FACT facilitates chromatin transcription by RNA polymerases I and III

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Efficient transcription elongation from a chromatin template requires RNA polymerases (Pols) to negotiate nucleosomes. Our biochemical analyses demonstrate that RNA Pol I can transcribe through nucleosome templates and that this requires structural rearrangement of the nucleosomal core particle. The subunits of the histone chaperone FACT (facilitates chromatin transcription), SSRP1 and Spt16, co-purify and co-immunoprecipitate with mammalian Pol I complexes. In cells, SSRP1 is detectable at the rRNA gene repeats. Crucially, siRNA-mediated repression of FACT subunit expression in cells results in a significant reduction in 47S pre-rRNA levels, whereas synthesis of the first 40 nt of the rRNA is not affected, implying that FACT is important for Pol I transcription elongation through chromatin. FACT also associates with RNA Pol III complexes, is present at the chromatin of genes transcribed by Pol III and facilitates their transcription in cells. Our findings indicate that, beyond the established role in Pol II transcription, FACT has physiological functions in chromatin transcription by all three nuclear RNA Pols. Our data also imply that local chromatin dynamics influence transcription of the active rRNA genes by Pol I and of Pol III-transcribed genes.

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Introduction

Eukaryotic gene expression can be regulated at various key steps in the transcription cycle, including the recruitment of the RNA polymerase (Pol) to the gene promoter, transcription initiation, promoter escape, elongation and termination, and the rate of RNA synthesis is dependent upon the efficiency by which RNA Pols negotiate nucleosomes in chromatin (Saunders et al, 2006; Li et al, 2007). At physiological salt concentrations, nucleosomes impose a strong block to elongation of RNA Pol II transcription in vitro (Kireeva et al, 2002). Pol II chromatin transcription is facilitated by histone chaperones, such as FACT (facilitates chromatin transcription; Orphanides et al, 1998), which aid Pol II passage by destabilizing the nucleosome through the transient release of a histone H2A–H2B dimer (Orphanides et al, 1999; Belotserkovskaya et al, 2003), consistent with the observed rapid exchange of these dimers in cells (Kimura and Cook, 2001). RNA Pol III possesses an intrinsic ability to transcribe through a mono-nucleosome by translocation of the nucleosome to a position upstream of its original site during polymerase passage without disruption of the majority of histone octamer–DNA contacts (Studitsky et al, 1997). Our initial aim was to identify RNA Pol I-associated proteins and activities important for rRNA gene transcription through chromatin in vitro and in cells.

Transcription of the major ribosomal RNAs by Pol I is a key determinant of ribosome biogenesis, driving cell growth and proliferation in eukaryotes. Of the hundreds of copies of rRNA genes present in each cell, only a proportion are transcribed in actively growing cells of yeast or humans (~50% of the rDNA repeats are transcribed in interphase cells; Conconi et al, 1989) and the cellular control of Pol I transcription involves adjustments both to the number of rRNA genes actively engaged in transcription and the rate of transcription from each active gene (reviewed in Grummt, 2003; Moss, 2004; Russell and Zomerdijk, 2005). Chromatin context influences the transcriptional activity of the rDNA genes (reviewed in Birch and Zomerdijk, 2008) and, conversely, passage of the transcription machinery affects chromatin structure (Dammann et al, 1995).

Inactive or silent rDNA genes have a chromatin structure distinct from that of active rDNA genes. Inactive rDNA repeats are in a chromatin state relatively refractory to psoralen crosslinking and organized in regular nucleosomal arrays similar to those observed for bulk chromatin (Conconi et al, 1989; Dammann et al, 1993). They are maintained in a silent state through association of the nucleolar remodelling complex (NoRC) with the rDNA promoter in mammalian cells (Strohner et al, 2001; Santoro et al, 2002). The actively transcribed rDNA repeats associated with nascent rRNA reside in a psoralen-accessible (‘open’) chromatin state lacking...
regular nucleosomal arrays (Conconi et al., 1989; Dammann et al., 1993). Active rDNA repeats in Saccharomyces cerevisiae have relatively few (Jones et al., 2007), if any (Merz et al., 2008), histones associated with the transcribed regions, yet there is evidence for a dynamic nucleosomal arrangement in both budding yeast and the slime mould Physarum (French et al., 2003; Thiriet and Hayes, 2005) and for the association of chromatin remodelling activities at actively transcribed RNA genes in yeast (Schneider et al., 2006; Jones et al., 2007). In mammalian rDNA, nucleosomes are present at the rDNA promoter regions of both active and inactive repeats though, significantly, the active and silent mammalian rDNA promoters are distinguishable by differential nucleosome positioning (Langst et al., 1998; Li et al., 2006), histone modifications and DNA methylation (Santoro et al., 2002; Nemeth et al., 2008). The nucleosomal arrangement at the transcribed regions of the mammalian rRNA genes is currently unknown. One contributory influence on the chromatin status of mammalian rDNA genes is Pol I transcription factor UBF, a protein with multiple HMG boxes, with the abilities to stimulate promoter escape (Panov et al., 2006a) and modulate Pol I transcription elongation rates (Stefanovsky et al., 2006), as well as, to decondense rDNA chromatin (Chen et al., 2004; Mais et al., 2005; Wright et al., 2006).

