *Mol Cell Biol*, **15**, 5294-5303.

Davis, A.H., Reudelhuber, T.L. and Garrard, W.T. (1983) Varigated chromatin structures of mouse ribosomal RNA genes. *J Mol Biol*, **167**, 133-155.

de La Serna, I.L., Carlson, K.A. and Imbalzano, A.N. (2001) Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. *Nat Genet*, **27**, 187-190.

Deuring, R., Fanti, L., Armstrong, J.A., Sarte, M., Papoulas, O., Prestel, M., Daubresse, G., Verardo, M., Moseley, S.L., Berloco, M., Tsukiyama, T., Wu, C., Pimpinelli, S. and Tamkun, J.W. (2000) The ISWI chromatin-remodeling protein is required for gene expression and the maintenance of higher order chromatin structure in vivo. *Mol Cell*, **5**, 355-365.

Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K. and Zhou, M.M. (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature*, **399**, 491-496.

Dhillon, N. and Kamakaka, R.T. (2002) Breaking through to the other side: silencers and barriers. *Curr Opin Genet Dev*, **12**, 188-192.

Dingwall, A.K., Beek, S.J., McCallum, C.M., Tamkun, J.W., Kalpana, G.V., Goff, S.P. and Scott, M.P. (1995) The Drosophila snr1 and brm proteins are related to yeast SWI/SNF proteins and are components of a large protein complex. *Mol Biol Cell*, **6**, 777-791.

Doerks, T., Copley, R. and Bork, P. (2001) DDT -- a novel domain in different transcription and chromosome remodeling factors. *Trends Biochem Sci*, **26**, 145-146.

Dousset, T., Wang, C., Verheggen, C., Chen, D., Hernandez-Verdun, D. and Huang, S. (2000) Initiation of nucleolar assembly is independent of RNA polymerase I transcription. *Mol Biol Cell*, **11**, 2705-2717.

Dunaief, J.L., Strober, B.E., Guha, S., Khavari, P.A., Alin, K., Luban, J., Begemann, M., Crabtree, G.R. and Goff, S.P. (1994) The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell*, **79**, 119-130.

Dundr, M., Hoffmann-Rohrer, U., Hu, Q., Grummt, I., Rothblum, L.I., Phair, R.D. and Misteli, T. (2002) A kinetic framework for a mammalian RNA polymerase in vivo. *Science*, **298**, 1623-1626.

Ebbert, R., Birkmann, A. and Schuller, H.J. (1999) The product of the SNF2/SWI2 paralogue INO80 of Saccharomyces cerevisiae required for efficient expression of various yeast structural genes is part of a high-molecular-weight protein complex. *Mol Microbiol*, **32**, 741-751.

Eberharter, A. (2004) Requirement of conserved structural motifs of Acf1 in nucleosome mobilization. *Embo J*, **in press**.

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

Eberharter, A., Ferrari, S., Längst, G., Straub, T., Imhof, A., Varga-Weisz, P., Wilm, M. and Becker, P.B. (2001) Acf1, the largest subunit of CHRAC, regulates ISWI-induced nucleosome remodelling. *Embo J*, **20**, 3781-3788.

Eberharter, A., Längst, G. and Becker, P.B. (2003) A Nucleosome Sliding Assay for Chromatin Remodeling Factors. *Methods Enzymol*, **377**, 344-353.

Eisen, J.A., Sweder, K.S. and Hanawalt, P.C. (1995) Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. *Nucleic Acids Res*, **23**, 2715-2723.

Elfring, L.K., Daniel, C., Papoulas, O., Deuring, R., Sarte, M., Moseley, S., Beek, S.J., Waldrip, W.R., Daubresse, G., DePace, A., Kennison, J.A. and Tamkun, J.W. (1998) Genetic analysis of brahma: the Drosophila homolog of the yeast chromatin remodeling factor SWI2/SNF2. *Genetics*, **148**, 251-265.

Elfring, L.K., Deuring, R., McCallum, C.M., Peterson, C.L. and Tamkun, J.W. (1994) Identification and characterization of *Drosophila* relatives of the yeast transcriptional activator SNF2/SWI2. *Mol. Cell. Biol.*, **14**, 2225-2234.

Evers, R. and Grummt, I. (1995) Molecular coevolution of mammalian ribosomal gene terminator sequences and the transcription termination factor TTF-I. *Proc Natl Acad Sci U S A*, **92**, 5827-5831.

Evers, R., Smid, A., Rudloff, U., Lottspeich, F. and Grummt, I. (1995) Different domains of the murine RNA polymerase I-specific termination factor mTTF-I serve distinct functions in transcription termination. *Embo J*, **14**, 1248-1256.

Fahrner, J.A. and Baylin, S.B. (2003) Heterochromatin: stable and unstable invasions at home and abroad. *Genes Dev*, **17**, 1805-1812.

Fan, H.Y., He, X., Kingston, R.E. and Narlikar, G.J. (2003) Distinct strategies to make nucleosomal DNA accessible. *Mol Cell*, **11**, 1311-1322.

Fath, S., Milkereit, P., Podtelejnikov, A.V., Bischler, N., Schultz, P., Bier, M., Mann, M. and Tschochner, H. (2000) Association of yeast RNA polymerase I with a nucleolar substructure active in rRNA synthesis and processing. *J Cell Biol*, **149**, 575-590.

Fauvarque, M.O., Laurenti, P., Boivin, A., Bloyer, S., Griffin-Shea, R., Bourbon, H.M. and Dura, J.M. (2001) Dominant modifiers of the polyhomeotic extra-sex-combs phenotype induced by marked P element insertional mutagenesis in Drosophila. *Genet Res*, **78**, 137-148.

Fazzio, T.G., Kooperberg, C., Goldmark, J.P., Neal, C., Basom, R., Delrow, J. and Tsukiyama, T. (2001) Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol Cell Biol*, **21**, 6450-6460.

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.

Felsenfeld, G. and Groudine, M. (2003) Controlling the double helix. *Nature*, **421**, 448-453.

Feng, Q. and Zhang, Y. (2001) The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. *Genes Dev*, **15**, 827-832.

Finch, J.T. and Klug, A. (1976) Solenoidal model for superstructure in chromatin. *Proc Natl Acad Sci U S A*, **73**, 1897-1901.

Finnin, M.S., Donigian, J.R., Cohen, A., Richon, V.M., Rifkind, R.A., Marks, P.A., Breslow, R. and Pavletich, N.P. (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature*, **401**, 188-193.

Fischle, W., Wang, Y. and Allis, C.D. (2003) Histone and chromatin cross-talk. *Curr Opin Cell Biol*, **15**, 172-183.

Fisher, A.G. and Merkenschlager, M. (2002) Gene silencing, cell fate and nuclear organisation. *Curr Opin Genet Dev*, **12**, 193-197.

Flaus, A. and Owen-Hughes, T. (2003) Mechanisms for nucleosome mobilization. *Biopolymers*, **68**, 563-578.

