DNA sequence encoded repression of rRNA gene transcription in chromatin

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ABSTRACT

Eukaryotic genomes are packaged into nucleosomes that occlude DNA from interacting with most DNA-binding proteins. Nucleosome positioning and chromatin organization is critical for gene regulation. We have investigated the mechanism by which nucleosomes are positioned at the promoters of active and silent rRNA genes (rDNA). The reconstitution of nucleosomes on rDNA results in sequence-dependent nucleosome positioning at the rDNA promoter that mimics the chromatin structure of silent rRNA genes *in vivo*, suggesting that active mechanisms are required to reorganize chromatin structure upon gene activation. Nucleosomes are excluded from positions observed at active rRNA genes, resulting in transcriptional repression on chromatin. We suggest that the repressed state is the default chromatin organization of the rDNA and gene activation requires ATP-dependent chromatin remodelling activities that move the promoter-bound nucleosome about 22-bp upstream. We suggest that nucleosome remodelling precedes promoter-dependent transcriptional activation as specific inhibition of ATP-dependent chromatin remodelling suppresses the initiation of RNA Polymerase I transcription *in vitro*. Once initiated, RNA Polymerase I is capable of elongating through reconstituted chromatin without apparent displacement of the nucleosomes. The results reveal the functional cooperation of DNA sequence and chromatin remodelling complexes in nucleosome positioning and in establishing the epigenetic active or silent state of rRNA genes.

INTRODUCTION

The genes that encode rRNA synthesis are tandemly repeated and the number of rRNA gene repeats varies from <100 to >10 000 between organisms (1). The high number of genes does only in part reflect the cellular demand for rRNAs, as only a fraction of these repeats is used for rRNA synthesis at any given time. In metabolically active human or mouse cells, approximately half of the ~400 rRNA gene copies are transcriptionally active and the other half is silent. Previous studies have established that the distinct epigenetic states can be distinguished by specific histone modifications, DNA methylation and nucleosome positions (2,3). On active genes, the promoter maintains a nucleosome positioned at −157 to −2 relative to the transcription start site, whereas on silent genes, the promoter-bound nucleosome is shifted 22-bp further downstream (3). Nucleosome positions correlate with gene activity, but it is not known whether nucleosome positions are a consequence or a prerequisite of gene activation or repression and which mechanisms determine nucleosome positioning.

Nucleosome cores, the basic packaging units of chromatin, contain a 147-bp stretch of DNA that is sharply bent and tightly wrapped around a histone octamer (4). Nucleosomal organization is generally repressive as it generally inhibits the access of specific DNA-binding factors. Eukaryotic genomes are packaged into regularly spaced arrays of nucleosomes; however, the spacing between nucleosomes varies among species and cell types (5). Despite this regularity, genome-wide studies reveal that regulatory regions exhibit common nucleosomal positioning patterns (6–8). Moreover, transcription factor-binding sites are enriched about 7-fold in the linker regions of nucleosomes (9). Therefore, it seems that gene activation could be more sensitive to signalling pathways than it would be if the binding sites were sequestered in positioned nucleosomes.

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However, this simplified view of gene regulation in chromatin cannot account for all activator binding. Studies have shown that factor binding and gene activation depends on posttranslational modifications of histones and activities that change chromatin structures, suggesting that nucleosome positioning is important for gene regulation *in vivo* (10,11).

Nucleosome positioning refers to two fundamental relationships between the histone octamer and the DNA wrapped around it. Rotational positioning defines the relative orientation of the DNA helix on the histone octamer surface with a 10-bp helical periodicity. The translational position of a nucleosome refers to the specific 147-bp sequence covered by the histone octamer. Recent genome-wide studies have shown that promoter nucleosomes frequently adopt specific positions *in vivo* (12). It was argued that DNA sequence has a major role in establishing these nucleosome positions, suggesting that evolution has selected for specific, default arrangements of promoter nucleosomes (13). This view was contradicted by Struhl and colleagues, showing that nucleosome positioning results from statistical positioning from a barrier near the promoter following transcription initiation events (14). Finally, recent studies indicate that ATP-dependent chromatin remodelling complexes move nucleosomes away from default positioning sequences and have the capability to position nucleosomes on DNA (15). For instance, yeast Isw2 mutants alter the positions of nucleosomes over Ssn6/Tup1 repressed genes (16) and yeast RSC (SWI/SNF family) mutants increase histone occupancy on RNA Polymerase III promoters and alter nucleosome positions on many RNA Polymerase II genes (17).

Chromatin remodelling complexes, which couple the energy of ATP hydrolysis with the movement and displacement of nucleosomes from DNA (11), are highly abundant proteins, with about one remodelling complex being present per 10 nucleosomes (18,19). These complexes have a molecular motor belonging to the Snf2-like ATPases including the Snf2, ISWI, Mi-2, Chd1, Ino80, ERCC6, ALC1, CHD7, Swr1, RAD54 and Lsh subfamilies (20). Each subfamily consists of at least one to six similar ATPases, many of which have been shown to remodel nucleosomes, transfer histone octamers in trans, and generate superhelical torsion in DNA as reviewed previously (11,21).

Packaging into chromatin results in transcriptional repression of the rRNA genes *in vitro*. The addition of the Transcription Termination Factor I (TTF-I) to the transcription extract results in TTF-I binding to the promoter proximal terminator (T_0) of the rRNA gene and transcriptional activation. TTF-I binding and transcriptional activation correlate with ATP-dependent nucleosome re-positioning, placing a nucleosome on the promoter (22,23). The chromatin remodelling machine CSB was shown to be recruited by TTF-I and to activate rRNA transcription *in vivo* (24). Gene activation required the ATPase activity of CSB and linked the H3K9 methyltransferase activity of G9a and HP1γ to the active rDNA loci. However, a recent study showed that TTF-I binds similarly well to active and inactive rRNA genes *in vivo* (25). TTF-I is also responsible for rDNA repression

due to its interaction with the chromatin remodelling complex NoRC and the positioning of a nucleosome between −133 and +22 relative to the transcription start site (3,26,27). Accordingly, TTF-I binding and nucleosome remodelling can either lead to gene activation or transcriptional repression. In this study, we addressed the default chromatin structure of the murine rRNA gene and the order and dynamic changes of rRNA genes during the process of gene activation. RNA Polymerase I can efficiently elongate through nucleosomes if transcription was initiated on a nucleosome-free promoter template indicating that the enzyme is capable of transcribing through a nucleosome without disrupting the core particle. In contrast, transcription initiation by RNA Polymerase I (Pol-I) is inhibited by the presence of nucleosomes. High resolution analysis of nucleosome occupancies and computer modelling of the chromatin structure revealed that murine rRNA genes reconstituted into chromatin lack nucleosomes positioned within the area of −153 to +2 corresponding to the nucleosome position observed at active genes. Therefore, sequence-dependent nucleosome positioning disfavours the formation of this nucleosome, and thus the formation of transcription initiation complexes on reconstituted chromatin cannot occur. Transcriptional activation is brought about by switching nucleosome positions, i.e. moving the promoter-bound nucleosome from the repressive to the active position. The inhibition of chromatin remodelling by phosphokanamycin (PK) prevents nucleosome repositioning, and hence represses Pol-I transcription on chromatin templates but not on free rDNA. We suggest that sequence-dependent nucleosome positioning determines the repressed state of the rRNA genes in chromatin, and that therefore nucleosome remodelling and nucleosome positioning precede gene activation.