Passage of the polymerase through a chromatin template is a potential control point for Pol I transcription, whether at active rRNA repeats or during de novo activation of previously silent repeats. Here, we provide evidence that the histone chaperone FACT, a heterodimer of Spt16 and SSRP1 (Orphaneides et al., 1999), associates with Pol I and with rRNA gene repeats in cells. We demonstrate that a Pol I complex with associated FACT has the capacity to transcribe through mono- and poly-nucleosomal templates in vitro. Crucially, down-regulation of FACT subunit expression in cells leads to a reduction in pre-rRNA synthesis due to a decrease in Pol I transcription elongation. Collectively, our data suggest a role for Pol I-associated FACT, as a histone chaperone, in passage of Pol I through chromatin templates in cells. Furthermore, our findings are highly suggestive of the presence of nucleosomes on the transcribed regions of the active mammalian rRNA genes.

We extended these studies to include an analysis of RNA Pol III-transcribed genes. Chromatin immunoprecipitation (ChIP) and siRNA experiments suggest that FACT also associates with Pol III genes and facilitates Pol III transcription in chromatin.

Our data complement previous findings that implicated FACT in Pol II transcription (Orphaneides et al., 1998; Belotserkovskaya et al., 2003; Saunders et al., 2003), thereby suggesting a role for histone chaperone FACT in chromatin transcription by all three nuclear RNA Pols.

**Results**

**Pol I is able to transcribe through nucleosomes in vitro**

A mono-nucleosomal template was generated using recombinant Xenopus histones and a 178-bp DNA fragment containing the MMTV nucleosome positioning sequence (NPS) A and an additional 31 bp of DNA to allow an entry point for the polymerase (Figure 1A, diagram and lane 1). Pol I\(\alpha\), purified from HeLa cell nuclear extracts in a multistep process that separates Pol I\(\alpha\) from the less abundant Pol I\(\beta\) complex, and then further purified over a Mono-S column (Miller et al., 2001), was incubated with the mono-nucleosomal template in a transcription reaction mix. Pol I\(\alpha\) was able to transcribe through the nucleosome to yield a full-length transcript of 178 nt (Figure 1B, lane 1). This 178 nt transcript resulted from Pol I transcription of the mono-nucleosomal template (N; Figure 1A, lane 1), rather than from transcription of residual nucleosome-free DNA (RF; Figure 1A, lane 1; <5% of total DNA) in the mono-nucleosomal preparation. Evidence to support this conclusion includes the finding that restriction enzyme AvaII digestion of all the residual nucleosome-free DNA into fragments of 122 and 56 bp (Figure 1A, lane 2; the nucleosome protects the mono-nucleosomal template, N, from digestion) did not significantly affect production of the 178 nt transcripts (Figure 1C, compare lanes 1 and 2) or, detectably, yield transcripts of 122 and 56 nt. (Note that, at the relatively low concentration of AvaII used, the nucleosome-free DNA, F, is only partially digested, hence there is substantial production of the full-length 178 nt transcripts in addition to the synthesis of 122 and 56 nt transcripts from the AvaII-digested fragments; Figure 1C, lane 3.)

Pol I transcription from the nucleosomal template (N) was consistently 10-fold less than that from the same amount of nucleosome-free DNA template (F) (Figure 1B, compare lanes 1 and 2), suggesting that the nucleosome presents a significant but not insurmountable barrier to efficient transcription elongation by Pol I.

We next asked whether Pol I could transcribe through a template with an array of nucleosomes. A poly-nucleosomal template was generated, comprising a DNA template that includes twelve SS rDNA nucleosome-positioning sequences and histone octamers (Figure 1D). The nucleosomal composition of this template was analysed by MNase digestion, which produced a DNA ladder upon digestion of poly-nucleosomal DNA (Figure 1D, lane 3). Pol I\(\alpha\) was able to transcribe the poly-nucleosomal template (poly-N) progressively to yield a full-length transcript of 2496 nt (Figure 1E, lane 1). The efficiency of transcription was ~10-fold less on the poly-nucleosomal template (poly-N) compared with that on the same amount of the equivalent nucleosome-free template (F) (Figure 1F, compare lanes 1 and 2) and similar, therefore, to the efficiency of transcription on the mono-nucleosomal template. Substitution of AMP-PNP for ATP reduced overall transcription levels as AMP-PNP is less readily utilized by Pol I, but the ratio of transcription from the poly-nucleosomal compared with the nucleosome-free template remained approximately 1:10, implying that no ATP-dependent chromatin remodelling activity is required for chromatin transcription by Pol I in vitro (Figure 1F, compare lanes 3 and 4). Collectively, the data suggest that the Pol I\(\alpha\) complex has the ability to negotiate nucleosomes in transcription.

