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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

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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 rRNA 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 (Orphanides *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, downregulation 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 (Orphanides *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 Iα, purified from HeLa cell nuclear extracts in a multistep process that separates Pol Iα from the less abundant Pol Iβ 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 Iα 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 *Ava*II 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 *Ava*II 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 *Ava*II-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 5S 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 Iα was able to transcribe the poly-nucleosomal template (poly-N) processively to yield a full-length transcript of 2496 nt (Figure 1E, lane 1). The efficiency of transcription was $\sim$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 Iα 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^3) to crosslink histones within a pre-formed nucleosomal core particle. Nucleosomal DNA templates crosslinked with BS^3 (Figure 2A, lane 2) were incubated with Pol Iα in an end-to-end transcription assay.

Nucleosomal templates mock-treated with BS[3], treated with BS[3] 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 Iα and with Pol I, II and III complexes from HeLa cell nuclear extracts

The ability of Pol Iα 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 Iα 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

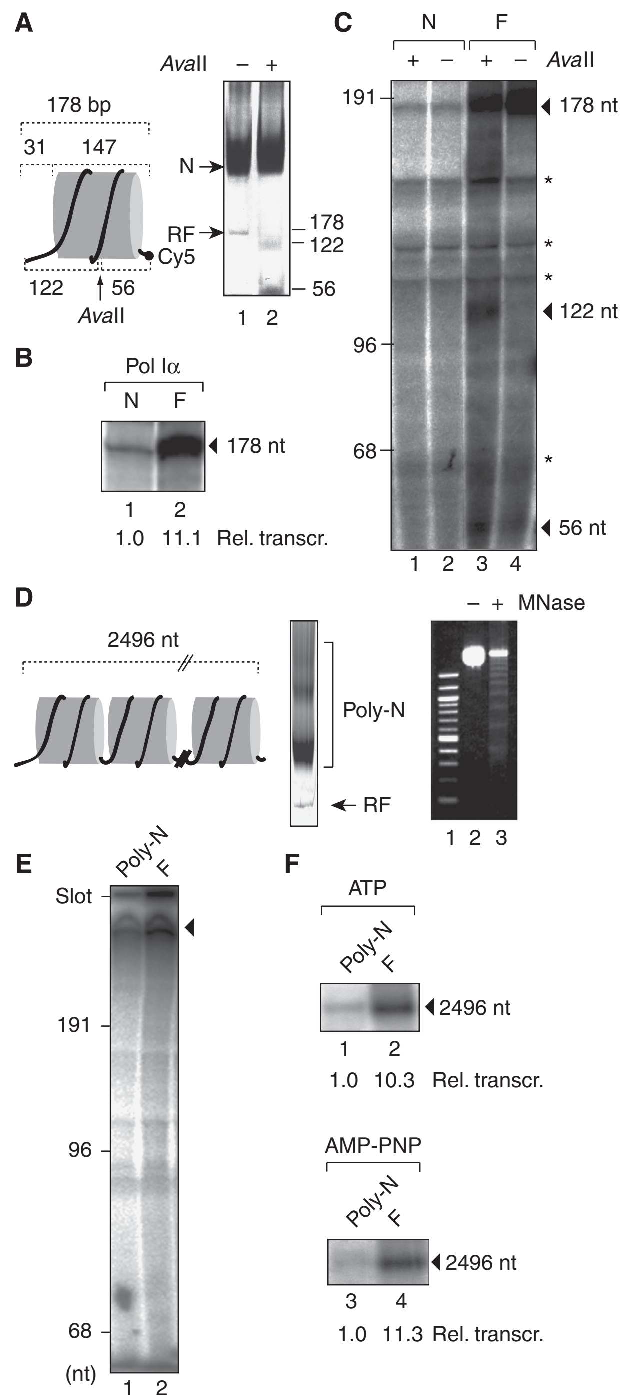

Figure 1 Pol Iα can transcribe through nucleosomal DNA *in vitro*. (**A**) Diagram of the reconstituted mono-nucleosomal template. A 3′-end Cy5-labelled 178-bp DNA fragment containing the MMTV nucleosome positioning sequence A and an additional 31 bp was reconstituted with recombinant histone octamers and analysed by 5% native polyacrylamide gel electrophoresis and Cy5 imaging (lane 1; N, mono-nucleosomal DNA; RF, residual nucleosome-free DNA). The presence of the nucleosome protects the *Ava*II site from digestion; *Ava*II digestion yields 122- and 56-bp DNA fragments (at the bottom of the gel) from the residual nucleosome-free DNA (lane 2), as revealed following SYPRO Green staining. (**B**) Mono-nucleosomal (N, lane 1) or nucleosome-free (F, lane 2) DNA templates