French, S.L., Osheim, Y.N., Cioci, F., Nomura, M. and Beyer, A.L. (2003) In exponentially growing Saccharomyces cerevisiae cells, rRNA synthesis is determined by the summed RNA polymerase I loading rate rather than by the number of active genes. *Mol Cell Biol*, **23**, 1558-1568.

Fyodorov, D.V., Blower, M.D., Karpen, G.H. and Kadonaga, J.T. (2004) Acf1 confers unique activities to ACF/CHRAC and promotes the formation rather than disruption of chromatin in vivo. *Genes Dev*, **18**, 170-183.

Fyodorov, D.V. and Kadonaga, J.T. (2002a) Binding of Acf1 to DNA involves a WAC motif and is important for ACF-mediated chromatin assembly. *Mol Cell Biol*, **22**, 6344-6353.

Fyodorov, D.V. and Kadonaga, J.T. (2002b) Dynamics of ATP-dependent chromatin assembly by ACF. *Nature*, **418**, 897-900.

Gasser, R., Koller, T. and Sogo, J.M. (1996) The stability of nucleosomes at the replication fork. *J Mol Biol*, **258**, 224-239.

Gelbart, M.E., Rechsteiner, T., Richmond, T.J. and Tsukiyama, T. (2001) Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. *Mol Cell Biol*, **21**, 2098-2106.

Gerber, J.K., Gogel, E., Berger, C., Wallisch, M., Muller, F., Grummt, I. and Grummt, F. (1997) Termination of mammalian rDNA replication: polar arrest of replication fork movement by transcription termination factor TTF-I. *Cell*, **90**, 559-567.

Goldmark, J.P., Fazzio, T.G., Estep, P.W., Church, G.M. and Tsukiyama, T. (2000) The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. *Cell*, **103**, 423-433.

Gonzalez, I.L. and Sylvester, J.E. (1995) Complete sequence of the 43-kb human ribosomal DNA repeat: analysis of the intergenic spacer. *Genomics*, **27**, 320-328.

Grant, P.A., Duggan, L., Cote, J., Roberts, S.M., Brownell, J.E., Candau, R., Ohba, R., Owen-Hughes, T., Allis, C.D., Winston, F., Berger, S.L. and Workman, J.L. (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev*, **11**, 1640-1650.

Grewal, S.I. and Elgin, S.C. (2002) Heterochromatin: new possibilities for the inheritance of structure. *Curr Opin Genet Dev*, **12**, 178-187.

Grewal, S.I. and Moazed, D. (2003) Heterochromatin and epigenetic control of gene expression. *Science*, **301**, 798-802.

Grozdanov, P., Georgiev, O. and Karagyozov, L. (2003) Complete sequence of the 45-kb mouse ribosomal DNA repeat: analysis of the intergenic spacer. *Genomics*, **82**, 637-643.

Gruenbaum, Y., Szyf, M., Cedar, H. and Razin, A. (1983) Methylation of replicating and post-replicated mouse L-cell DNA. *Proc Natl Acad Sci U S A*, **80**, 4919-4921.

Grummt, I. (1999) Regulation of mammalian ribosomal gene transcription by RNA polymerase I. *Prog Nucleic Acid Res Mol Biol*, **62**, 109-154.

Grummt, I. (2003) Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes Dev*, **17**, 1691-1702.

Grummt, I., Kuhn, A., Bartsch, I. and Rosenbauer, H. (1986a) A transcription terminator located upstream of the mouse rDNA initiation site affects rRNA synthesis. *Cell*, **47**, 901-911.

Grummt, I., Maier, U., Ohrlein, A., Hassouna, N. and Bachellerie, J.P. (1985) Transcription of mouse rDNA terminates downstream of the 3' end of 28S RNA and involves interaction of factors with repeated sequences in the 3' spacer. *Cell*, **43**, 801-810.

Grummt, I. and Pikaard, C.S. (2003) Epigenetic silencing of RNA polymerase I transcription. *Nat Rev Mol Cell Biol*, **4**, 641-649.

Grummt, I., Rosenbauer, H., Niedermeyer, I., Maier, U. and Ohrlein, A. (1986b) A repeated 18 bp sequence motif in the mouse rDNA spacer mediates binding of a nuclear factor and transcription termination. *Cell*, **45**, 837-846.

Grüne, T., Brzeski, J., Eberharter, A., Clapier, C.R., Corona, D.F., Becker, P.B. and Muller, C.W. (2003) Crystal structure and functional analysis of a nucleosome recognition module of the remodeling factor ISWI. *Mol Cell*, **12**, 449-460.

Grunstein, M. (1997) Histone acetylation in chromatin structure and transcription. *Nature*, **389**, 349-352.

Gu, W. and Roeder, R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595-606.

Guschin, D., Geiman, T.M., Kikyo, N., Tremethick, D.J., Wolffe, A.P. and Wade, P.A. (2000) Multiple ISWI ATPase complexes from xenopus laevis. Functional conservation of an ACF/CHRAC homolog. *J Biol Chem*, **275**, 35248-35255.

Haaf, T., Hayman, D.L. and Schmid, M. (1991) Quantitative determination of rDNA transcription units in vertebrate cells. *Exp Cell Res*, **193**, 78-86.

Hakimi, M.A., Bochar, D.A., Schmiesing, J.A., Dong, Y., Barak, O.G., Speicher, D.W., Yokomori, K. and Shiekhattar, R. (2002) A chromatin remodelling complex that loads cohesin onto human chromosomes. *Nature*, **418**, 994-998.

Hamiche, A., Kang, J.G., Dennis, C., Xiao, H. and Wu, C. (2001) Histone tails modulate nucleosome mobility and regulate ATP-dependent nucleosome sliding by NURF. *Proc Natl Acad Sci U S A*, **98**, 14316-14321.

Hamiche, A., Sandaltzopoulos, R., Gdula, D.A. and Wu, C. (1999) ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF. *Cell*, **97**, 833-842.

Hancock, R. (2000) A new look at the nuclear matrix. *Chromosoma*, **109**, 219-225.

Hannan, R.D., Cavanaugh, A., Hempel, W.M., Moss, T. and Rothblum, L. (1999) Identification of a mammalian RNA polymerase I holoenzyme containing components of the DNA repair/replication system. *Nucleic Acids Res*, **27**, 3720-3727.

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

Hassan, A.H., Prochasson, P., Neely, K.E., Galasinski, S.C., Chandy, M., Carrozza, M.J. and Workman, J.L. (2002) Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. *Cell*, **111**, 369-379.

Haushalter, K.A. and Kadonaga, J.T. (2003) Chromatin assembly by DNA-translocating motors. *Nat Rev Mol Cell Biol*, **4**, 613-620.

Havas, K., Whitehouse, I. and Owen-Hughes, T. (2001) ATP-dependent chromatin remodeling activities. *Cell Mol Life Sci*, **58**, 673-682.