**Pol I transcription is inhibited by the presence of crosslinked histones**

To test whether Pol I might negotiate the nucleosomal barrier through rearrangement of histones in the octamer DNA, we used a homo-bifunctional crosslinking reagent bis(sulphosuccinimidyl) suberate (BS\(\delta\)) to crosslink histones within a pre-formed nucleosomal core particle. Nucleosomal DNA templates crosslinked with BS\(\delta\) (Figure 2A, lane 2) were incubated with Pol I\(\alpha\) in an end-to-end transcription assay.
Nucleosomal templates mock-treated with BS₃, treated with BS₃ inactivated by glycine treatment or untreated were used as controls. There was a marked inhibition of nucleosomal transcription by Pol I on the crosslinked nucleosomal templates (Figure 2B, lanes 1 and 2 and graph), in comparison with the controls (Figure 2B, lanes 3–8 and graph). These results suggest that structural rearrangement of the nucleosomal core particle is necessary to enable Pol I passage.

**FACT associates with purified Pol Ix and with Pol I, II and III complexes from HeLa cell nuclear extracts**

The ability of Pol Ix to transcribe through a nucleosome, by way of structural rearrangement of the nucleosomal core particle, could be an intrinsic property of core Pol I or the activity of an associated protein or proteins. Pol Ix was purified as described previously (Miller et al., 2001) and the peak Pol I fraction from the Mono-S column was separated into its component proteins on a denaturing gel and SYPRO Ruby-stained (Figure 3A). Bands migrating at ~130 and ~80 kDa, with intensities similar to those for the largest and second largest subunits of Pol I (hRPA190 and hRPA135), were identified by mass spectrometry as subunits Spt16 (SUPT16H, an orthologue of S. cerevisiae SPT16/CDC68; Malone et al., 1991; Rowley et al., 1991) and SSRP1 (structure specific recognition protein 1; Bruhn et al., 1992), respectively, of the histone chaperone FACT (Orphanides et al., 1999). The gradient salt elution profiles from the Mono-S column were similar for FACT subunit SSRP1, Pol I subunit hRPA19 (AC19) and Pol I transcriptional activity...
Figure 2 Pol I transcription of a mono-nucleosomal template is inhibited by crosslinking of the histones within the nucleosome. (A) Crosslinking of the histones in the octamer of the mono-nucleosomal template with the homo-bifunctional crosslinker bis(sulphosuccinimidyl) suberate (BS3). Histones from the BS3-treated (lane 2) and untreated (lane 3) mono-nucleosomal templates were analysed on a 4–12% gradient denaturing protein gel, SYPRO Ruby-stained. The positions of free histones and crosslinked histone octamers are marked. Lane 1 contains a protein size marker. (B) Duplicate transcription reactions contained the mono-nucleosomal 178 bp DNA template (0.3 μg) pretreated with BS3 (+, lanes 1 and 2), inactivated BS3 (+, lanes 3 and 4) or crosslinking buffer alone (−, lanes 5 and 6), or untreated (control C, lanes 7 and 8). Radiolabelled transcripts were analysed by 7.5 M urea 11% polyacrylamide gel electrophoresis and phosphorimaging. The position of the full-length transcript (178 nt) from a representative experiment is indicated (as determined using a radiolabelled RNA size marker). The graph shows the results of duplicate reactions from two independent experiments; average transcript (178 nt) levels are expressed in arbitrary units (AU) and the ranges are indicated.

(Figure 3B), suggesting the co-purification of FACT with Pol Iα. That these FACT subunits had not been noted previously in highly purified mammalian Pol I complexes (for example, Matsui et al., 1976; Rose et al., 1988; Song et al., 1994; Hanada et al., 1996; Seither et al., 1997; Hannan et al., 1998; Yamamoto et al., 2004) might reflect differences in the way the complexes were purified.

Therefore, to determine whether indeed FACT associates with Pol I complexes, we analysed FACT co-immunoprecipitation with Pol I from Pol Iα fractions and, also, from nuclear extracts. Pol Iα was incubated with antibodies specific to SSRP1, or with control nonspecific IgGs, and immunoprecipitated complexes on the antibody beads were assayed for Pol I transcription activity. The SSRP1-specific antibodies immunoprecipitated Pol I transcription activity (Figure 3C), suggesting that the SSRP1 subunit of FACT is in a complex with Pol I. This transcriptional activity was lower than that of the input (see IgG supernatant), probably because the polymerase was tethered to beads. There was little Pol I activity detectable in the supernatant following immunoprecipitation with SSRP1- (Figure 3C) or Spt16-specific antibodies (data not shown), suggesting that the immunoprecipitation was efficient and association with Pol Iα is stoichiometric; the SYPRO Ruby staining pattern of FACT and Pol I subunits (Figure 3A) is consistent with such stoichiometry.

To determine whether Spt16 can associate with Pol I, Pol Iα was incubated with Spt16-specific antibodies and immunoprecipitated were immunoblotted using PAF53-specific antibodies. The Spt16-specific antibodies co-immunoprecipitated the Pol I-specific subunit PAF53 (Hanada et al., 1996; Seither et al., 1997; Figure 3D, lane 2), suggesting that the Spt16 subunit of FACT associates with Pol I.

To examine the association of FACT with Pol I in the context of a more complex protein mixture, Pol I was immunoprecipitated from nuclear extracts of HeLa cells expressing Flag-tagged Pol I-specific subunit CAST/hPAF49 (Panov et al., 2006b) and Flag-antibody immunoprecipitates were analysed by immunoblotting using Spt16- and PAF53-specific antibodies. Spt16 was detectable in the Pol I immunoprecipitates (Figure 3E, lane 3). Therefore, FACT associates both with purified Pol Iα complexes and with Pol I complexes from HeLa cell nuclear extracts.