were incubated with Pol Iα in a transcription reaction mix. Radiolabelled full-length transcripts (178 nt) were analysed on a 7.5 M urea 11% polyacrylamide gel and quantified with the aid of a phosphorimager. Transcript levels are expressed relative to the levels of transcripts detected in the nucleosomal transcription reactions (set at 1.0; rel. transcr.). (**C**) The 178 nt transcripts generated in Pol Iα transcription reactions with a mono-nucleosomal template are not due to transcription of residual nucleosome-free DNA in that reaction. Pol Iα transcription reactions (as in (B)) through a mono-nucleosomal (N, lanes 1 and 2) or nucleosome-free (F, lanes 3 and 4) DNA template were performed in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of *Ava*II. Transcripts were analysed as in (B); full-length transcripts of 178 nt and smaller transcripts of 122 and 56 nt, produced from *Ava*II-digested DNA templates, are indicated on the right of the gel image. RNA size markers are shown on the left of the gel image. Bands marked with an asterisk (*) are nonspecific background signals, largely independent of the level of Pol I transcription. (**D**) Diagram of the reconstituted poly-nucleosomal template. A linear DNA fragment of 2496 bp containing twelve 5S nucleosome-positioning sequences was reconstituted into chromatin (Poly-N) and a sample was analysed on a native 2% polyacrylamide–1% agarose composite gel, stained with SYPRO Green and scanned for fluorescence (left panel; RF indicates the residual nucleosome-free DNA following the reconstitution reaction). The Poly-N DNA, untreated (lane 2, right panel) or limit-digested with MNase (lane 3, right panel), was analysed on a 1.2% agarose gel and ethidium bromide-stained. A DNA size marker (100 bp ladder) was run in lane 1 (right panel). (**E**) Pol Iα transcription reactions with poly-nucleosomal (Poly-N, lane 1) or an equivalent amount of nucleosome-free (F, lane 2) DNA template (0.3 μg). Radiolabelled transcripts were analysed on an 8% denaturing polyacrylamide gel and detected by phosphorimaging. The full-length transcript signal is indicated. (**F**) Pol Iα transcription reactions with poly-nucleosomal (Poly-N, lanes 1 and 3) or non-nucleosomal (F, lanes 2 and 4) DNA templates (0.3 μg), included either NTPs (lanes 1 and 2) or an NTP mixture with a non-hydrolysable analogue of ATP, AMP-PNP, substituted for ATP (lanes 3 and 4). Radiolabelled transcripts (2496 nt) were analysed on a 1.2% formaldehyde-agarose gel and detected by phosphorimaging. Transcript levels were quantified and expressed as in (B).