Heitz, E. (1928) Das Heterochromatin der Moose. *I Jb Wiss Bot 1928*, **69**, 762-818.

Heix, J. and Grummt, I. (1995) Species specificity of transcription by RNA polymerase I. *Curr Opin Genet Dev*, **5**, 652-656.

Henderson, S. and Sollner-Webb, B. (1986) A transcriptional terminator is a novel element of the promoter of the mouse ribosomal RNA gene. *Cell*, **47**, 891-900.

Henikoff, S., Furuyama, T. and Ahmad, K. (2004) Histone variants, nucleosome assembly and epigenetic inheritance. *Trends Genet*, **20**, 320-326.

Hirschhorn, J.N., Brown, S.A., Clark, C.D. and Winston, F. (1992) Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev*, **6**, 2288-2298.

Hirschler-Laszkiewicz, I., Cavanaugh, A., Hu, Q., Catania, J., Avantaggiati, M.L. and Rothblum, L.I. (2001) The role of acetylation in rDNA transcription. *Nucleic Acids Res*, **29**, 4114-4124.

Hoffmann-Rohrer, U. and Labaere, P. (eds.). (2000) *Lab FAQS, Find a Quick Solution*. Roche Bolecular Biochemicals.

Horn, P.J. and Peterson, C.L. (2001) The bromodomain: a regulator of ATP-dependent chromatin remodeling? *Front Biosci*, **6**, D1019-1023.

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

Hsu, J.Y., Sun, Z.W., Li, X., Reuben, M., Tatchell, K., Bishop, D.K., Grushcow, J.M., Brame, C.J., Caldwell, J.A., Hunt, D.F., Lin, R., Smith, M.M. and Allis, C.D. (2000) Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. *Cell*, **102**, 279-291.

Huang, Y. (2002) Transcriptional silencing in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Nucleic Acids Res*, **30**, 1465-1482.

Iben, S., Tschochner, H., Bier, M., Hoogstraten, D., Hozak, P., Egly, J.M. and Grummt, I. (2002) TFIIH plays an essential role in RNA polymerase I transcription. *Cell*, **109**, 297-306.

Iida, T. and Araki, H. (2004) Noncompetitive counteractions of DNA polymerase epsilon and ISW2/yCHRAC for epigenetic inheritance of telomere position effect in Saccharomyces cerevisiae. *Mol Cell Biol*, **24**, 217-227.

Iizuka, M. and Smith, M.M. (2003) Functional consequences of histone modifications. *Curr Opin Genet Dev*, **13**, 154-160.

Ito, T., Bulger, M., Pazin, M.J., Kobayashi, R. and Kadonaga, J.T. (1997) ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. *Cell*, **90**, 145-155.

Ito, T., Levenstein, M.E., Fyodorov, D.V., Kutach, A.K., Kobayashi, R. and Kadonaga, J.T. (1999) ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. *Genes Dev*, **13**, 1529-1539.

Jacobs, S.A. and Khorasanizadeh, S. (2002) Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science*, **295**, 2080-2083.

Jacobson, R.H., Ladurner, A.G., King, D.S. and Tjian, R. (2000) Structure and function of a human TAFII250 double bromodomain module. *Science*, **288**, 1422-1425.

Jaenisch, R. and Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*, **33 Suppl**, 245-254.

Jansa, P. and Grummt, I. (1999) Mechanism of transcription termination: PTRF interacts with the largest subunit of RNA polymerase I and dissociates paused transcription complexes from yeast and mouse. *Mol Gen Genet*, **262**, 508-514.

Jansa, P., Mason, S.W., Hoffmann-Rohrer, U. and Grummt, I. (1998) Cloning and functional characterization of PTRF, a novel protein which induces dissociation of paused ternary transcription complexes. *Embo J*, **17**, 2855-2864.

Jantzen, H.M., Admon, A., Bell, S.P. and Tjian, R. (1990) Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. *Nature*, **344**, 830-836.

Jeanmougin, F., Wurtz, J.M., Le Douarin, B., Chambon, P. and Losson, R. (1997) The bromodomain revisited [letter]. *Trends Biochem Sci*, **22**, 151-153.

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

Johnson, E.M., Sterner, R. and Allfrey, V.G. (1987) Altered nucleosomes of active nucleolar chromatin contain accessible histone H3 in its hyperacetylated forms. *J Biol Chem*, **262**, 6943-6946.

Jones, M.H., Hamana, N., Nezu, J. and Shimane, M. (2000) A novel family of bromodomain genes. *Genomics*, **63**, 40-45.

Kadam, S. and Emerson, B.M. (2002) Mechanisms of chromatin assembly and transcription. *Curr Opin Cell Biol*, **14**, 262-268.

Kadam, S., McAlpine, G.S., Phelan, M.L., Kingston, R.E., Jones, K.A. and Emerson, B.M. (2000) Functional selectivity of recombinant mammalian SWI/SNF subunits. *Genes Dev*, **14**, 2441-2451.

Kang, J.G., Hamiche, A. and Wu, C. (2002) GAL4 directs nucleosome sliding induced by NURF. *Embo J*, **21**, 1406-1413.

Kassabov, S.R., Henry, N.M., Zofall, M., Tsukiyama, T. and Bartholomew, B. (2002) High-resolution mapping of changes in histone-DNA contacts of nucleosomes remodeled by ISW2. *Mol Cell Biol*, **22**, 7524-7534.

Katan-Khaykovich, Y. and Struhl, K. (2002) Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes Dev*, **16**, 743-752.

Kehle, J., Beuchle, D., Treuheit, S., Christen, B., Kennison, J.A., Bienz, M. and Muller, J. (1998) dMi-2, a hunchback-interacting protein that functions in polycomb repression. *Science*, **282**, 1897-1900.

Kelley, D.E., Stokes, D.G. and Perry, R.P. (1999) CHD1 interacts with SSRP1 and depends on both its chromodomain and its ATPase/helicase-like domain for proper association with chromatin. *Chromosoma*, **108**, 10-25.

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.

Khorasanizadeh, S. (2004) The nucleosome: from genomic organization to genomic regulation. *Cell*, **116**, 259-272.

Kim, J., Sif, S., Jones, B., Jackson, A., Koipally, J., Heller, E., Winandy, S., Viel, A., Sawyer, A., Ikeda, T., Kingston, R. and Georgopoulos, K. (1999) Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. *Immunity*, **10**, 345-355.

Kitagawa, H., Fujiki, R., Yoshimura, K., Mezaki, Y., Uematsu, Y., Matsui, D., Ogawa, S., Unno, K., Okubo, M., Tokita, A., Nakagawa, T., Ito, T., Ishimi, Y., Nagasawa, H., Matsumoto, T., Yanagisawa, J. and Kato, S. (2003) The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell*, **113**, 905-917.