Previously, we have isolated and biochemically defined the much less abundant initiation-competent Pol Iβ complexes, characterized by the presence of hRRN3, which bridges the interaction between promoter-bound SL1 and Pol I (Miller et al., 2001). To test whether or not FACT could be found associated with these Pol I complexes, we used hRRN3-specific antibodies to immunoprecipitate Pol Iβ from an intermediate Pol I chromatography fraction that contains both Pol Iα and Pol Iβ (0.2 M DEAE fraction; Miller et al., 2001) and analysed the immunoprecipitates using Spt16- RPA190- and PAF53-specific antibodies in immunoblotting. The low-abundance Pol Iβ complexes were immunoprecipitated specifically (Figure 3F, compare lane 4 with lane 1), but no hSpt16 was detectable in the Pol Iβ immunoprecipitates (Figure 3F, lane 4), despite the presence of hSpt16 in the fraction that contains both forms of Pol I (Figure 3F, lane 2). These data suggest that FACT is not associated stoichiometrically with Pol Iβ, implying that FACT could become associated with Pol I following initiation of transcription.

The association of FACT with the human Pol I complex (Figure 3A–D) and the interaction of FACT with Pol II detected in Drosophila cell extracts (Saunders et al., 2003) led to the possibility that FACT might also interact with Pol III. We tested whether immunoprecipitation of FACT, using SSRP1-specific antibodies would co-precipitate the three nuclear RNA Pols from HeLa nuclear extracts. Immunoprecipitates were analysed by immunoblotting using antibodies specific to Pol I subunit CAST, the Pol II largest subunit (C-terminal domain) and Pol III subunit RPC5. The subunits of all three polymerases were detectable in the SSRP1 immunoprecipitates.
though a greater proportion of the Pol I subunits in the nuclear extract co-immunoprecipitated with FACT, relative to the proportions of the co-immunoprecipitating Pol II and III subunits (Figure 3G, lane 3). Control immunoprecipitations, which included ethidium bromide or DNase I (Tan et al., 2006), suggested that the association of FACT with the RNA Pols was independent of DNA (data not shown). The association of FACT with all three nuclear RNA Pols leads us to speculate that FACT might interact with a conserved subunit shared by the three, as has been observed for yeast chromatin remodeler RSC (Soutourina et al., 2006).

**SSRP1-specific antibodies selectively inhibit nucleosomal transcription by Pol I**

FACT facilitates transcription by Pol II through nucleosomes (Orphanides et al., 1998, 1999; Belotserkovskaya et al., 2003) and could perform a similar role in Pol I transcription. Preincubation of Pol I with SSRP1-specific antibodies

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**Figure 3** FACT associates with purified Pol Ix and with Pol I, II and III complexes from HeLa cell nuclear extract. (A) Mono-S fraction of Pol Ix, purified from HeLa cell nuclear extracts in a multistep process as detailed in the Materials and methods, was separated into its subunits and associated proteins on a 4–12% gradient denaturing protein gel and SYPRO-Ruby stained (see also Panov et al., 2006b). The bands representing the SSRP1 (structure-specific recognition protein, ~80 kDa) and Spt16 (~130 kDa) subunits of FACT, as identified by mass spectrometric analysis, and the positions of nine of the core Pol I subunits (following the nomenclature in Panov et al., 2006b) are indicated. (B) Pol Ix gradient salt-eluted fractions from the Mono-S column were analysed for Pol I transcription activity in a nonspecific transcription assay (graph, fractions 15–29; inclusion of 0.1 mg/ml α-amanitin does not affect RNA synthesis activity; Miller et al., 2001) and by immunoblotting (fractions 18–26) using antibodies specific for SSRP1 (upper panel) and for Pol I subunit hRPA19 (lower panel). (C) Antibodies specific for SSRP1 or control IgG were used for immunoprecipitation of Pol Ix (peak activity from the Mono-S column) and the immunoprecipitates (IP, beads) and supernatants (Sup, following IP) were analysed for Pol I transcription activity in a nonspecific transcription assay. Transcript levels from two independent experiments were quantified, expressed as a percentage of maximum, set at 100%, and plotted as the average and range. (D) Antibodies specific for Spt16 (lanes 1 and 2) or control IgG (lanes 3 and 4) were used for immunoprecipitation from Pol Ix (as above) and the immunoprecipitates (IP, lanes 2 and 4) and supernatants (Sup, lanes 1 and 3) were analysed by immunoblotting using antibodies specific for Pol I subunit hPAF53. (E) Pol Ix was immunoprecipitated through the Flag epitope from nuclear extracts of HeLa cells expressing Flag-tagged Pol I subunit CAST. Flag antibody immunoprecipitates (Flag-IP, lane 3), the supernatants (10%) of the immunoprecipitation (Sup, lane 2) and control immunoprecipitates (IgG-IP, lane 1) were immunoblotted with antibodies specific for Spt16 and Pol I subunit PAf5. (F) Pol Ipp was immunoprecipitated through hRNN3 from a chromatography fraction containing both Pol Ix and Ipp (0.2 M KCl DEAE fraction; Miller et al., 2001). hRNN3-antibody immunoprecipitates (lane 4), the supernatant (10%) left following immunoprecipitation (lane 2), control immunoprecipitation with IgG (lane 1) and a control fraction (C) of purified Pol Ix (containing FACT, lane 3) were immunoblotted with antibodies specific for Spt16 and human Pol I subunits RPA190 and PAf53. (G) Immunoprecipitation of FACT and associated RNA polymerases from HeLa cell nuclear extracts. Immunoprecipitation (IP) was performed with the SSRP1 mouse monoclonal (10D1) antibody (lane 3) or with control mouse IgG (lane 2). Immunocomplexes were boiled in SDS sample buffer and subsequently analysed by SDS-PAGE and immunoblotting using antibodies specific for FACT subunits SSRP1 and hSpt16, Pol I subunit CAST, Pol II largest subunit CTD4H8 and Pol III subunit RPC5. In lane 1, 1.5% of the input (In) nuclear extract was loaded.
impaired the ability of Pol I to transcribe the mono-nucleosomal template (Figure 4, lane 2 and graph). This inhibition of transcription is specific for the nucleosomal template (N) as SSRP1-antibody preincubation of Pol I did not affect transcription from the nucleosome-free template (F) (Figure 4, lane 1 and graph). These data suggest a role for FACT in facilitating transcription by Pol I (at near-physiological salt concentrations) of nucleosomal templates.