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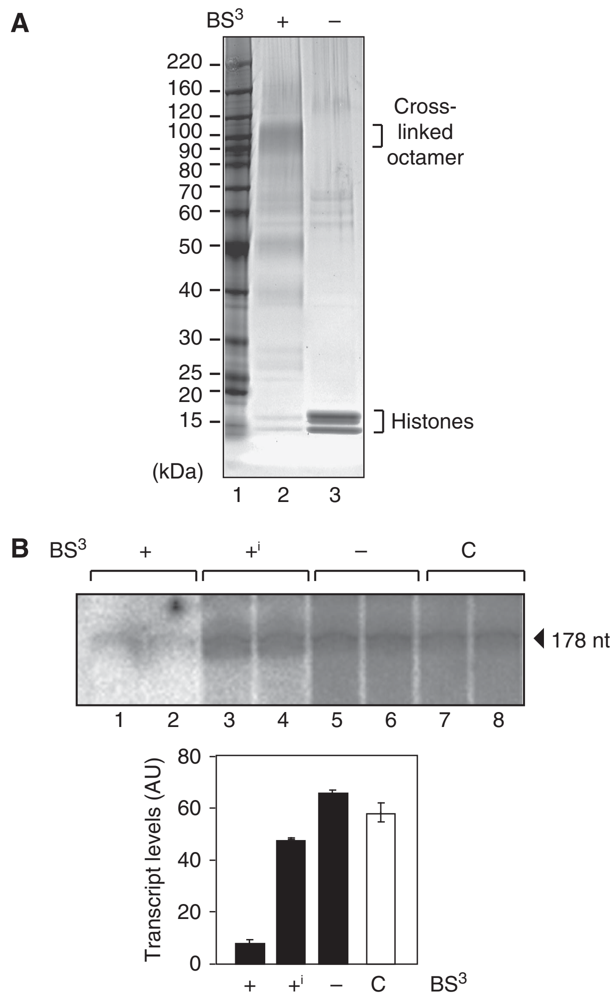

Figure 2 Pol I transcription of a mono-nucleosomal template is inhibited by crosslinking of the histones within the nucleosome. (**A**) Crosslinking of the histones in the octamer of the mono-nucleosomal template with the homo-bifunctional crosslinker bis(sulphosuccinimidyl) suberate (BS^3). Histones from the BS^3-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 BS^3 (+, lanes 1 and 2), inactivated BS^3 ($+^i$ 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 immunoprecipitates 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 Iα 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,

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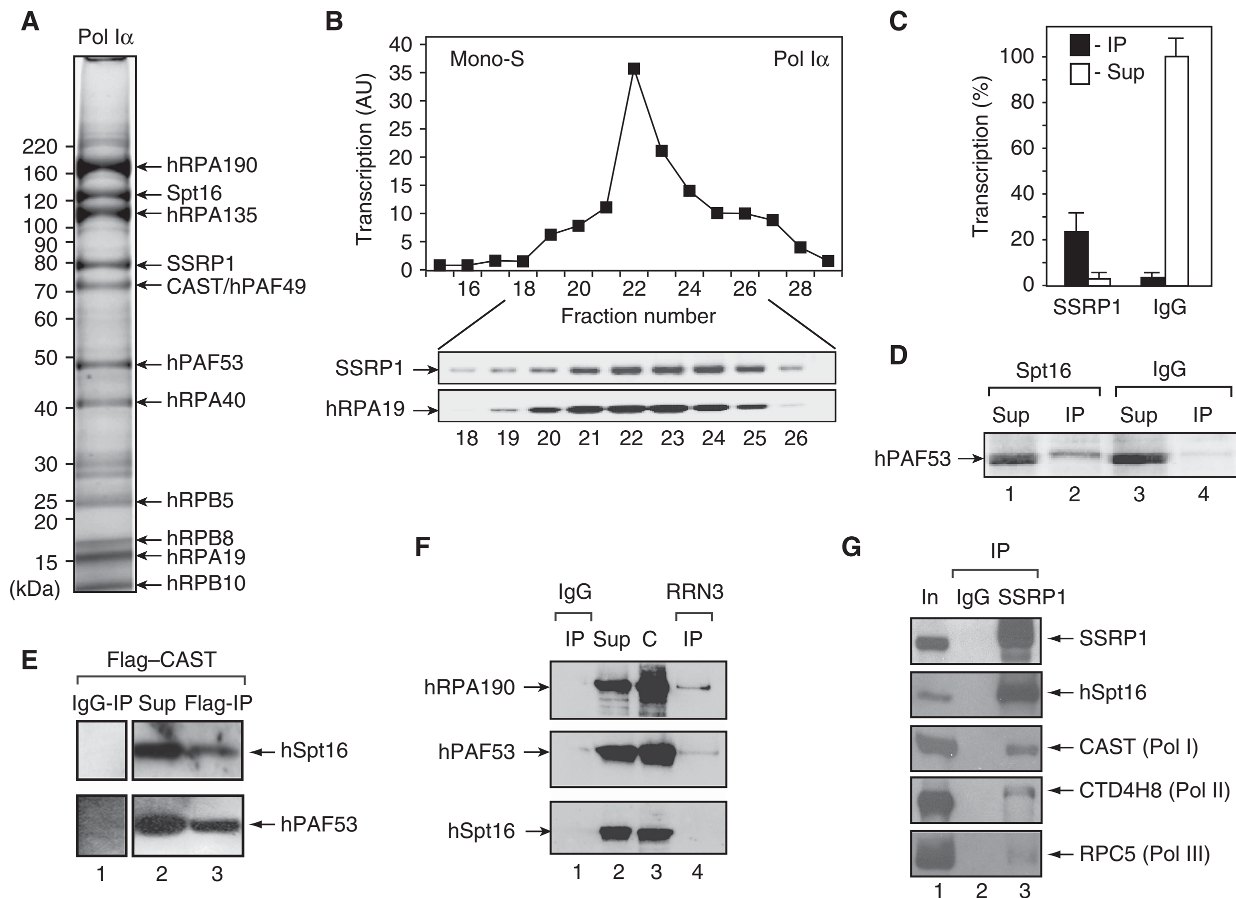