Klempnauer, K.H., Fanning, E., Otto, B. and Knippers, R. (1980) Maturation of newly replicated chromatin of simian virus 40 and its host cell. *J Mol Biol*, **136**, 359-374.

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.

Koipally, J., Renold, A., Kim, J. and Georgopoulos, K. (1999) Repression by Ikaros and Aiolos is mediated through histone deacetylase complexes. *Embo J*, **18**, 3090-3100.

Korber, P. and Hörz, W. (2004) SWRred not shaken; mixing the histones. *Cell*, **117**, 5-7.

Kornberg, R.D. (1974) Chromatin structure: a repeating unit of histones and DNA. *Science*, **184**, 868-871.

Kouzarides, T. (2002) Histone methylation in transcriptional control. *Curr Opin Genet Dev*, **12**, 198-209.

Krebs, J.E., Fry, C.J., Samuels, M.L. and Peterson, C.L. (2000) Global role for chromatin remodeling enzymes in mitotic gene expression. *Cell*, **102**, 587-598.

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.

Krogan, N.J., Peng, W.T., Cagney, G., Robinson, M.D., Haw, R., Zhong, G., Guo, X., Zhang, X., Canadien, V., Richards, D.P., Beattie, B.K., Lalev, A., Zhang, W., Davierwala, A.P., Mnaimneh, S., Starostine, A., Tikuisis, A.P., Grigull, J., Datta, N., Bray, J.E., Hughes, T.R., Emili, A. and Greenblatt, J.F. (2004) High-definition macromolecular composition of yeast RNA-processing complexes. *Mol Cell*, **13**, 225-239.

Kuhn, A. and Grummt, I. (1992) Dual role of the nucleolar transcription factor UBF: trans-activator and antirepressor. *Proc Natl Acad Sci U S A*, **89**, 7340-7344.

Kuhn, A., Voit, R., Stefanovsky, V., Evers, R., Bianchi, M. and Grummt, I. (1994) Functional differences between the two splice variants of the nucleolar transcription factor UBF: the second HMG box determines specificity of DNA binding and transcriptional activity. *Embo J*, **13**, 416-424.

Kukimoto, I., Elderkin, S., Grimaldi, M., Oelgeschlager, T. and Varga-Weisz, P.D. (2004) The histone-fold protein complex CHRAC-15/17 enhances nucleosome sliding and assembly mediated by ACF. *Mol Cell*, **13**, 265-277.

Kulkens, T., van, d.S.C., Dekker, A.F., van, H.H. and Planta, R.J. (1992) A system to study transcription by yeast RNA polymerase I within the chromosomal context: functional analysis of the ribosomal DNA enhancer and the RBP1/REB1 binding sites. *Embo J*, **11**, 4665-4674.

Kurdistani, S.K. and Grunstein, M. (2003) Histone acetylation and deacetylation in yeast. *Nat Rev Mol Cell Biol*, **4**, 276-284.

Kurdistani, S.K., Tavazoie, S. and Grunstein, M. (2004) Mapping global histone acetylation patterns to gene expression. *Cell*, **117**, 721-733.

Kwon, H., Imbalzano, A.N., Khavari, P.A., Kingston, R.E. and Green, M.R. (1994) Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex. *Nature*, **370**, 477-481.

Lachner, M. and Jenuwein, T. (2002) The many faces of histone lysine methylation. *Curr Opin Cell Biol*, **14**, 286-298.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, **410**, 116-120.

Lachner, M., O'Sullivan, R.J. and Jenuwein, T. (2003) An epigenetic road map for histone lysine methylation. *J Cell Sci*, **116**, 2117-2124.

Längst, G. and Becker, P.B. (2001a) ISWI induces nucleosome sliding on nicked DNA. *Mol Cell*, **8**, 1085-1092.
Längst, G. and Becker, P.B. (2001b) Nucleosome mobilization and positioning by ISWI-containing chromatin-remodeling factors. *J Cell Sci*, **114**, 2561-2568.
Längst, G. and Becker, P.B. (2004) Nucleosome remodeling: one mechanism, many phenomena? *Biochim Biophys Acta*, **1677**, 58-63.
Längst, G., Becker, P.B. and Grummt, I. (1998) TTF-I determines the chromatin architecture of the active rDNA promoter. *Embo J*, **17**, 3135-3145.
Längst, G., Blank, T.A., Becker, P.B. and Grummt, I. (1997a) 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.
Längst, G., Schatz, T., Langowski, J. and Grummt, I. (1997b) Structural analysis of mouse rDNA: coincidence between nuclease hypersensitive sites, DNA curvature and regulatory elements in the intergenic spacer. *Nucleic Acids Res*, **25**, 511-517.
Laurent, B.C., Treich, I. and Carlson, M. (1993) The yeast SNF2/SWI2 protein has DNA-stimulated ATPase activity required for transcriptional activation. *Genes Dev*, **7**, 583-591.
Lawrence, R.J., Earley, K., Pontes, O., Silva, M., Chen, Z.J., Neves, N., Viegas, W. and Pikaard, C.S. (2004) A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. *Mol Cell*, **13**, 599-609.
Lazdins, I.B., Delannoy, M. and Sollner-Webb, B. (1997) Analysis of nucleolar transcription and processing domains and pre-rRNA movements by in situ hybridization. *Chromosoma*, **105**, 481-495.
Lazzaro, M.A. and Picketts, D.J. (2001) Cloning and characterization of the murine Imitation Switch (ISWI) genes: differential expression patterns suggest distinct developmental roles for Snf2h and Snf2l. *J Neurochem*, **77**, 1145-1156.
Leary, D.J. and Huang, S. (2001) Regulation of ribosome biogenesis within the nucleolus. *FEBS Lett*, **509**, 145-150.
LeRoy, G., Loyola, A., Lane, W.S. and Reinberg, D. (2000) Purification and characterization of a human factor that assembles and remodels chromatin. *J Biol Chem*, **275**, 14787-14790.
LeRoy, G., Orphanides, G., Lane, W.S. and Reinberg, D. (1998) Requirement of RSF and FACT for transcription of chromatin templates in vitro [see comments]. *Science*, **282**, 1900-1904.
Leung, A.K. and Lamond, A.I. (2003) The dynamics of the nucleolus. *Crit Rev Eukaryot Gene Expr*, **13**, 39-54.
Lorch, Y., Zhang, M. and Kornberg, R.D. (1999) Histone octamer transfer by a chromatin-remodeling complex. *Cell*, **96**, 389-392.
Loyola, A., Huang, J.Y., LeRoy, G., Hu, S., Wang, Y.H., Donnelly, R.J., Lane, W.S., Lee, S.C. and Reinberg, D. (2003) Functional analysis of the subunits of the chromatin assembly factor RSF. *Mol Cell Biol*, **23**, 6759-6768.
Lucchini, R. and Sogo, J.M. (1995) Replication of transcriptionally active chromatin. *Nature*, **374**, 276-280.
Lucchini, R., Wellinger, R.E. and Sogo, J.M. (2001) Nucleosome positioning at the replication fork. *Embo J*, **20**, 7294-7302.
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.
Lusser, A. and Kadonaga, J.T. (2003) Chromatin remodeling by ATP-dependent molecular machines. *Bioessays*, **25**, 1192-1200.
Macleod, D. and Bird, A. (1983) Transcription in oocytes of highly methylated rDNA from Xenopus laevis sperm. *Nature*, **306**, 200-203.
Martens, J.A. and Winston, F. (2002) Evidence that Swi/Snf directly represses transcription in S. cerevisiae. *Genes Dev*, **16**, 2231-2236.
Martens, J.A. and Winston, F. (2003) Recent advances in understanding chromatin remodeling by Swi/Snf complexes. *Curr Opin Genet Dev*, **13**, 136-142.
Martinez-Balbas, M.A., Tsukiyama, T., Gdula, D. and Wu, C. (1998) Drosophila NURF-55, a WD repeat protein involved in histone metabolism. *Proc Natl Acad Sci U S A*, **95**, 132-137.
McConnell, A.D., Gelbart, M.E. and Tsukiyama, T. (2004) Histone fold protein Dls1p is required for Isw2-dependent chromatin remodeling in vivo. *Mol Cell Biol*, **24**, 2605-2613.
McStay, B. and Reeder, R.H. (1990) An RNA polymerase I termination site can stimulate the adjacent ribosomal gene promoter by two distinct mechanisms in Xenopus laevis. *Genes Dev*, **4**, 1240-1251.
Mellor, J. and Morillon, A. (2004) ISWI complexes in Saccharomyces cerevisiae. *Biochim Biophys Acta*, **1677**, 100-112.
Milkereit, P. and Tschochner, H. (1998) A specialized form of RNA polymerase I, essential for initiation and growth-dependent regulation of rRNA synthesis, is disrupted during transcription. *Embo J*, **17**, 3692-3703.