MATERIALS AND METHODS

Proteins and extracts

The purification of histones, the expression and purification of remodelling complexes and TTF-I and the synthesis of PK is described in the Supplementary Data.

Chromatin reconstitution and analysis

Chromatin assembly and purification, nucleosome mapping and nucleosome remodelling reactions were performed as described in the Supplementary Data. For high resolution mapping of nucleosome positions, purified nucleosomal arrays (300 ng) were digested with 1.5 U of MNase (Sigma) for 40 s. Reactions were conducted in 10 mM Tris–HCl (pH 7.6), 80 mM KCl, 1.5 mM MgCl$_2$, 10% glycerol, 0.5 mM ATP and 200 ng/μl of bovine serum albumin. Reactions were stopped by the addition of 0.2 volumes of stop buffer [4% sodium dodecyl sulfate (SDS), 100 mM EDTA]. DNA was purified and analysed by a primer extension reaction (denaturation, 5 min at 95°C; annealing, 2 min at 56°C; extension, 1 min at 72°C) using radioactively labelled oligonucleotides that hybridized to the rDNA promoter. DNA fragments were

resolved on 8% sequencing gels and quantified with a PhosphorImager and Aida software.

High resolution mapping of nucleosome positions was performed with mononucleosomal DNA reconstituted on the murine rDNA fragment encompassing the DNA sequence from position –175 to +155 relative to the transcription start site. Reconstituted chromatin was purified and incubated with MNase as described above. DNA molecules corresponding to the protected nucleosomal DNA were gel-purified and cloned with the perfectly blunt end cloning kit (Novagen) into the Plasmid pT7Blue. A total of 75 individual clones were isolated and sequenced to reveal nucleosome positions.

ATPase assay

Phosphoenolpyruvate (PEP), pyruvate kinase (PK), lactate dehydrogenase (LDH), and NADH were purchased from Sigma. The regeneration system consisting of PEP and PK converts ADP back to ATP. The resulting pyruvate is subsequently converted to lactate by LDH, thereby oxidizing NADH. The decline of NADH absorbance at 340 nm was measured with a Tecan GeniosPro plate reader, revealing the rate of ATP hydrolysis in real time (28). A typical reaction contained 6 mM PEP, 150 U PK/LDH, 0.3 mM NADH, 3 mM ATP, 2 µg of DNA, the extract and the inhibitor in a volume of 100 µl.

In vitro transcription assays

Transcription was performed on short rDNA fragments spanning murine rDNA sequences from –170 to +155 and –170 to +317, and on a rDNA minigene (pMrWT-T) representing a fusion of mouse promoter and terminator sequences. pMrWT-T contains 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 (from +57 to +3643) containing the 10 transcription termination elements (T_1–T_{10}). The promoter and the terminator elements were separated by 686 bp (26). Chromatin was reconstituted with the salt assembly method and purified as described in the Supplementary Data. Transcription experiments were performed as described (29). Transcripts were purified and analysed on 4.5% polyacrylamide gels.

RESULTS AND DISCUSSION

The rDNA promoter is repressed by sequence-dependent nucleosome positioning

Ribosomal RNA genes in higher eukaryotes are amplified to fulfil cellular requirements for pre-rRNA synthesis. The actively transcribed genes are densely packaged with elongating RNA Polymerase I, suggesting a severe disturbance of the chromatin structure. But still, actively transcribed ribosomal genes in higher eukaryotes remain associated with histones, although they may not maintain an intact nucleosomal rDNA structure (30–32). Nevertheless, studies on rDNA gene regulation in the context of chromatin and RNA Polymerase I elongation

through nucleosomes are meaningful studies, since rDNA is fully nucleosomal after DNA replication. Sogo and colleagues did beautifully show that *Saccharomyces cerevisiae* rDNA is perfectly reconstituted into nucleosomes after the passage of the replication fork (33). They also showed that transcription activation correlates with the loss of a psoralen-inaccessible structure at the rRNA gene promoter. Consequently, these data suggest that at least the first round of transcription has to be initiated on a nucleosomal promoter, and the RNA Polymerase I has to elongate through an array of nucleosomes.

Packaging of rRNA genes into chromatin leads to transcriptional repression that can be relieved by the addition of the Transcription Termination Factor-I (TTF-I) (23). Since recent reports showed a dual role for TTF-I in gene activation and repression of rRNA genes that are correlated with two distinct nucleosome positions on the rRNA promoter (3), we decided to study the mechanism of chromatin dependent transcriptional repression, TTF-I dependent re-activation and the associated chromatin states in high resolution.

To examine the relationship between chromatin remodelling and gene activation, we assayed the ability of Pol-I to initiate specific transcription on reconstituted nucleosomal templates *in vitro*. To monitor the impact of sequence dependent nucleosome positioning on rRNA promoter activity, a mononucleosome was reconstituted on an end-labelled DNA fragment containing the murine rDNA promoter and 155 bp of transcribed sequence (Figure 1A). Nucleosomal DNA was hydrolysed with *Rsa*I to select for nucleosomes located within the rDNA promoter and purified via gel filtration chromatography. Nucleosome reconstitution resulted in the assembly of ∼30% nucleosome, as visualized by the levels of hydrolysed free DNA (Figure 1A, lane 3 and Figure 1B, lanes 5–7). As a control, the nucleosome assembly reaction was incubated with heat-inactivated *Rsa*I and purified in parallel. While free DNA and the nucleosome assembly reaction directed the synthesis of the 155-nt transcripts (Figure 1B, lanes 1–4), equivalent amounts of *Rsa*I-cleaved nucleosomal template did not promote transcription (lanes 5–7). Thus, nucleosomes covering the rDNA promoter impair specific transcription initiation.

Active genes are correlated with a nucleosome occupying a specific position over the promoter (dyad axis at position −77) and repressed genes are correlated with a repressive nucleosome having the dyad axis located about 22 bp more downstream at position –55 (3). We asked whether transcriptional repression *in vitro* is an effect of sequence dependent nucleosome positioning and how these positions correlate with the *in vivo* situation.