Figure 3 FACT associates with purified Pol Iα and with Pol I, II and III complexes from HeLa cell nuclear extract. (**A**) Mono-S fraction of Pol Iα, purified from HeLa cell nuclear extracts in a multistep process as detailed in the Materials and methods, was separated into its subunits and associated 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 Iα 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 Iα (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 Iα (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 I 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 PAF53. (**F**) Pol Iβ was immunoprecipitated through hRRN3 from a chromatography fraction containing both Pol Iα and Iβ (0.2 M KCl DEAE fraction; Miller *et al*, 2001). hRRN3-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 Iα (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.

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 remodeller RSC (Soutourina *et al*, 2006).

SSRP1-specific antibodies selectively inhibit nucleosomal transcription by Pol Iα

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 Iα with SSRP1-specific antibodies

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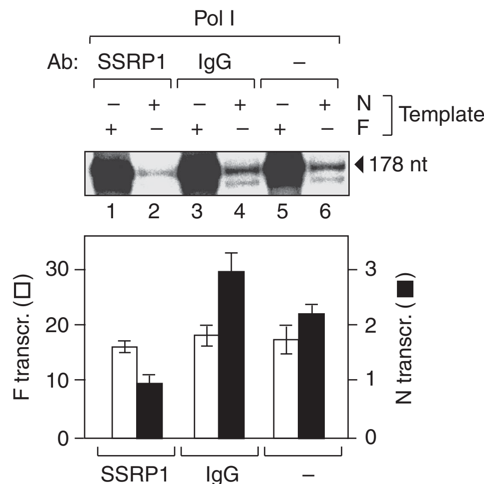

Figure 4 SSRP1-specific antibodies selectively inhibit nucleosomal transcription by Pol Iα. Pol Iα was pre-incubated with SSRP1 antibodies (1 μg, sc-25382; lanes 1 and 2), with control rabbit IgGs (lanes 3 and 4) or in buffer alone (lanes 5 and 6) prior to incubation with mono-nucleosomal templates (N; lanes 2, 4 and 6) or nucleosome-free templates (F; lanes 1, 3 and 5) in a transcription reaction mix. Radiolabelled transcripts were analysed by 7.5 M urea 11 % polyacrylamide gel electrophoresis and phosphorimaging. The two RNA bands around 178 nt are the products of bi-directional end-to-end transcription; due to the asymmetrical nature of the nucleosomal template, Pol I favours one DNA-end over the other. The 178-nt transcripts levels of two independent experiments were quantified (in arbitrary units) with the aid of a phosphorimager. The data of duplicate samples are presented as the average and range. Note the two scales for the *Y* axis; transcription signals (in arbitrary units) from the nucleosomal template (N, black bars) are approximately 10 times lower than those from the equivalent nucleosome-free DNA template (F, white bars).

impaired the ability of Pol Iα 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 Iα 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 nucleoli (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.