Miller, G., Panov, K.I., Friedrich, J.K., Trinkle-Mulcahy, L., Lamond, A.I. and Zomerdijk, J.C. (2001) hRRN3 is essential in the SL1-mediated recruitment of RNA Polymerase I to rRNA gene promoters. *Embo J*, **20**, 1373-1382.

Miller, O.L., Jr. and Beatty, B.R. (1969) Visualization of nucleolar genes. *Science*, **164**, 955-957.

Min, J., Zhang, Y. and Xu, R.M. (2003) Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. *Genes Dev*, **17**, 1823-1828.

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.

Mizuguchi, G., Tsukiyama, T., Wisniewski, J. and Wu, C. (1997) Role of Nucleosome Remodeling Factor NURF in Transcriptional Activation of Chromatin. *Cell*, **1**, 141-150.

Mohrmann, L., Langenberg, K., Krijgsveld, J., Kal, A.J., Heck, A.J. and Verrijzer, C.P. (2004) Differential targeting of two distinct SWI/SNF-related Drosophila chromatin-remodeling complexes. *Mol Cell Biol*, **24**, 3077-3088.

Morrow, B.E., Johnson, S.P. and Warner, J.R. (1989) Proteins that bind to the yeast rDNA enhancer. *J Biol Chem*, **264**, 9061-9068.

Mosgoeller, W., Schofer, C., Steiner, M., Sylvester, J.E. and Hozak, P. (2001) Arrangement of ribosomal genes in nucleolar domains revealed by detection of "Christmas tree" components. *Histochem Cell Biol*, **116**, 495-505.

Moss, T. (2004) At the crossroads of growth control; making ribosomal RNA. *Curr Opin Genet Dev*, **14**, 210-217.

Moss, T. and Stefanovsky, V.Y. (2002) At the center of eukaryotic life. *Cell*, **109**, 545-548.

Muchardt, C. and Yaniv, M. (1993) A human homologue of Saccharomyces cerevisiae SNF2/SWI2 and Drosophila brm genes potentiates transcriptional activation by the glucocorticoid receptor. *Embo J*, **12**, 4279-4290.

Murawsky, C.M., Brehm, A., Badenhorst, P., Lowe, N., Becker, P.B. and Travers, A.A. (2001) Tramtrack69 interacts with the dMi-2 subunit of the Drosophila NuRD chromatin remodelling complex. *EMBO Rep*, **2**, 1089-1094.

Murray, K. (1964) The Occurrence of Epsilon-N-Methyl Lysine in Histones. *Biochemistry*, **127**, 10-15.

Muth, V., Nadaud, S., Grummt, I. and Voit, R. (2001) Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. *Embo J*, **20**, 1353-1362.

Mutskov, V.J., Russanova, V.R., Dimitrov, S.I. and Pashev, I.G. (1996) Histones associated with non-nucleosomal rat ribosomal genes are acetylated while those bound to nucleosome-organized gene copies are not. *J Biol Chem*, **271**, 11852-11857.

Nakagawa, T., Bulger, M., Muramatsu, M. and Ito, T. (2001) Multistep chromatin assembly on supercoiled plasmid DNA by nucleosome assembly protein-1 and ATP-utilizing chromatin assembly and remodeling factor. *J Biol Chem*, **276**, 27384-27391.

Nan, X., Meehan, R.R. and Bird, A. (1993) Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Res*, **21**, 4886-4892.

Narlikar, G.J., Phelan, M.L. and Kingston, R.E. (2001) Generation and interconversion of multiple distinct nucleosomal states as a mechanism for catalyzing chromatin fluidity. *Mol Cell*, **8**, 1219-1230.

Németh, A. (2002) Functional analysis of the N-terminal domain of mTTF-I and its interacting partner Tip5. Semmelweis University, Budapest.

Németh, A., Strohner, R., Grummt, I. and Längst, G. (2004) The chromatin remodeling complex NoRC and TTF-I cooperate in the regulation of the mammalian rRNA genes in vivo. *Nucleic Acids Res*, **32**, 4091-4099.

Ng, H.H. and Bird, A. (1999) DNA methylation and chromatin modification. *Curr Opin Genet Dev*, **9**, 158-163.

Ng, H.H., Ciccone, D.N., Morshead, K.B., Oettinger, M.A. and Struhl, K. (2003) Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. *Proc Natl Acad Sci U S A*, **100**, 1820-1825.

Ng, H.H., Robert, F., Young, R.A. and Struhl, K. (2002) Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. *Genes Dev*, **16**, 806-819.

O'Sullivan, A.C., Sullivan, G.J. and McStay, B. (2002) UBF binding in vivo is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat. *Mol Cell Biol*, **22**, 657-668.

Okuwaki, M. and Verreault, A. (2004) Maintenance DNA methylation of nucleosome core particles. *J Biol Chem*, **279**, 2904-2912.

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

Olins, D.E. and Olins, A.L. (2003) Chromatin history: our view from the bridge. *Nat Rev Mol Cell Biol*, **4**, 809-814.