We mapped nucleosome positioning at a high resolution to gain insight into the molecular mechanism underlying transcriptional repression. Nucleosomal rDNA (from–175 to +155) was hydrolysed with micrococcal nuclease (MNase), and the protected nucleosomal DNA subsequently isolated, cloned and sequenced (Figure 1C). The position of the dyad axis of 75 independent clones, with a mean length of 147 bp (± 11 bp), was plotted against the rDNA sequence. The positions of the nucleosomes were not randomly distributed along the rDNA fragment, but
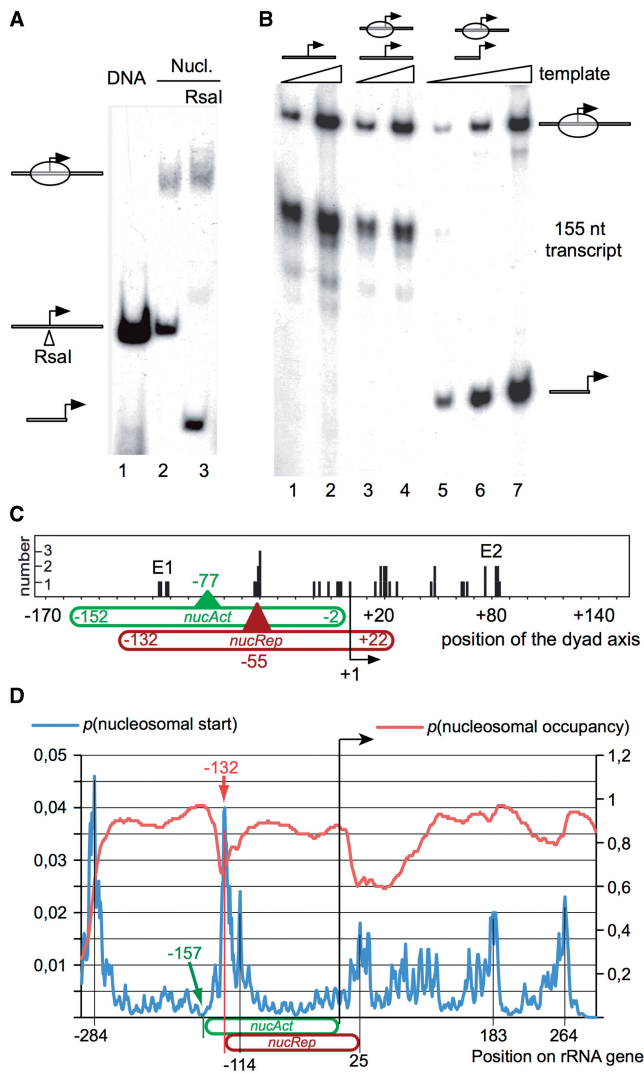
**Figure 1.** rRNA genes are switched off in chromatin. (**A**) Reconstitution of mononucleosomes on a 330 bp (–175 to +155) rDNA fragment. The end-labelled DNA was reconstituted into mononucleosomes by salt dialysis reconstitution (lanes 1 and 2) and analysed by native PAGE. Nucleosomal DNA molecules harbouring a nucleosome on the transcription start site were selected by digestion with the restriction enzyme *Rsa*I (lane 3). The positions of the nucleosomal DNA, the undigested and digested DNA fragment are indicated. (**B**) Transcription assay with free and nucleosomal rDNA fragments. Increasing amounts of free DNA (lanes 1 and 2), a mixture of free DNA and nucleosomal DNA (lanes 3 and 4) and *Rsa*I-selected nucleosomal DNA (lanes 5–7) were incubated with the transcription extract. The radioactive labelled transcripts were analysed by native PAGE. The nucleosomal templates used for the transcription reactions are shown above the gel. The positions of the undigested or nucleosomal rDNA fragment, the digested free DNA and the 155-nt-long transcript are indicated on the right. (**C**) Analysis of nucleosome positions on the rDNA promoter. Mononucleosomal templates (–175 to +155) were digested with MNase, and the protected nucleosomal DNA was gel-purified, cloned and sequenced. The graph shows the positions of the nucleosomal dyad axis. The positions of the *nucAct* and *nucRep* nucleosomes observed in vivo are indicated with the 5′, 3′ and dyad axis positions relative to the rRNA gene transcription start site. E1 and E2 indicate the dyad axis positions of nucleosomes located at the end of the DNA fragment. (**D**) Prediction of nucleosome positioning by the probability of nucleosome occupancy and the probability of encountering a nucleosomal start site. rRNA sequences from position –5000 to +5000 relative to the transcription start site were used for computational analysis at http://genie.weizmann.ac.il/pubs/nucleosomes06/ (13).

rather clustered within seven areas (Figure 1C). Two of these clusters (E1 and E2) result from DNA end effects, artificially aligning the nucleosomal positions relative to the ends of the linear DNA fragment. A significant number of nucleosomes were positioned between nt −130 to +25 (dyad axis at position −55), precisely matching the position of nucleosomes (*nucRep*) at silent rRNA genes *in vivo* (3). More important, we did not observe nucleosomes occupying the corresponding active position detected *in vivo* (*nucAct;* from −157 to −2 with the location of the dyad axis at about −77), suggesting that rDNA sequence disfavours nucleosome reconstitution on this site. The absence of nucleosomes covering the active position indicates that repressed rRNA genes represent the default state of rDNA. Sequence-dependent nucleosome positioning signals have been shown to be relatively weak and to function as combinatorial elements in conjunction with larger areas of neighbouring DNA sequences (13). Therefore, the multitude of nucleosomal positions in addition to the preferential nucleosome binding sites at DNA ends (E1 and E2; Figure 1C) are expected on short DNA fragments. In contrast, larger DNA elements that have the potential to phase nucleosomal arrays may have a more defined chromatin structure (Figures 1D and 6).

Whole-genome nucleosome mapping studies and biophysical analyses of histone–DNA interactions have revealed that DNA sequence plays an important role in determining nucleosome positions *in vitro* and *in vivo* (13,34). To assess the contribution of the underlying DNA sequence in guiding and phasing nucleosomes on DNA, we analysed nucleosome occupancy on the rDNA promoter with an algorithm implemented by Jon Widom and colleagues (13). This algorithm takes into account the fact that neighbouring high affinity nucleosome-binding sites could create boundaries for nucleosome positioning and alignment (Figure 1D). This analysis reveals that upstream rDNA sequences contain strong nucleosome positioning sites, including a high probability for a nucleosome start site at position –132 (Figure 1D, red arrow). Interestingly, this prediction perfectly matches the *nucRep* nucleosome (dyad axis at –55) that occupies silent rDNA repeats *in vivo* and was detected in our mapping study (Figure 1C). In addition, the algorithm predicts a very low probability for a nucleosome start site at position –157 (Figure 1D, green arrow), corresponding to *nucAct* (dyad axis at –77), which occupies active promoters *in vivo*. This result is also perfectly mirrored by our nucleosome mapping studies, showing a gap of nucleosome positions on the rRNA gene promoter at this site (Figure 1C). The absence of *nucAct* in cell-free nucleosome assembly reactions and computer predictions (Figure 1C and D) strongly suggest that the lack of nucleosomes covering the activating nucleosome position reflect gene