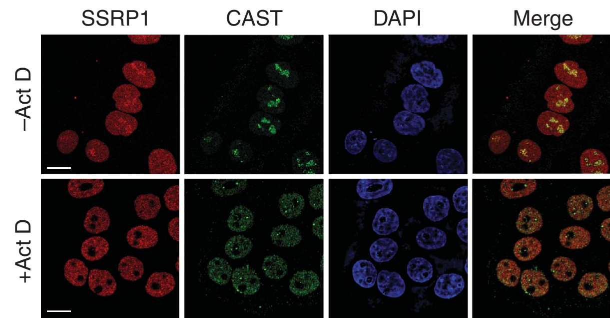

Figure 5 Subcellular localization of FACT and Pol I. Indirect immunofluorescence analysis was performed on HeLa cells to observe localization of endogenous SSRP1 (red) and CAST/hPAF49 (green). Before immunostaining, HeLa cells were cultured in the presence of 50 ng/ml actinomycin D for 30 min to inhibit Pol I activity (+Act D) or in its absence (–Act D). Nuclear DNA was stained by DAPI (blue). Images were captured by laser scanning confocal microscopy and single sections are shown (scale bar is 10 μm).

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, $tRNA^{Tyr}$ 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

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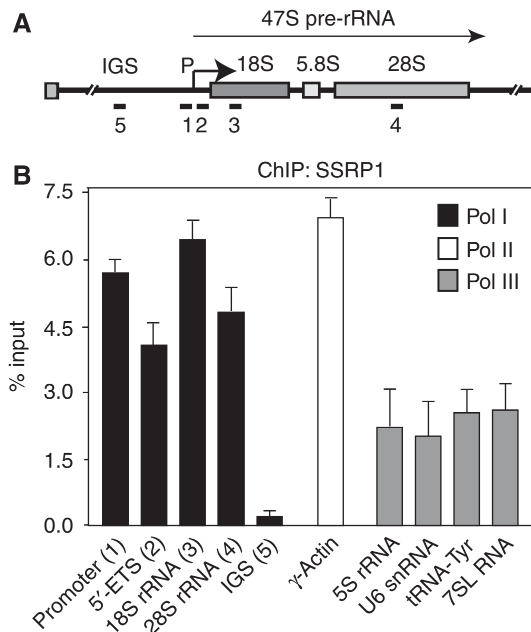

Figure 6 FACT is present at the chromatin of genes transcribed by all three nuclear RNA polymerases. (**A**) Schematic representation of the rDNA repeat region indicating the positions of the PCR primers used in chromatin immunoprecipitation (ChIP) analysis. (**B**) ChIP assays were performed on formaldehyde crosslinked chromatin from HeLa cells using antibodies specific for FACT subunit SSRP1 (monoclonal 10D1) or control mouse antibodies (IgG). Primer sets used in the quantitative real-time PCR for the Pol I-transcribed rDNA repeat were as follows: the ribosomal DNA promoter (promoter, 1), 5′-ETS (2), 18S (3) or 28S (4) gene region, or the non-transcribed intergenic spacer (IGS; 5) of the rDNA repeat (indicated in (A)). PCR primers also included those that detect the γ-actin gene transcribed by Pol II and four Pol III-transcribed genes (5S rRNA, U6 snRNA, $tRNA^{Tyr}$ and 7SL RNA). The bar graph shows the relative levels of bound DNA in the SSRP1 immunoprecipitates, as determined by quantitative RT–PCR; results from three independent ChIP experiments combined are expressed as a percentage of input chromatin (and standard deviation), normalized to control IgG samples.

immunofluorescence, following a short BrUTP pulse-labelling (Leung *et al*, 2004) of newly synthesized RNA in the nucleoli of HeLa cells in which SSRP1 expression was downregulated by the SSRP1-specific dsRNA (Figure 7C). Notably, there was no detectable effect of SSRP1-siRNA on synthesis of the first 40 nt of the pre-rRNA transcript (Figure 7D). Reductions in 47S pre-rRNA levels similar to those observed with synthetic siRNA for SSRP1 (Figure 7B) were obtained in a different RNAi experimental set-up, in which expression plasmids encoding an SSRP1- or Spt16-specific dsRNA were transfected into HeLa cells (Figure 7E).