Olson, M.O., Dundr, M. and Szebeni, A. (2000) The nucleolus: an old factory with unexpected capabilities. *Trends Cell Biol*, **10**, 189-196.

Osheim, Y.N., Mougey, E.B., Windle, J., Anderson, M., O'Reilly, M., Miller, O.L., Jr., Beyer, A. and Sollner-Webb, B. (1996) Metazoan rDNA enhancer acts by making more genes transcriptionally active. *J Cell Biol*, **133**, 943-954.

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.

Owen, D.J., Ornaghi, P., Yang, J.C., Lowe, N., Evans, P.R., Ballario, P., Neuhaus, D., Filetici, P. and Travers, A.A. (2000) The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. *Embo J*, **19**, 6141-6149.

Palmer, D.K., O'Day, K., Trong, H.L., Charbonneau, H. and Margolis, R.L. (1991) Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc Natl Acad Sci U S A*, **88**, 3734-3738.

Papoulas, O., Beek, S.J., Moseley, S.L., McCallum, C.M., Sarte, M., Shearn, A. and Tamkun, J.W. (1998) The Drosophila trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. *Development*, **125**, 3955-3966.

Paro, R. and Hogness, D.S. (1991) The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of Drosophila. *Proc Natl Acad Sci U S A*, **88**, 263-267.

Paule, M.R. (ed.). (1998) *Transcription of Ribosomal RNA Genes by Eukaryotic RNA Polymerase I*. Springer Verlag and R.G. Landes Company, Austin, Texas USA.

Paule, M.R. and White, R.J. (2000) Survey and summary: transcription by RNA polymerases I and III. *Nucleic Acids Res*, **28**, 1283-1298.

Pazin, M.J., Sheridan, P.L., Cannon, K., Cao, Z., Keck, J.G., Kadonaga, J.T. and Jones, K.A. (1996) NF-kappa B-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro. *Genes Dev*, **10**, 37-49.

Pederson, T. (1998) The plurifunctional nucleolus. *Nucleic Acids Res*, **26**, 3871-3876.

Pederson, T. (2004) The spatial organization of the genome in mammalian cells. *Curr Opin Genet Dev*, **14**, 203-209.

Pelletier, G., Stefanovsky, V.Y., Faubladier, M., Hirschler-Laszkiewicz, I.I., Savard, J., Rothblum, L.I., Cote, J. and Moss, T. (2000) Competitive recruitment of CBP and Rb-HDAC regulates UBF acetylation and ribosomal transcription. *Mol Cell*, **6**, 1059-1066.

Perry, C.A. and Annunziato, A.T. (1991) Histone acetylation reduces H1-mediated nucleosome interactions during chromatin assembly. *Exp Cell Res*, **196**, 337-345.

Peters, A.H., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J.H. and Jenuwein, T. (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell*, **12**, 1577-1589.

Peterson, C.L. (2000) ATP-dependent chromatin remodeling: going mobile. *FEBS Lett*, **476**, 68-72.

Peterson, C.L. (2002) HDAC's at work: everyone doing their part. *Mol Cell*, **9**, 921-922.

Peterson, C.L., Dingwall, A. and Scott, M.P. (1994) Five SWI/SNF gene products are components of a large multisubunit complex required for transcriptional enhancement [see comments]. *Proc Natl Acad Sci U S A*, **91**, 2905-2908.

Phelan, M.L., Schnitzler, G.R. and Kingston, R.E. (2000) Octamer transfer and creation of stably remodeled nucleosomes by human SWI-SNF and its isolated ATPases. *Mol Cell Biol*, **20**, 6380-6389.

Phillips, D.M. (1963) The presence of acetyl groups of histones. *Biochem J*, **87**, 258-263.

Pikaard, C.S. (2000) Nucleolar dominance: uniparental gene silencing on a multi-megabase scale in genetic hybrids. *Plant Mol Biol*, **43**, 163-177.

Poot, R.A., Dellaire, G., Hulsmann, B.B., Grimaldi, M.A., Corona, D.F., Becker, P.B., Bickmore, W.A. and Varga-Weisz, P.D. (2000) HuCHRAC, a human ISWI chromatin remodelling complex contains hACF1 and two novel histone-fold proteins. *Embo J*, **19**, 3377-3387.

Prior, C.P., Cantor, C.R., Johnson, E.M., Littau, V.C. and Allfrey, V.G. (1983) Reversible changes in nucleosome structure and histone H3 accessibility in transcriptionally active and inactive states of rDNA chromatin. *Cell*, **34**, 1033-1042.

Putter, V. and Grummt, F. (2002) Transcription termination factor TTF-I exhibits contrahelicase activity during DNA replication. *EMBO Rep*, **3**, 147-152.

Ragvin, A., Valvatne, H., Erdal, S., Arskog, V., Tufteland, K.R., Breen, K., AM, O.Y., Eberharter, A., Gibson, T.J., Becker, P.B. and Aasland, R. (2004) Nucleosome binding by the bromodomain and PHD finger of the transcriptional cofactor p300. *J Mol Biol*, **337**, 773-788.

Rammstein. (1995) Herzeleid. Motor Music GMBH, Hamburg.

Rammstein. (2001) Mutter. Motor Music GMBH, Hamburg.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D. and Jenuwein, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature*, **406**, 593-599.

Reuter, G. and Spierer, P. (1992) Position effect variegation and chromatin proteins. *Bioessays*, **14**, 605-612.

Rhodes, D. and Laskey, R.A. (1989) Assembly of nucleosomes and chromain *in vitro*. *Methods Enzymol*, **170**, 575-585.

Richmond, E. and Peterson, C.L. (1996) Functional analysis of the DNA-stimulated ATPase domain of yeast SWI2/SNF2. *Nucleic Acids Res*, **24**, 3685-3692.

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

Robyr, D., Suka, Y., Xenarios, I., Kurdistani, S.K., Wang, A., Suka, N. and Grunstein, M. (2002) Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell*, **109**, 437-446.

Saez-Vasquez, J. and Pikaard, C.S. (1997) Extensive purification of a putative RNA polymerase I holoenzyme from plants that accurately initiates rRNA gene transcription in vitro. *Proc Natl Acad Sci U S A*, **94**, 11869-11874.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning : a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sambrook, J. and Rusell, J. (2001) *Molecular cloning : a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sander, E.E. and Grummt, I. (1997) Oligomerization of the transcription termination factor TTF-I: implications for the structural organization of ribosomal transcription units. *Nucleic Acids Res*, **25**, 1142-1147.

Sander, E.E., Mason, S.W., Munz, C. and Grummt, I. (1996) The amino-terminal domain of the transcription termination factor TTF-I causes protein oligomerization and inhibition of DNA binding. *Nucleic Acids Res*, **24**, 3677-3684.