The graph displays a window of the calculated predictions, ranging from position –300 to +300 within the rDNA sequence. Peaks of high *p*(nucleosomal start) values, indicating a high probability for a nucleosomal start site, are indicated. The two nucleosomal positions identified on the rRNA gene *in vivo* are indicated [*nucAct* –157 to –2 (green); the repressive nucleosome position –132 to +22 (red)].
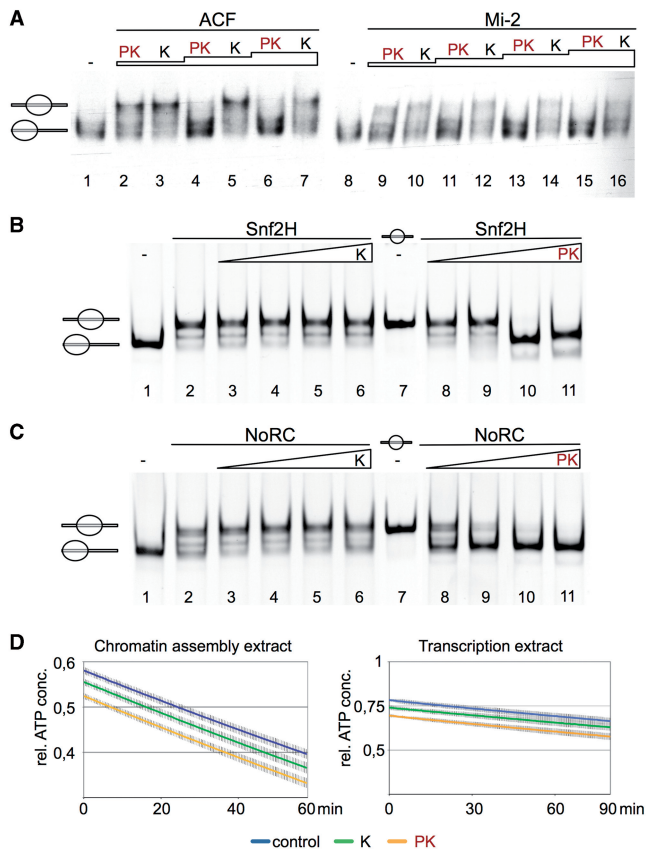
Figure 2. PK inhibits nucleosome remodelling. (**A**) Purified mononucleosomes, positioned at the border of the rDNA promoter fragment, were incubated with ACF (lanes 2–7), Mi-2 (lanes 9–16) and increasing concentrations of K and PK (100–800 μM) as indicated. Nucleosome remodelling reactions were incubated for 60 min and analysed by native PAGE. The positions of the nucleosomes on the DNA fragment are indicated. (**B**) Nucleosome remodelling reactions performed as in (**A**), except that nucleosomes occupied the border of the 601 DNA fragment, and the remodeller Snf2H was analysed. Lane 7 serves as a control, showing the '601' nucleosome reconstituted on a central position of the DNA fragment. (**C**) Nucleosome remodelling reaction performed as described in (**B**), except that the recombinant remodelling complex NoRC was analysed. (**D**) Measurement of ATP hydrolysis rates in the *Drosophila* embryo extract and the murine transcription extract. Extracts were incubated with 500 μM K or PK, and the rate of ATP hydrolysis was measured. The standard deviation is shown for every time point.

repression *in vitro* and identifies the repressed state as the default activity state of rDNA *in vivo*.

Active mechanisms that lead to a positioning of all nucleosomes at the repressive site *in vivo* may ensure the maintenance of the repressed state. These results do also imply that nucleosome positions have to be actively changed in order to activate rRNA gene expression, i.e. moving the *nucRep* nucleosome to the *nucAct* site. ATP dependent nucleosome movement and positioning is very likely achieved by specific chromatin remodelling complexes targeted to the rRNA gene promoter (15).

PK inhibits chromatin remodelling complexes

The above-mentioned observations prompted us to assess the role of chromatin dynamics in the process of gene activation and nucleosome positioning. No study to date did

monitor the direct involvement and functional role of chromatin remodelling complexes in DNA dependent processes. In order to assay the order of events and the dynamics of gene activation we established an experimental system allowing us to uncouple chromatin remodelling from transcription. We therefore tested the capability of Phosphokanamycin (PK), a potent inhibitor of the Swi/Snf remodelling complex (35), in the repression of other classes of chromatin remodelling machines. First, we examined the effect of PK on the activity of the remodelling enzymes ACF and Mi-2, using the 'nucleosome sliding' assay that monitors ATP-dependent changes in nucleosome positions (36) (Supplementary Figure S4). Both ACF and Mi-2 have been shown to relocate mononucleosomes from the end to more central positions of the 248-bp murine rDNA fragment (36,37) (Supplementary Figure S4B). The addition of increasing amounts of kanamycin A (K) did not affect nucleosome remodelling (Figure 2A), whereas identical concentrations of PK inhibited nucleosome remodelling in a dose-dependent manner. Furthermore, PK inhibited the activity of Snf2H and the chromatin remodelling complex NoRC, a Snf2H-containing complex that silences rRNA genes (26,38) (Figure 2B and C). Our experiments show that PK is a potent inhibitor of Swi/Snf complexes as well as the ISWI and Chd subfamilies of chromatin remodelling ATPases.

To exclude the possibility that PK has a global inhibitory effect on all kind of ATPases, we monitored the ATPase activity of chromatin assembly and nuclear extracts by an enzymatic assay that couples ATP hydrolysis to NADH consumption (Figure 2D). No obvious differences in ATP hydrolysis were observed in the absence and presence of PK, indicating that, while PK selectively inhibits the activity of chromatin remodelling ATPases, it does not affect the activity of other ATPases in the extracts. In addition, transcription initiation on free DNA and elongation by Pol-I is not affected (Figure 5B).

PK inactivates nucleosome remodelling

Using extracts from *Drosophila* embryos that efficiently deposit nucleosomes with regular spacing (39), we reconstituted chromatin on artificial murine rDNA minigenes to study the effect of PK on chromatin dynamics (Figure 3A). We used this crude chromatin assembly system to prove whether PK can be used as a global inhibitor of all kind of chromatin remodelling activities that are present in the extract. As PK might affect the assembly of chromatin in the extract, we added PK after chromatin assembly had been completed. K and PK were added after 4 h, and did not change the chromatin structure of the rDNA minigenes, as revealed by MNase digestion and visualization of the nucleosomal ladder (Figure 3A).