Our preliminary data suggest that in transcription reactions with mono-nucleosomal templates, Pol I facilitates the loss of Cy3-labelled H2B from the template (see Supplementary Figure S1). These data are also consistent with the ascribed histone chaperone activity of FACT, which destabilizes the histone octamer and facilitates the release of a single histone H2A–H2B dimer from the nucleosome to promote passage by transcribing Pol II (Orphanides et al., 1998, 1999; Belotserkovskaya et al., 2003). We conclude that FACT might operate similarly to facilitate chromatin transcription by Pol I.

FACT is associated with transcriptionally active rRNA genes in the nucleolus

To further characterize the association of FACT with Pol I, we examined the subcellular distribution of endogenous FACT in mammalian cells. Immunostaining analysis revealed that a fraction of SSRP1 resides in the nucleolus (Figure 5), with a distribution similar to that of Pol I subunit CAST/hPAF49. Treatment of cells with a low concentration of actinomycin D, which primarily inhibits Pol I transcription (Perry and Kelley, 1970), resulted in the disappearance of the FACT subunit from the nucleoli (Figure 5), with a similar pattern of redistribution observed for CAST/hPAF49, which underwent translocation from the nucleolus to the nucleoplasm. These data suggest that nucleolar localization of FACT requires ongoing rDNA transcription and the presence of Pol I in the nucleolus. Furthermore, the data are consistent with a role for FACT in modifying nucleosomal structures at the active rRNA genes in vivo, and support the possibility of a direct link between FACT and Pol I transcription in cells.

FACT is associated with the chromatin of Pol I- and III-transcribed genes in cells

To explore whether FACT is associated with the rDNA chromatin in cells, the rDNA occupancy of FACT was examined by ChIP. Crosslinked chromatin was precipitated with an SSRP1-specific monoclonal antibody. Subsequent PCR reactions used primers for the amplification of the rDNA promoter, three regions within the transcribed sequence of human rDNA (5′-ETS, 18S and 28S) and a sequence within the non-transcribed intergenic spacer (IGS) (Figure 6A). The ChIP results suggest that FACT is associated with the promoter and the transcribed regions of the rDNA repeats (Figure 6B). SSRP1 occupancy of the transcribed regions of the rDNA repeat was comparable to that of the Pol II-transcribed γ-actin gene. Furthermore, FACT was found at various Pol III-transcribed genes, including the 5S rRNA, U6 snRNA, rRNA175 and 7SL RNA genes, with type 1 (5S rRNA gene), 2 (tRNA genes) and 3 (U6 snRNA gene) promoters (Figure 6B).

Downregulation of FACT expression decreases Pol I and Pol III transcription in cells

The involvement of FACT in rRNA synthesis by Pol I in cells was further examined by downregulation of SSRP1 expression through RNA interference. RNAi-mediated downregulation of SSRP1 (to <20% of control; Figure 7A, lane 4) led to a reduction in 47S pre-rRNA levels (Figure 7B, lanes 3 and 4 compared with lanes 1 and 2, respectively) of about two-fold post-transfection of SSRP1-specific siRNAs into HeLa cells (Figure 7B, graph). Furthermore, a substantial decrease in nascent pre-rRNA levels was observed by...
FACT in Pol I and III chromatin transcription

In recent years, it has become increasingly clear that in many genes Pol II is stalled at promoter proximal regions, poised for transcriptional activation, and that the expression of genes is therefore regulated post-initiation at the steps of promoter escape and elongation of transcription (Saunders et al., 2006; Muse et al., 2007; Zeitlinger et al., 2007). In the expression of the mammalian rRNA genes, post-initiation events are also important, as activator UBF has been shown to facilitate promoter escape by Pol I (Panov et al., 2006a), and binding of UBF throughout the rDNA repeat chromatin modulates elongation of Pol I transcription (Stefanovsky et al., 2006). The rate of rRNA synthesis and the linked processing events (Schneider et al., 2007) depend upon the efficiency by which Pol I negotiates rDNA chromatin, and our identification of the histone chaperone FACT as a Pol I

in facilitating Pol I transcription elongation through rDNA chromatin.