Sandmeier, J.J., French, S., Osheim, Y., Cheung, W.L., Gallo, C.M., Beyer, A.L. and Smith, J.S. (2002) RPD3 is required for the inactivation of yeast ribosomal DNA genes in stationary phase. *Embo J*, **21**, 4959-4968.

Santisteban, M.S., Kalashnikova, T. and Smith, M.M. (2000) Histone H2A.Z regulats transcription and is partially redundant with nucleosome remodeling complexes [In Process Citation]. *Cell*, **103**, 411-422.

Santoro, R. and Grummt, I. (2001) Molecular mechanisms mediating methylation-dependent silencing of ribosomal gene transcription. *Mol Cell*, **8**, 719-725.

Santoro, R., Li, J. and Grummt, I. (2002) The nucleolar remodeling complex NoRC mediates heterochromatin formation and silencing of ribosomal gene transcription. *Nat Genet*, **32**, 393-396.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J. and Kouzarides, T. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature*, **419**, 407-411.

Schatz, T. and Langowski, J. (1997) Curvature and sequence analysis of eukaryotic promoters. *J Biomol Struct Dyn*, **15**, 265-275.

Scheer, U. and Hock, R. (1999) Structure and function of the nucleolus. *Curr Opin Cell Biol*, **11**, 385-390.

Scherl, A., Coute, Y., Deon, C., Calle, A., Kindbeiter, K., Sanchez, J.C., Greco, A., Hochstrasser, D. and Diaz, J.J. (2002) Functional proteomic analysis of human nucleolus. *Mol Biol Cell*, **13**, 4100-4109.

Schnapp, A. and Grummt, I. (1991) Transcription complex formation at the mouse rDNA promoter involves the stepwise association of four transcription factors and RNA polymerase I. *J Biol Chem*, **266**, 24588-24595.

Schnapp, A. and Grummt, I. (1996) Purification, assay, and properties of RNA polymerase I and class I-specific transcription factors in mouse. *Methods Enzymol*, **273**, 233-248.

Schnapp, A., Pfleiderer, C., Rosenbauer, H. and Grummt, I. (1990) A growth-dependent transcription initiation factor (TIF-IA) interacting with RNA polymerase I regulates mouse ribosomal RNA synthesis. *Embo J*, **9**, 2857-2863.

Schnapp, G., Schnapp, A., Rosenbauer, H. and Grummt, I. (1994) TIF-IC, a factor involved in both transcription initiation and elongation of RNA polymerase I. *Embo J*, **13**, 4028-4035.

Schotta, G., Ebert, A., Dorn, R. and Reuter, G. (2003) Position-effect variegation and the genetic dissection of chromatin regulation in Drosophila. *Semin Cell Dev Biol*, **14**, 67-75.

Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D. and Jenuwein, T. (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev*, **18**, 1251-1262.

Schreiber, S.L. and Bernstein, B.E. (2002) Signaling network model of chromatin. *Cell*, **111**, 771-778.

Schroth, G.P., Siino, J.S., Cooney, C.A., Th'ng, J.P., Ho, P.S. and Bradbury, E.M. (1992) Intrinsically bent DNA flanks both sides of an RNA polymerase I transcription start site. Both regions display novel electrophoretic mobility. *J Biol Chem*, **267**, 9958-9964.

Schultz, D.C., Friedman, J.R. and Rauscher, F.J., 3rd. (2001) Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev*, **15**, 428-443.

Schwanbeck, R., Xiao, H. and Wu, C. (2004) Spatial contacts and nucleosome step movements induced by the NURF chromatin remodeling complex. *J Biol Chem*.

Seither, P., Iben, S. and Grummt, I. (1998) Mammalian RNA polymerase I exists as a holoenzyme with associated basal transcription factors. *J Mol Biol*, **275**, 43-53.

Shen, X., Mizuguchi, G., Hamiche, A. and Wu, C. (2000) A chromatin remodelling complex involved in transcription and DNA processing. *Nature*, **406**, 541-544.

Sif, S., Saurin, A.J., Imbalzano, A.N. and Kingston, R.E. (2001) Purification and characterization of mSin3A-containing Brg1 and hBrm chromatin remodeling complexes. *Genes Dev*, **15**, 603-618.

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.

Sirri, V., Roussel, P. and Hernandez-Verdun, D. (1999) The mitotically phosphorylated form of the transcription termination factor TTF-1 is associated with the repressed rDNA transcription machinery. *J Cell Sci*, **112**, 3259-3268.

Smid, A., Finsterer, M. and Grummt, I. (1992) Limited proteolysis unmasks specific DNA-binding of the murine RNA polymerase I-specific transcription termination factor TTFI. *J Mol Biol*, **227**, 635-647.

Sogo, J.M., Ness, P.J., Widmer, R.M., Parish, R.W. and Koller, T. (1984) Psoralen-crosslinking of DNA as a probe for the structure of active nucleolar chromatin. *J Mol Biol*, **178**, 897-919.

Stancheva, I., Lucchini, R., Koller, T. and Sogo, J.M. (1997) Chromatin structure and methylation of rat rRNA genes studied by formaldehyde fixation and psoralen cross-linking. *Nucleic Acids Res*, **25**, 1727-1735.

Stein, A., Whitlock, J.P., Jr. and Bina, M. (1979) Acidic polypeptides can assemble both histones and chromatin in vitro at physiological ionic strength. *Proc Natl Acad Sci U S A*, **76**, 5000-5004.

Stokes, D.G., Tartof, K.D. and Perry, R.P. (1996) CHD1 is concentrated in interbands and puffed regions of Drosophila polytene chromosomes. *Proc Natl Acad Sci U S A*, **93**, 7137-7142.

Stopka, T. and Skoultchi, A.I. (2003) The ISWI ATPase Snf2h is required for early mouse development. *Proc Natl Acad Sci U S A*, **100**, 14097-14102.

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature*, **403**, 41-45.

Strohner, R. (2000) Tip5, a novel TTF-I interacting protein: cloning, biochemical purification and functional analysis. *DKFZ- Molecularbiology of the cell II*. Ruprecht-Karls-University, Heidelberg.

Strohner, R., Németh, A., Jansa, P., Hofmann-Rohrer, U., Santoro, R., Längst, G. and Grummt, I. (2001) NoRC--a novel member of mammalian ISWI-containing chromatin remodeling machines. *Embo J*, **20**, 4892-4900.

Strohner, R., Németh, A., Nightingale, K.P., Grummt, I., Becker, P.B. and Längst, G. (2004) Recruitment of the nucleolar remodeling complex NoRC establishes ribosomal DNA silencing in chromatin. *Mol Cell Biol*, **24**, 1791-1798.

Sudarsanam, P. and Winston, F. (2000) The Swi/Snf family nucleosome-remodeling complexes and transcriptional control. *Trends Genet*, **16**, 345-351.

Sun, Z.W. and Allis, C.D. (2002) Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature*, **418**, 104-108.