The activation of rDNA transcription on chromatin templates requires binding of the transcription factor TTF-I to its target site 170-bp upstream of the transcription start site (23). As shown by indirect end-labelling assays, the addition of recombinant TTF-I to pre-assembled chromatin induces two MNase-sensitive sites
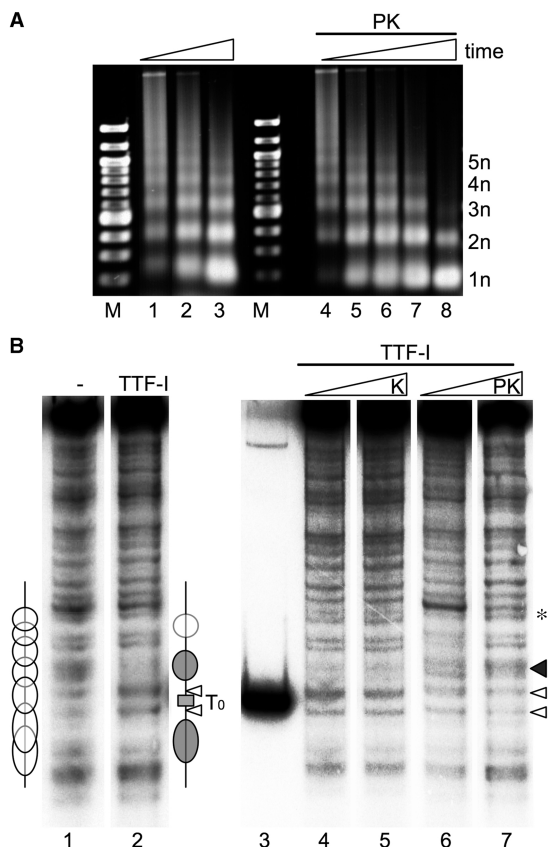
Figure 3. PK inhibits nucleosome remodelling at the rDNA promoter. (**A**) MNase digestion of chromatin assembled on the rDNA minigene (pMrWT-T). Chromatin reconstituted with the *Drosophila* extract was digested with MNase for 0.5–3 min (lanes 1–3) or for 0.5–6 min (lanes 4–8) in the presence of 600 μM PK. The nucleosomal ladder (1n-5n) and the DNA marker (M; 1kb ladder) are indicated. (**B**) Chromatin assembled on pMrWT-T was incubated in the absence or presence of TTF-I and partially digested with MNase. Purified DNA was digested with EcoRI, separated on an agarose gel and transferred onto a nylon membrane. Chromatin configuration around the TTF-I-binding site (T_0) was visualized by indirect end-labelling (lanes 1 and 2). Chromatin remodelling was monitored in the presence of K (lanes 4 and 5; 300 and 600 μM) or PK (lanes 6 and 7, 300 and 600 μM). The position of the TTF-I-binding site is indicated by SalI digestion of the template DNA (lane 3). Open circles mark non-positioned nucleosomes, whereas the gray circles indicate positioned nucleosomes. The position of the TTF-I-binding site (gray box), MNase-protected DNA regions (black triangles) and MNase-sensitive regions (white triangles) are indicated. The strong band in lane 6 (marked with an asterisk) arises due to the relatively lower MNase digestion of this sample.

adjacent to the TTF-I-binding site, and positions nucleosomes upstream and downstream of the binding site, thereby protecting these regions from MNase digestion (Figure 3B, lane 2). A similar chromatin structure is established in the presence of K, which does not interfere with the activity of the chromatin remodelling complexes (lanes 4 and 5). Significantly, this specific nucleosomal pattern is not observed in the presence of PK (lanes 6 and 7), indicating that PK does efficiently inhibit nucleosome remodelling activities in the chromatin assembly extract. In addition, PK activity does not affect the overall structure of chromatin and has no global inhibitory effect on other ATPases present in the transcription

and chromatin assembly extracts (Figures 3A and 2D). Accordingly PK, as a global inhibitor of chromatin remodelling activities, serves as a potent tool to uncouple chromatin dynamics and transcription initiation to study the dynamics and interdependency of both processes.

RNA Polymerase I transcribes through nucleosomes without displacing them

Chromatin associated factors like the FACT complex have been shown to participate in transcription elongation through nucleosomes (40,41). Our subsequent experiments require nucleosomal arrays that allow the assembly of high quality chromatin, enabling TTF-I dependent transcriptional activation. Therefore, we had to test whether we are able to uncouple the repressive effect of chromatin on transcription initiation from transcription elongation through nucleosomes. To study the mechanism of transcription initiation we had to exclude repressive effects of nucleosomes on transcription elongation. In addition, we realized that no study did address RNA Polymerase I elongation through nucleosomes and their fate in a defined mononucleosomal system in sufficient detail.

To study transcription elongation we have developed a system to monitor RNA Polymerase I (Pol-I) transcription on naked and nucleosomal templates, visualizing the DNA template and the transcript in the same reaction (Figure 4A). Nucleosomes were reconstituted by salt dialysis on a radio-labelled DNA fragment containing rDNA sequences from nt –175 to +317 relative to the transcription start site. As DNA packaged into nucleosomes is not digested by restriction enzymes, mononucleosomes located downstream of the transcription start site can be selected by restriction digestion with *Sma*I, which cleaves murine rDNA at nucleotide +155 (Figure 4A).

Nucleosome assembly reactions were performed at a histone to DNA ratio of 1:2, resulting in a significant fraction of free DNA and reconstituted mononucleosomal DNA (Figure 4B). Higher histone to DNA ratios result in the formation of dinucleosomal templates (Supplementary Figure S2A). Mononucleosomal templates give rise to a variety of distinct complexes as visualized by native gel electrophoresis (Figure 4B, lane 3 and lane 5). Individual complexes represent rDNA fragments with nucleosomes assembled at different sites on the DNA. Slow migrating complexes do exhibit nucleosomes preferentially positioned at central positions and fast migrating complexes have nucleosomes reconstituted close to the ends of the DNA fragment (42).

In order to monitor nucleosomal occupancy at different sites of the rDNA fragment, nucleosomes were either incubated with *Ava*II (cleaving at position –15; lane 4) or *Sma*I (cleaving at position +155; lane 6) and analysed by native gel electrophoresis. Free DNA was hydrolysed, whereas nucleosomal DNA was partially digested, changing the migration behaviour of only a subset of nucleosomal species. The patterns of nucleosomes hydrolysed with *Ava*II or *Sma*I are distinct. The
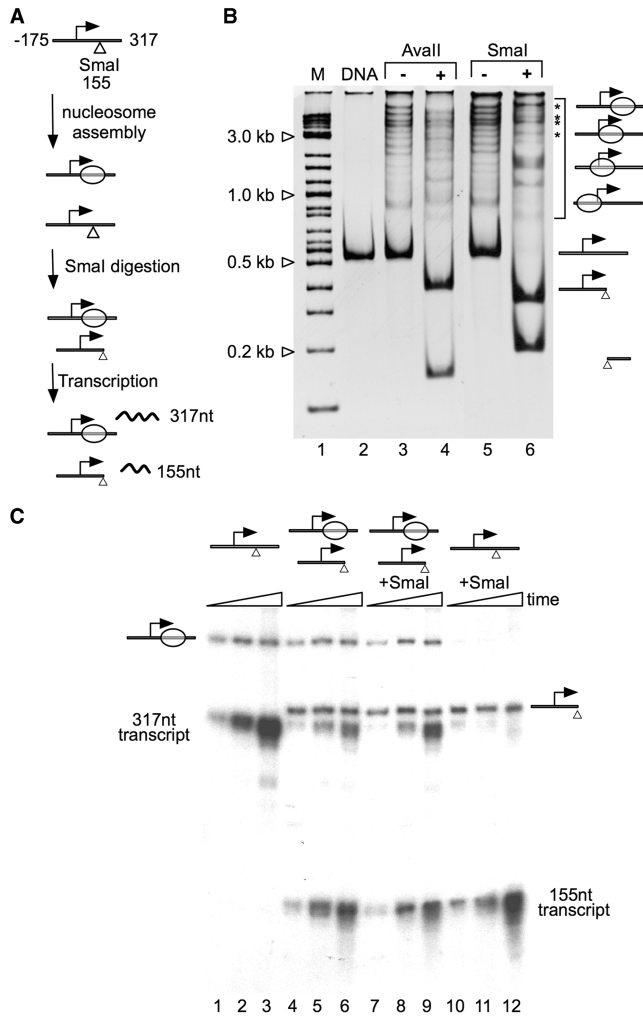
Figure 4. Pol-I elongates through a nucleosome. (**A**) Scheme of the nucleosome assembly, selection and analysis procedure. The end-labelled, 492-bp rDNA fragment was reconstituted into mononucleosomes and the templates containing nucleosomes within the transcribed region were selected by digestion of the DNA with *Sma*I. *Sma*I-sensitive templates give rise to a 155-nt RNA transcript, whereas *Sma*I-resistant, nucleosomal templates give rise to a 317-nt long transcript. (**B**) The rDNA fragment (lane 2) was reconstituted into nucleosomes at a histone:DNA ratio of about 1:2 (lanes 3 and 5). Nucleosome assembly reactions were incubated with *Ava*II (−15) and *Sma*I (+155) as indicated and the resulting nucleoprotein complexes were resolved by native polyacrylamide gel electrophoresis. The sizes of the DNA marker (lane 1) are indicated and a rough scheme of DNA and nucleoprotein complexes hydrolysed with the restriction enzymes is shown on the left. The box indicates reconstituted mononucleosomes occupying different positions and the stars mark nucleosomes with a protected *Sma*I site. (**C**) Transcription reactions contained 10 ng of free DNA (lanes 1–3), purified, *Sma*I-digested nucleosomal DNA (lanes 4–6), and selected nucleosomal DNA transcribed in the presence of *Sma*I (lanes 7–9) and free DNA transcribed in the presence of *Sma*I (lanes 10–12). Transcription reactions were incubated for 10–40 min at 30°C and the DNA/RNA was analysed on 5% polyacrylamide gels. The positions of the free DNA cleaved with *Sma*I and the nucleosomal *Sma*I-resistant DNA fragments are indicated. Transcript lengths and the templates are marked. Nucleosomal templates (ovals represent nucleosomes) and enzymes added to the reactions are indicated on top.