Collectively, these results suggest that FACT has a specific function in rDNA transcription by Pol I and, crucially, that FACT appears to function at a stage following pre-initiation complex formation, initiation of transcription and transcription of the first 40 nt. The observed downregulation of rRNA synthesis is not, then, an indirect effect of decreased Pol II transcription leading to reduced availability of Pol I transcription initiation components. The finding that siRNA-mediated downregulation of FACT subunit expression reduces transcription elongation by Pol I through the rRNA genes is consistent with a role for FACT as a histone chaperone 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 $tRNA^{Tyr}$ 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 nucleolar 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

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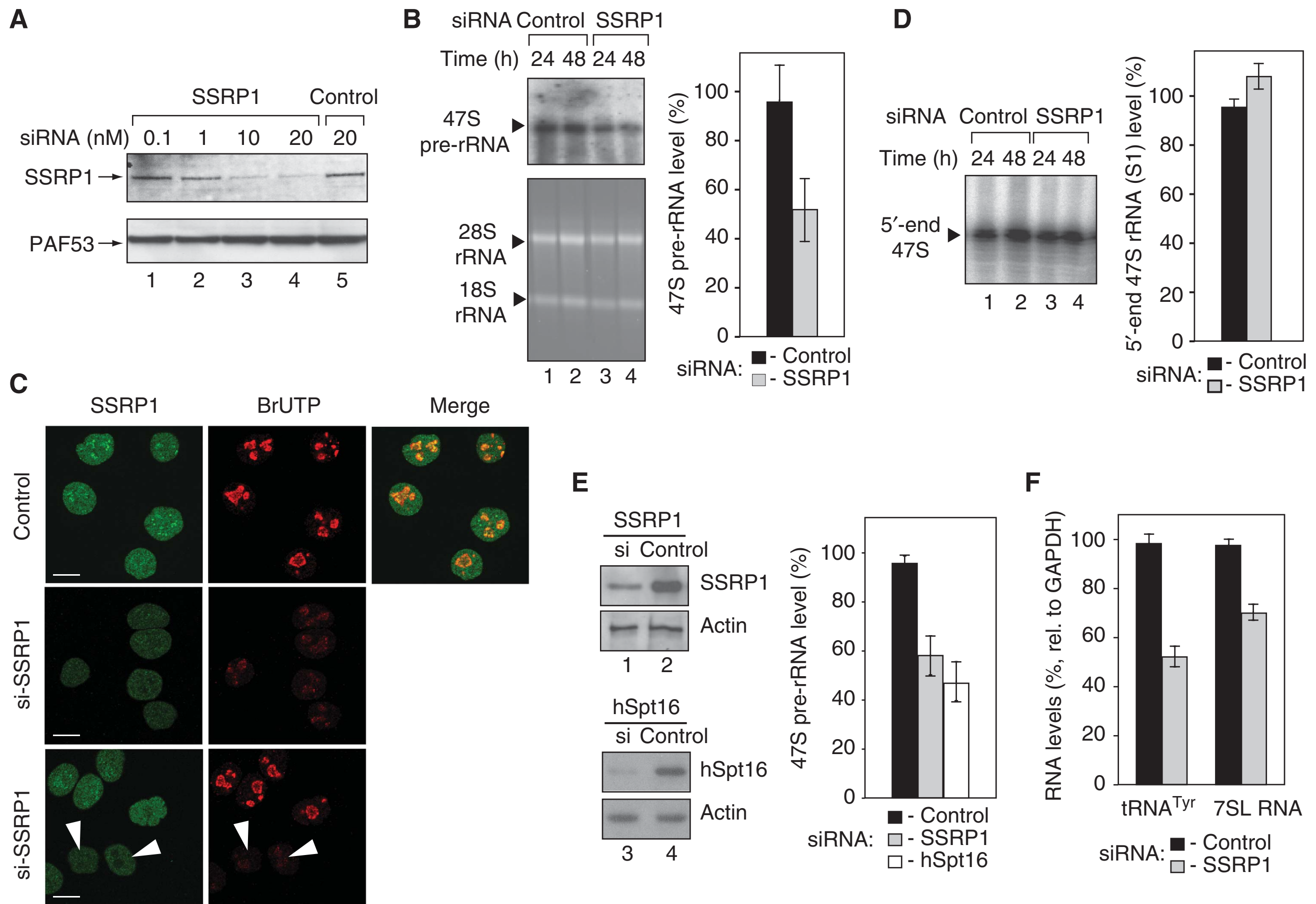