Tagami, H., Ray-Gallet, D., Almouzni, G. and Nakatani, Y. (2004) Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell*, **116**, 51-61.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992) brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell*, **68**, 561-572.

Tate, P., Lee, M., Tweedie, S., Skarnes, W.C. and Bickmore, W.A. (1998) Capturing novel mouse genes encoding chromosomal and other nuclear proteins. *J Cell Sci*, **111 (Pt 17)**, 2575-2585.

Taunton, J., Hassig, C.A. and Schreiber, S.L. (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p [see comments]. *Science*, **272**, 408-411.

Tong, J.K., Hassig, C.A., Schnitzler, G.R., Kingston, R.E. and Schreiber, S.L. (1998) Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. *Nature*, **395**, 917-921.

Trachtulcova, P., Frydlova, I., Janatova, I. and Hasek, J. (2004) The absence of the Isw2p-Itc1p chromatin-remodelling complex induces mating type-specific and Flo11p-independent invasive growth of Saccharomyces cerevisiae. *Yeast*, **21**, 389-401.

Tran, H.G., Steger, D.J., Iyer, V.R. and Johnson, A.D. (2000) The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. *Embo J*, **19**, 2323-2331.

Tsukiyama, T. (2002) The in vivo functions of ATP-dependent chromatin-remodelling factors. *Nat Rev Mol Cell Biol*, **3**, 422-429.

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.

Tsukiyama, T., Daniel, C., Tamkun, J. and Wu, C. (1995) ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. *Cell*, **83**, 1021-1026.

Tsukiyama, T., Palmer, J., Landel, C.C., Shiloach, J. and Wu, C. (1999) Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in Saccharomyces cerevisiae. *Genes Dev*, **13**, 686-697.

Tsukiyama, T. and Wu, C. (1995) Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell*, **83**, 1011-1020.

Turner, B.M. (2002) Cellular memory and the histone code. *Cell*, **111**, 285-291.

Turner, B.M., Birley, A.J. and Lavender, J. (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. *Cell*, **69**, 375-384.

Vaquero, A., Loyola, A. and Reinberg, D. (2003) The constantly changing face of chromatin. *Sci Aging Knowledge Environ*, **2003**, RE4.

Varga-Weisz, P.D., Wilm, M., Bonte, E., Dumas, K., Mann, M. and Becker, P.B. (1997) Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II. *Nature*, **388**, 598-602.

Vary, J.C., Jr., Gangaraju, V.K., Qin, J., Landel, C.C., Kooperberg, C., Bartholomew, B. and Tsukiyama, T. (2003) Yeast Isw1p forms two separable complexes in vivo. *Mol Cell Biol*, **23**, 80-91.

Vavra, K.J., Colavito-Shepanski, M. and Gorovsky, M.A. (1982) Histone acetylation and the deoxyribonuclease I sensitivity of the Tetrahymena ribosomal gene. *Biochemistry*, **21**, 1772-1781.

Vermaak, D., Ahmad, K. and Henikoff, S. (2003) Maintenance of chromatin states: an open-and-shut case. *Curr Opin Cell Biol*, **15**, 266-274.

Vignali, M., Hassan, A.H., Neely, K.E. and Workman, J.L. (2000) ATP-dependent chromatin-remodeling complexes. *Mol Cell Biol*, **20**, 1899-1910.

Voit, R., Kuhn, A., Sander, E.E. and Grummt, I. (1995) Activation of mammalian ribosomal gene transcription requires phosphorylation of the nucleolar transcription factor UBF. *Nucleic Acids Res*, **23**, 2593-2599.

Wade, P.A. (2001) Methyl CpG-binding proteins and transcriptional repression. *Bioessays*, **23**, 1131-1137.

Wade, P.A., Jones, P.L., Vermaak, D. and Wolffe, A.P. (1998) A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. *Curr Biol*, **8**, 843-846.

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, A., Kurdistani, S.K. and Grunstein, M. (2002) Requirement of Hos2 histone deacetylase for gene activity in yeast. *Science*, **298**, 1412-1414.

Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P.A., Biggar, S.R., Muchardt, C., Kalpana, G.V., Goff, S.P., Yaniv, M., Workman, J.L. and Crabtree, G.R. (1996a) Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *Embo J*, **15**, 5370-5382.

Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B.R. and Crabtree, G.R. (1996b) Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev*, **10**, 2117-2130.

Whitehouse, I., Flaus, A., Cairns, B.R., White, M.F., Workman, J.L. and Owen-Hughes, T. (1999) Nucleosome mobilization catalysed by the yeast SWI/SNF complex. *Nature*, **400**, 784-787.

Whitehouse, I., Stockdale, C., Flaus, A., Szczelkun, M.D. and Owen-Hughes, T. (2003) Evidence for DNA translocation by the ISWI chromatin-remodeling enzyme. *Mol Cell Biol*, **23**, 1935-1945.

Winston, F. and Carlson, M. (1992) Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. *Trends Genet*, **8**, 387-391.

Wolffe, A.P. (1998) *Chromatin structure & function.* Academic Press, London.

Woodage, T., Basrai, M.A., Baxevanis, A.D., Hieter, P. and Collins, F.S. (1997) Characterization of the CHD family of proteins. *Proc Natl Acad Sci U S A*, **94**, 11472-11477.

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

Woodcock, D.M., Simmons, D.L., Crowther, P.J., Cooper, I.A., Trainor, K.J. and Morley, A.A. (1986) Delayed DNA methylation is an integral feature of DNA replication in mammalian cells. *Exp Cell Res*, **166**, 103-112.

Xiao, H., Sandaltzopoulos, R., Wang, H.M., Hamiche, A., Ranallo, R., Lee, K.M., Fu, D. and Wu, C. (2001) Dual functions of largest NURF subunit NURF301 in nucleosome sliding and transcription factor interactions. *Mol Cell*, **8**, 531-543.

Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Cote, J. and Wang, W. (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell*, **2**, 851-861.

Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P. and Kohwi-Shigematsu, T. (2002) SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature*, **419**, 641-645.

Yuan, X., Zhao, J., Zentgraf, H., Hoffmann-Rohrer, U. and Grummt, I. (2002) Multiple interactions between RNA polymerase I, TIF-IA and TAF(I) subunits regulate preinitiation complex assembly at the ribosomal gene promoter. *EMBO Rep*, **3**, 1082-1087.

Zhang, Y., LeRoy, G., Seelig, H.P., Lane, W.S. and Reinberg, D. (1998) The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell*, **95**, 279-289.

Zheng, C. and Hayes, J.J. (2003) Structures and interactions of the core histone tail domains. *Biopolymers*, **68**, 539-546.

Zhou, Y., Santoro, R. and Grummt, I. (2002) The chromatin remodeling complex NoRC targets HDAC1 to the ribosomal gene promoter and represses RNA polymerase I transcription. *Embo J*, **21**, 4632-4640.