We also explored the possibility of a role for FACT in facilitating chromatin transcription of Pol III-transcribed genes by analysing the effects of RNAi-mediated downregulation of SSRP1 on the transcription of these genes. The data indicate that downregulation of SSRP1 also reduced the levels of tRNA\textsuperscript{\textgamma}r and 7SL RNA (Figure 7F), suggesting the involvement of FACT in Pol III transcription in cells.

In summary, we have demonstrated an important role for RNA Pol I-associated FACT in rRNA gene transcription through chromatin in vitro and in cells. Furthermore, we have provided evidence of a role for FACT in RNA Pol III transcription through chromatin in cells.

**Discussion**

Although FACT has been regarded as a general chromatin structure modulator for transcription (Reinberg and Sims, 2006), its involvement in this process beyond Pol II-dependent chromatin transcription elongation had remained largely uncharacterized. A potential nuclear function of FACT could be inferred from previous studies that demonstrated its presence in the nucleoli of mammalian cells (Andersen et al., 2002) and its association with the nucleolar organizer of Drosophila polytene chromosomes (Saunders et al., 2003).

Although consistent with a role for FACT in rDNA transcription, the presence of FACT in nucleoli might also reflect its roles in rDNA replication (Tan et al., 2006), DNA damage detection and response (Bruhn et al., 1992; Keller et al., 2001; Yarnell et al., 2001) and/or DNA repair (Heo et al., 2008). A recent report indicated that FACT and nucleolin, which possesses a similar histone chaperone activity (Angelov et al., 2006), are each sufficient to drive efficient Pol I transcription of an in vitro assembled chromatin template (Rickards et al., 2007). Yet evidence linking FACT to Pol I-dependent transcription in cells was missing. Here, we have provided evidence for the physical and functional interaction of FACT with Pol I complexes, as well as a physiological role for FACT in rRNA gene expression regulation in cells. Our data also suggest a role for FACT in transcription through chromatin of Pol III-transcribed genes. Therefore, this study extends the known biological roles for this histone chaperone and chromatin remodelling complex to chromatin transcription by all three nuclear RNA Pols. Moreover, our findings imply that local chromatin dynamics have a critical impact on the expression of Pol I- and III-transcribed genes.

In recent years, it has become increasingly clear that in many genes Pol II is stalled at promoter proximal regions, poised for transcriptional activation, and that the expression of genes is therefore regulated post-initiation at the steps of promoter escape and elongation of transcription (Saunders et al., 2006; Muse et al., 2007; Zeitlinger et al., 2007). In the expression of the mammalian rRNA genes, post-initiation events are also important, as activator UBF has been shown to facilitate promoter escape by Pol I (Panov et al., 2006a), and binding of UBF throughout the rDNA repeat chromatin modulates elongation of Pol I transcription (Stefanovsky et al., 2006). The rate of rRNA synthesis and the linked processing events (Schneider et al., 2007) depend upon the efficiency by which Pol I negotiates rDNA chromatin, and our identification of the histone chaperone FACT as a Pol I
cofactor highlights the importance of chromatin dynamics in ribosomal DNA transcription. Indeed, various chromatin remodelers and modifiers with a function in Pol I-dependent transcription have recently been identified (reviewed in Birch and Zomerdijk, 2008), including NoRC, an ATP-dependent nucleosome remodeler (SNF2; Strohner et al, 2001; Li et al, 2006), which can additionally recruit histone deacetylases and DNA methyltransferases such as DNA methyltransferase 1 and 3 (Santoro et al, 2002; Zhou et al, 2002; Zhou and Grummt, 2005; Espada et al, 2007); WICH, a chromatin remodelling complex containing WSTF (Williams syndrome transcription factor) and SNF2h (Percipalle et al, 2006);
Cockayne syndrome group B (CSB) protein, a member of the SWI/SNF family of ATP-dependent chromatin remodelling activities (Bradsher et al., 2002); Tip60, a histone acetyl transferase complex (Halkidou et al., 2004) and histone methyltransferase G9a (Yuan et al., 2007b). Furthermore, chromatin transcription by mammalian Pol I appears to require the histone chaperone activities provided by nucleolin (Angevlo et al., 2006; Rickards et al., 2007) and nucleophosmin (B23; Okuwaki et al., 2001; Murano et al., 2008), as well as the activity of FACT. The involvement of multiple factors, with the potential for functional redundancy, both ensures the robustness of the system and implies that chromatin remodelling is crucial for transcription elongation by Pol I in cells.