cleavage sites are separated by 170 bp and discriminate between nucleosomes located over the promoter and the transcribed region of the rDNA fragment respectively. Importantly, *Ava*II and *Sma*I digestion do not hydrolyse all nucleosomal complexes, showing that a significant fraction of the nucleosomes are located at these sites. Nucleosomal DNA was purified via gelfiltration chromatography in order to remove excess restriction enzymes (Supplementary Figure S2B). In addition, a detailed analysis of the nucleosome assembly reactions using biotinylated DNA templates (Supplementary Figure S3) showed that after assembly all histones were reconstituted into nucleosomes, and therefore a contamination of free histones affecting our analysis could be excluded.

Transcription of the full-length rDNA template yields 317-nt run-off transcripts, while 155-nt transcripts are synthesized after cleavage with *Sma*I (Figure 4C, lanes 1–3 and 10–12). To compare transcription on nucleosomal and naked rDNA templates, nucleosomes were reconstituted as described above yielding ∼30% of rDNA packaged into mononucleosomes, as visualized by the ratio of full-length (*Sma*I site protected; nucleosomal) to *Sma*I-cleaved (free DNA) templates (Figure 4C; lanes 4–9). *Sma*I-sensitive templates result either from hydrolysis of free DNA or nucleosomal DNA with a nucleosome located upstream of the *Sma*I site, giving rise to 155-nt transcripts (lanes 4–9). Importantly, the templates with a protected *Sma*I cleavage site possess a nucleosome within the transcribed region. Transcription of the *Sma*I-selected nucleosomal DNA fraction yield 317-nt transcripts, indicating that Pol-I is capable of elongating through nucleosomes (lanes 4–9). Notably, the nucleosomal template (317-nt transcripts) is transcribed ∼4-fold less efficiently than free DNA, indicating that nucleosomes slow down transcription elongation or do affect transcription initiation from a distance.

In order to exclude the possibility that 317-nt transcripts were generated from small amounts of nucleosome-free, non-digested DNA, the transcription reactions were also performed in the presence of *Sma*I. *Sma*I addition at the onset of the transcription reaction results in the complete hydrolysis of the free DNA template, and the 317-nt transcript cannot be generated (Figure 4C, lanes 10–12). Addition of *Sma*I to the transcription reaction using the *Sma*I pre-treated nucleosomal template does not affect the synthesis of the 317-nt transcripts (Figure 4C, lanes 7–9), suggesting that Pol-I elongates through a nucleosome. In addition, co-transcriptional *Sma*I cleavage allows monitoring the fate of the nucleosomal DNA after the passage of the polymerase. If Pol-I elongation had evicted nucleosomes, the level of cleaved DNA should have increased. Yet, the level of transcripts and the ratio of digested versus non-digested template does not change (Figure 4C, lanes 7–9), indicating that Pol-I transcription does not generate nucleosome-free DNA. Although this assay does not reveal whether transcription elongation leads to partial disruption of the nucleosome, such as the loss of H2A/H2B dimers, it clearly demonstrates that Pol-I is capable of elongating through nucleosomes without displacing the complete histone octamers from DNA. Moreover, in contrast to
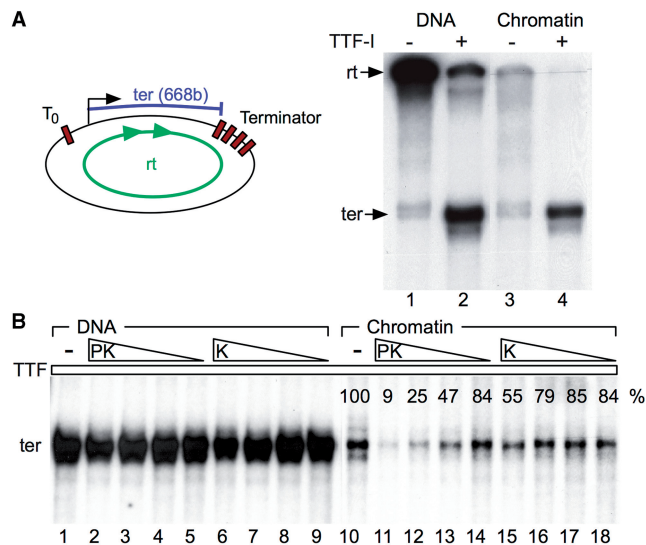
transcription by RNA Polymerase II or viral RNA polymerase, the nucleosomes apparently remain at, or close to, their original position (43,44).

PK is a chromatin-specific inhibitor of transcription

According to the *in vitro* transcription experiments on mono-nucleosomal DNA, chromatin mediated transcriptional repression acts on the level of transcription initiation (Figures 1 and 4), allowing us to study the regulation of initiation on nucleosomal arrays. Analyzing the order of events and the impact of chromatin dynamics on gene activation, we compared *in vitro* transcription reactions on free and nucleosomal murine rDNA minigenes (pMrWT-T; Supplemental Figure S6). DNA templates were reconstituted into chromatin and incubated with a partially purified nuclear extract from mouse cells (DEAE-280 fraction) that contains all the factors required for transcription initiation (29). Because this fraction lacks TTF-I, long read-through transcripts are synthesized on free DNA (Figure 5A; Supplementary Figure S6). In the presence of recombinant TTF-I, read-through transcripts are greatly decreased and short transcripts are synthesized that terminate 686-nt downstream of the initiation site (lane 2). Like on the mononucleosomal rDNA template (Figure 2, lane 5–7),

Figure 5. Nucleosome remodelling is required for transcriptional activation in chromatin. (**A**) TTF-I activates transcription of an rDNA minigene assembled into chromatin (pMrWT-T). Transcription of free DNA generates long read-through transcripts (rt) that are terminated (ter) in the presence of TTF-I (lanes 1 and 2). The same DNA reconstituted in chromatin was transcribed in the absence or presence of TTF-I (lanes 3 and 4). The radio-labelled RNA was analysed on native polyacrylamide gels. The scheme of the rDNA minigene, the read-through (rt) and the terminated transcript (ter) are shown on the left side. The read-through transcripts migrate close to the top of the gel. The absence of read through transcript on chromatin templates may be a result of different transcription termination efficiency of the free DNA and chromatin system. (**B**) Free DNA (lanes 1–9) and chromatin (lanes 10–18) were transcribed in the presence of TTF-I and decreasing concentrations of either K or PK. The position of the terminated transcripts (ter) is indicated. Quantification of the transcription levels relative to the TTF-I activated control (100%) are indicated above the chromatin dependent transcripts.

transcription is also repressed on the nucleosomal array, suggesting as well the lack of nucleosomes positioned at the *nucAct* site (Figure 1). Comparable to previous studies, the addition of TTF-I is required to activate transcription on rDNA reconstituted into chromatin (23) (Figure 5A; lanes 3 and 4), yielding exclusively short, terminated transcripts.

Next, we uncoupled chromatin remodelling from transcription initiation to monitor the requirement of nucleosome dynamics and the order of events during the activation process. We performed transcription reactions in the presence of increasing amounts of K and PK to specifically inhibit chromatin remodelling complexes during transcriptional activation (Figure 5B). Neither K nor PK significantly affect transcription on free DNA (lanes 1–9). On chromatin templates, however, clear differences are observed; increasing concentrations of PK, but not of K, result in a dose-dependent reduction of gene activation (lanes 11–18). Thus, we can conclude that a lack of chromatin remodelling activity inhibits gene activation, suggesting chromatin remodelling being a process indispensable for transcription activation on the nucleosomal rRNA genes. This result together with the observation that a nucleosome covers the *nucAct* position at active genes *in vivo*, implies that during gene activation *in vitro* a significant portion of nucleosomes is re-located to the *nucAct* position.

Nucleosome remodelling is an initial step in gene activation

The transcription experiments suggested active nucleosome remodelling to be required for gene activation. Next we analysed in high resolution the structural changes at the rRNA gene promoter during the process of gene activation. Nucleosome arrays, also used for the transcription experiments, were incubated with TTF-I, as well as with a partially purified transcription extract (TxE) in the absence and presence of K or PK (Figure 6). Nucleosome positions were analysed by partial MNase digestion and primer extension with a labelled oligonucleotide as described (26). The observed MNase cleavage sites correspond to the 3′ boundaries of nucleosomes, and the intensity of the peaks correlate with the fraction of nucleosomes occupying each position.

Major nucleosome positions are observed at locations +22, +58, +86 and +220 (3′ boundaries of the nucleosomes) (Figure 6, Input). In agreement with our previous results, the positions +22 (corresponding to *nucRep;* dyad axis at −55) and +86 were also mapped on the mononucleosomal rDNA fragment (Figure 1C). Identical nucleosomal positions in the different experimental systems, strengthens our suggestion that sequence-dependent features determine the repressive nucleosome configuration as the default state of the rRNA gene. The absence of the +58 nucleosome from the mononucleosomal template and its appearance in the nucleosomal array may be a result of boundary effects, as this nucleosome is in frame with the +220 nucleosome that could form a boundary, and guide positioning of the upstream nucleosome on the longer DNA template.
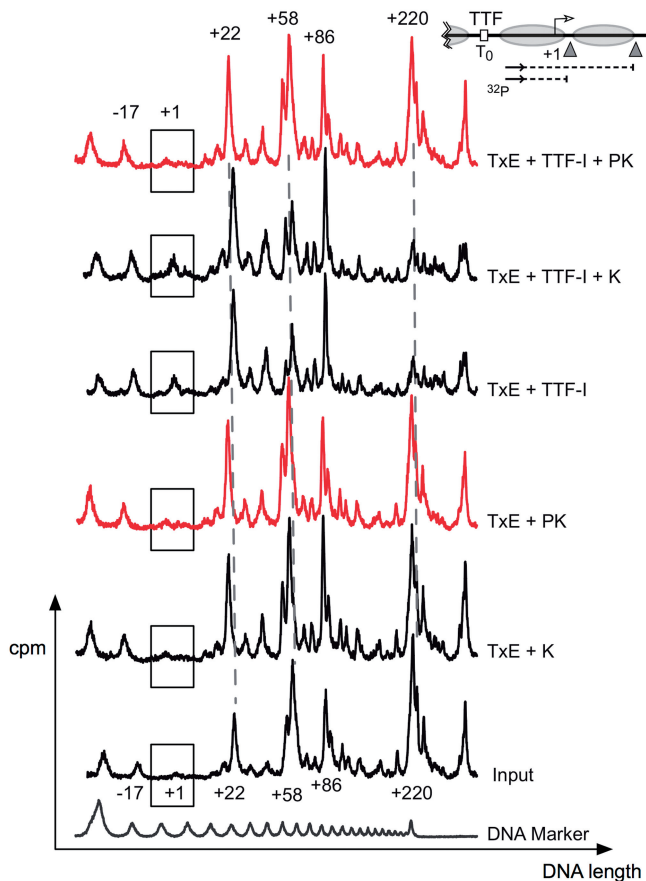
Figure 6. TTF-I-dependent chromatin dynamics at the rRNA gene promoter. Reconstituted nucleosomal arrays were incubated for 90 min with the TxE, TTF-I, K (600 µM) or PK (600 µM) as indicated. Nucleosome positions at the rDNA promoter were mapped by partial MNase digestion and primer extension of the purified DNA. DNA fragments were resolved on 8% sequencing gels and quantified with a PhosphorImager. The graph shows the positions (relative to the transcription start site, +1; site is marked by boxes) and relative intensities of the MNase cleavage sites corresponding to the 3′ boundaries of positioned nucleosomes. Boxes highlight the MNase cleavage sites around the transcription start site, correlating with the nucleosome position *nucAct*. The position of the oligonucleotide used for primer extension and the major MNase-sensitive sites on the rDNA are indicated. The scan of the DNA marker (10-bp ladder) is shown below the graphs.