New findings linking distinct histone tail modification patterns with active rDNA gene repeats further support the notion that rRNA expression is under epigenetic control (reviewed in Grummt and Pikaard, 2003; McStay, 2006; Birch and Zomerdiik, 2008); the promoters of actively transcribed mouse rRNA genes are hypomethylated (Santoro et al., 2002; Nemeth et al., 2008) and the associated histones are highly acetylated, with the opposite true of inactive gene promoters (Santoro et al., 2002). FACT activity has been shown to be influenced by histone modifications, such as trimethylation of H3K4, which marks active genes including those of the active rRNA gene repeats (Preuss and Pikaard, 2007), and monoubiquitination of H2A and H2B (Favre et al., 2006; Zhou et al., 2008). Furthermore, the chromatin remodeler CHD1, which binds H3K4me3 and cooperates with FACT (Sims et al., 2007), has been found at active rDNA repeats in yeast (Jones et al., 2007). It will be interesting to determine whether such histone modifications and interacting factors also influence rRNA gene expression through FACT. As FACT can be post-translationally modified, for example, by CK2 or PARP1 (Li et al., 2005; Heo et al., 2008), rRNA gene expression might also be rapidly modulated by such activities.

There are data to suggest that Pol III-transcribed genes are devoid of nucleosomes (Wittig and Wittig, 1982; Morse et al., 1992) and that Pol III has the intrinsic ability to transcribe a short mono-nucleosomal template in vitro without the need for additional activities (Studitsky et al., 1997). Nonetheless, a substantial number of studies suggest that chromatin remodelling and/or histone modification are required for initiation and elongation of Pol III transcription through nucleosomal arrays in vitro and in cells (Englander et al., 1993; Ura et al., 1997; Tse et al., 1998; Ng et al., 2002; Gelbart et al., 2005; Cavellan et al., 2006; Shivashawmy and Bhargava, 2006; Yuan et al., 2007a; Arimbasser and Bhargava, 2008). Furthermore, TFIIC, required at type I and 2 promoters of Pol III-transcribed genes, has intrinsic histone acetyltransferase activity (Hsieh et al., 1999; Kundu et al., 1999). Our study now implicates the histone chaperone FACT in the transcription by Pol III of small RNA genes in chromatin, thus further underscoring the importance of chromatin remodelling at actively transcribed Pol III genes. Previous immunofluorescence studies of FACT at Drosophila polytene chromosomes did not reveal FACT at the Pol III-transcribed SS rRNA gene cluster (Saunders et al., 2003). Perhaps this discrepancy is due in part to a low abundance of FACT at this cluster—in mammalian cells, our ChIP analyses suggest a lower occupancy of FACT at the Pol III-transcribed genes, compared with the Pol I- and Pol II-transcribed genes.

In Pol II transcription, some RNA Pol complexes were found associated with FACT, consistent with previous observations in Drosophila (Saunders et al., 2003), but the recruitment of FACT to active genes might be dependent primarily on its association with other chromatin remodelers, such as CHD1, and with trimethylation of histone H3 at Lys4 (Sims et al., 2005, 2007). The association of FACT with Pol III is probably also substoichiometric. In Pol I transcription, the unique stoichiometric association of FACT with Pol I complexes suggests that coupling of this histone chaperone to the Pol complexes may be required to achieve efficient rDNA transcription through chromatin, to meet the demand for the vast amounts of rRNAs necessary to support ribosome biogenesis during active cell growth and proliferation.

Although the exact nucleosomal nature of active mammalian rRNA chromatin is currently unknown, our findings imply that nucleosomal barriers are encountered by Pol I at active genes and/or at de novo activated rRNA genes during pioneering rounds of transcription. We propose that nucleosomal barriers can be overcome by Pol I-associated FACT activity, perhaps in conjunction with other FACT-like histone chaperones and chromatin remodelers, to allow for productive elongation of transcription and rRNA synthesis. Our data also strongly suggest that Pol III requires auxiliary factors with histone chaperone activity to facilitate transcription and passage of Pol III through nucleosomes in cells. Therefore, histone chaperone FACT could be universally involved in chromatin dynamics and transcription by all three nuclear RNA Pols in mammalian cells.

Materials and methods

Reconstitution of mono- and poly-nucleosomal DNA templates and the H2A–H2B dimer displacement assay

Preparation of mono- and poly-nucleosomal DNA templates is detailed in the Supplementary data. The 147 bp mouse mammary tumour virus NPS A plus an extra 31 bp 5’ of the positioning sequence (Flaus and Richmond, 1998) was used for the mono-nucleosomal template and a series of twelve SS rDNA NPSs was used to create the poly-nucleosomal template. Escherichia coli-expressed Xenopus laevis recombinant histones (Luger et al., 1997) were used to refold the octamer and these were reconstituted with the template DNA by salt dialysis. The mono-nucleosomal reconstituted templates were incubated with 5U of Avall (New England Biolabs) for 15 min at 37°C to digest the relatively small fraction of non-reconstituted DNA prior to and during the end-to-end transcription reaction.

Crosslinking of histones in the octamer of a mono-nucleosomal template with BS3 and the H2A–H2B dimer displacement assay are detailed in the Supplementary data.

Transcription assays

Transcription reactions with nucleosomal templates and nonspecific transcription reactions were performed essentially as described (Miller et al., 2001; Panov et al., 2006a) and are detailed in the Supplementary data. The radiolabelled RNA was analysed by denaturing gel electrophoresis (7.5 M urea and 8% polyacrylamide) with RNA markers (T3 and T7 RNA Pol body-labelled run-off transcripts). End-to-end transcript levels were quantified with the aid of a Fuji phosphorimager (and Aida software).

In the FACT antibody inhibition experiments, Pol I was preincubated with SSRP1-specific antibodies (Santa Cruz Biotechnology; sc-25382) or control rabbit IgG for 10 min at 30°C prior to the initiation of transcription on mono- or non-nucleosomal templates.

Pol I purification and mass spectrometry

Pol I was purified from HeLa nuclear extracts through a series of chromatographic steps, including Superose 6, DEAE Sepharose, SP
Seaphoros, Poros Heparin, and finally by a linear (0.15–0.35 M) KCl gradient elution from a Mono-S column (GE Healthcare) as described previously (Miller et al., 2001). Proteins in the Pol Ix peak fraction from the Mono-S column were size-fractionated in a 4–12% NuPAGE Bis–Tris gel (MES buffer; Invitrogen), and individual gel slices were dissected with trypsin and LysC. Tandem mass spectrometry (LC MS/MS) was used in peptide analysis and protein identification as described (Andersen et al., 2002).

**Immunoblotting, immunoprecipitation and immunofluorescence microscopy**

Antibodies specific for SSRP1 (Santa Cruz Biotechnology; sc-25382), Sp16 (sc-28734), PAF53 (Pol I subunit; BD Transduction Laboratories; P95220), hRP A9 (Pol I and III subunits; Miller et al., 2001) or hRN3 (affinity purified sheep polyclonal antibody; Miller et al., 2001) were used for immunoblotting and immunoprecipitation, and primary antibodies for SSRP1 (monoclonal antibody 10D1; Tan and Lee, 2004) and CAST/PAF49 (Bethyl Laboratories; A301-294A) were used in immunofluorescence microscopy as detailed in the Supplementary data.

The SSRP1 (10D1) monoclonal antibody was used for immunoprecipitation of FACT from HeLa cell nuclear extract, as detailed in the Supplementary data, and the blots were probed with antibodies for CAST (Pol I subunit; Bethyl Laboratories; A301-294A), RNA Pol II (mouse monoclonal antibody for the C-terminal domain of the largest subunit of Pol II; CTD4H8, Santa Cruz Biotechnology; sc-47701) and RPCS (Pol III subunit 5 or POLR3E; Abgent; AP1956c).

**RNA interference and analysis of rRNA synthesis**

RNA interference strategies are described in the Supplementary data. The 47S pre-rRNA levels were determined by northern blotting (probed with a 32P end-labelled oligonucleotide complementary to the 5′-end of the pre-rRNA; 81–125 relative to the transcription start site at +1; human rRNA sequence U13369) and S1 nuclease protection as described (James and Zomerdijk, 2004). Levels of the precursors for rRNA and 7SL RNA were determined by real-time PCR with an ABI Prism 7000 instrument using SYBR Green PCR Master Mix (both Applied Biosystems). Triplicate PCRs for each sample were carried out. Control ChIP assays with non specific antisera (IgG) were performed in each experiment. The relative proportions of immunoprecipitated DNA fragments were determined based on the threshold cycle (C) for each PCR product (Livak and Schmittgen, 2001). Data were quantitatively analysed according to the formula 2^−[ΔCt(Experiment)−ΔCt(Input)], which normalized the relative level of DNA (in relation to the input) specifically immunoprecipitated by the SSF1 antibody to that immunoprecipitated by the control IgG.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**A** (antisense); 7SL RNA gene (107–137 and 226–255), 5′-CCGCAATTGACAGCAGCCTCAATGAC-3′ (antisense); U6 snRNA gene (7–31 and 69–89), 5′-GCTTCGCGACGACATATCTAATAAT-3′ (antisense); 5′-AGCCATTTTGCGTGTCATCCTT-3′ (antisense); RNA701 gene (1–30 and 55–84, relative to the precursor transcript; intron: 38–58), 5′-GCTTCTGATAGCTCAGCTGGTAGAGCG-3′ (antisense); 5′-CCGAAATTGACAGCAGCCTCAATGAC-3′ (antisense); 7SL RNA gene (107–137 and 226–255), 5′-GTTGCCGACCTAACTGTCTACAAG-3′ (antisense); 5′-TATTCACAGGCCCATCCCACTACTAC-3′ (antisense). The primer sequences for the Tyr-rRNA and 7SL RNA genes were designed based on a previous report (Winter et al., 2000). PCR conditions: 25–27 cycles, with each cycle for 45 s at 94°C, for 45 s at 50–52°C and 40 s at 72°C. DNA samples from ChIP preparations were quantified by real-time PCR with an ABI Prism 7000 instrument using SYBR Green PCR Master Mix (both Applied Biosystems). Triplicate PCRs for each sample were carried out. Control ChIP assays with nonspecific antisera (IgG) were performed in each experiment. The relative proportions of immunoprecipitated DNA fragments were determined based on the threshold cycle (C) for each PCR product (Livak and Schmittgen, 2001). Data were quantitatively analysed according to the formula 2^−[ΔCt(Experiment)−ΔCt(Input)], which normalized the relative level of DNA (in relation to the input) specifically immunoprecipitated by the SSRP1 antibody to that immunoprecipitated by the control IgG.
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