Significantly, as seen for reconstituted mononucleosomes (Figure 1C, D), no nucleosomes cover the *nucAct* position (dyad axis at –77; 3′ boundary at ±1; Figure 6, Input—boxed area) found on active rDNA repeats *in vivo* (3). Interestingly, no MNase cleavage was observed between rDNA position −17 and +22 forming a large gap of 39 bp, explicitly avoiding the positioning of nucleosomes close to the *nucAct* site. We think that the lack of this nucleosomal position is a hallmark of repressed rRNA genes and conserved between the different experimental setups. Hence, we can re-enforce our suggestion that sequence-dependent features disfavour the reconstitution of a nucleosome at the *nucAct* site, correlating with transcriptional repression of the rRNA gene in chromatin.

Addition of murine TxE in the presence of K or PK does not significantly change nucleosomal occupancy patterns compared to the input (Figure 6; compare TxE+K to TxE+PK). The chromatin structure at the rRNA gene promoter is not changed by the Pol-I initiation factors and the remodelling complexes present in the extract. The transcription extract lacked TTF-I. Accordingly, these reactions remained transcriptionally repressed and the *nucAct* nucleosome does not emerge. This result is consistent with the transcription analysis performed at identical conditions (Figure 5). The addition of stoichiometric amounts of TTF-I (relative to template) to the transcription extract dramatically changes the picture of nucleosome positions (Figure 6, TxE + TTF-I). The fraction of nucleosomes occupying the sites +220 and +86 strongly decrease, whereas a minor but significant nucleosome positions appears whose 3′ boundary coincide with the transcription start site (Figure 6, TxE + TTF-I—boxed area). This MNase sensitive site coincides with the position of the *nucAct* nucleosome that is observed at the active rRNA gene promoter. The observed changes in chromatin structure correlate with active transcription, suggesting that this structural change over the promoter enables the binding of the transcription initiation factors and gene activation. It also suggests that the nucleosomal templates harbouring the *nucAct* nucleosome do correspond to the fraction of actively transcribed chromatin templates.

Still it is possible that MNase cleavage sites may appear due to initiation factor binding and do not reflect nucleosome positions. Therefore chromatin remodelling and transcription initiation was uncoupled by the addition of PK. The addition of K or PK had no effect on the structural changes at the rRNA promoter (Figure 6, compare TxE with TxE+K, TxE+PK—boxed areas). Upon addition of TTF-I an additional MNase sensitive site at position +1 appears (TxE + TTF-I + K—boxed area) suggesting TTF-I-dependent nucleosomal repositioning by chromatin remodelling activities present in the transcription extract. Blocking of chromatin remodelling activities by PK (Figures 3B and 5B) inhibits TTF-I induced nucleosomal rearrangement. Thus, the MNase sensitive site at +1, reflecting the nucleosomal boundary of the *nucAct,* does not appear (Figure 6, TxE+ TTF-I + PK—boxed area).

Interestingly, at conditions of active transcription the major fraction of nucleosomes remains positioned at the *nucRep* site (position +22, dyad axis –55), suggesting that only a minor fraction of chromatin templates are transcriptionally active. Indeed, calculating the relative transcript intensity compared to the radio-labelled DNA template suggests that only up to 5% of the templates are actively transcribed. This correlates very well with our observations and the low number of chromatin templates with nucleosomes at the *nucAct* site. In addition our results do also reflect the dual role of TTF-I in gene activation and repression (3,24,38,45). TTF-I binding to T_0 leads to strong changes in the overall chromatin structure, but it does not affect the positioning of the *nucRep* nucleosome and it enables, together with remodelling activities of the transcription extract, the formation of a former unfavoured nucleosome position (*nucAct*). In agreement with the role of TTF-I in gene repression and

activation, both nucleosomal positions coexist in our experimental assay (Figure 6). Our results suggest that the occupancy of nucleosomes at these sites may be directly influenced by TTF-I interacting factors in the extract that determine nucleosome positioning and the level of active or repressed rDNA templates.

In fact, although the low resolution analysis (Figure 3B) suggested a homogenous chromatin organization after the addition of TTF-I, aligning nucleosomes next to the TTF-I-binding site and correlating with transcription activation (22), the high resolution analysis shows now a more complex chromatin pattern reflecting the different functions of TTF-I. We therefore suggest that TTF-I binding induces gross changes in the overall chromatin structure, i.e. placing a nucleosome over the promoter region while still allowing different positions that either correlate with transcription (*nucAct*) or repression (*nucRep*). The recruitment of specific remodelers, such as NoRC, do then determine the absolute nucleosome positioning and gene activity. Sequence-dependent effects disfavour nucleosome reconstitution at the *nucAct* site, so that active nucleosome positioning mechanisms are required for rRNA gene activation. We suggest that the recruitment of specific chromatin remodelling complexes like NoRC and CSB (24,26) determine the exact local chromatin structure as well as the gene's repression or activation.

The regulatory potential of the two different nucleosome positions most probably determines the accessibility of the promoter sequences for initiation factor binding. Transcription initiation of the rRNA genes requires co-operative binding of TIF-IB and UBF to the core promoter and the upstream control element (UCE), which are separated by ∼140 bp (46). Nucleosomes covering the *nucRep* position would mask the binding site of TIF-IB, and probably inhibit sequence-specific binding. The nucleosome remodelling event positions the nucleosome such that the sequences of the core promoter and the UCE are located at the DNA entry/exit sites of *nucAct*. As a result, the DNA sequence is placed in close proximity on the surface of the histone octamer, suggesting that this configuration allows co-operative binding of these factors to the promoter, as well as subsequent formation of the initiation complex.

An additional important observation that can be drawn from our studies is addressing the action and activity of chromatin remodelling complexes. Our results show that nucleosomes are not constantly moving around, driven by the ATP-dependent chromatin remodelling complexes, but require specific triggers and dedicated remodelling complexes that define a rather static chromatin structure. If just nucleosome positions determine gene activity, dynamic chromatin (constant nucleosome movement by remodelling complexes) would not allow efficient gene repression. At a given time a nucleosome would be placed such that initiation factors can bind to its linker regions and for example activate transcription. In our experimental system we did not observe a randomization of chromatin structure by the remodelling complexes of the extract, but the initial structures remained stable. In contrast to the general view of the action of chromatin

remodelling machines we suggest that they do not constantly move nucleosomes, but that the machines require triggers to be recruited and activated and that they have the capability to position nucleosomes (15,24). We propose that chromatin remodeller-dependent *nucAct* positioning places the histone octamer in such a way that initiation factors can be bound to the promoter-bound nucleosome.

In summary, we suggest that sequence-dependent mechanisms organize the rRNA gene promoter in a repressed chromatin configuration by disfavouring the positioning of a nucleosome at the *nucAct* site. Nucleosome positions have to be switched via a two-step mechanism. First, the binding of TTF-I reorganizes the chromatin structure so it becomes competent for transcription activation. Second, the recruitment of specific remodelling complexes to the rRNA gene promoter establishes local nucleosome positions that either repress (NoRC) or activate (CSB) rRNA gene transcription. Regulation of rRNA gene activity depends on switching nucleosomal positions between *nucAct* and *nucRep* by chromatin remodelling complexes that position nucleosomes based on DNA sequence/structure features (15).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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