Figure 7 RNAi-mediated downregulation of FACT expression in cells leads to a decrease in the synthesis of RNAs from Pol I- and Pol III-transcribed genes. (**A**) Whole-cell extracts (20 μg) of HeLa cells transfected with various amounts of SSRP1-specific siRNAs (sc-37877; lanes 1–4) or with control siRNAs (control sc-36869, lane 5) were immunoblotted using antibodies specific for FACT subunit SSRP1 (top panel) or Pol I subunit PAF53 (bottom panel). (**B**) HeLa cells were transfected with 20 nM of SSRP1-specific siRNA (SSRP1, lanes 3 and 4) or control siRNA (control, lanes 1 and 2) as in (A), then total RNA was extracted from the cells at 24 h (lanes 1 and 3) and 48 h (lanes 2 and 4) post-transfections. The levels of 47S pre-rRNA were analysed in a northern blot probed with a ^{32}P-labelled DNA probe complementary to a region from +81 to +125 relative to the transcription start site (top panel). The levels of 28S and 18S rRNA were assessed in a 0.8% agarose denaturing gel, stained with ethidium bromide (bottom panel). The levels of 47S pre-rRNA (24 h post-transfections) of two independent experiments were quantified by phosphorimaging, normalized to 28S rRNA levels, calculated as a percentage relative to the maximum level (set at 100%) in the control siRNA-treated cells. The data of duplicate samples are presented as the average and range. (**C**) HeLa cells grown on coverslips were transfected with plasmids expressing control or SSRP1-specific siRNAs (si-SSRP1) and 54 h post-transfection nascent RNA synthesis was visualized through a 10 min BrUTP labelling of cells. Cells were probed with antibodies for SSRP1 (green) and BrdU (red). Within the same microscopy field, a mixture of cells can be seen in the bottom of the siSSRP1 panels; some cells have reduced levels of SSRP1 and BrUTP incorporation (white arrowheads), whereas others appear to have levels unaffected and hence serve as an internal reference. All images were generated at pre-set levels of brightness/contrast and laser intensity, by laser scanning confocal microscopy, and single sections are shown (scale bar is 10 μm). (**D**) Total RNA was extracted 24 h after HeLa cells were transfected with 20 nM of specific (SSRP1, grey bars) or control siRNA (control, black bars) as in (A). Samples were analysed by S1 nuclease protection with an oligonucleotide probe complementary to +1 to +40 of the 47S pre-rRNA. A representative phosphorimage of the protected 40 nt S1 probe is shown on the left. S1 signals from two experiments were quantified with the aid of a phosphorimager, normalized to 28S rRNA levels (as derived from the ethidium bromide image of the same samples analysed on denaturing agarose gels, see (B)), calculated as a percentage relative to the maximum level (set at 100%) in the control siRNA-treated cells. The data of duplicate samples are presented as the average and range. (**E**) RNAi-mediated downregulation of FACT expression in HeLa cells reduced pre-rRNA levels. RNAi-mediated downregulation of SSRP1 or hSpt16 expression was assessed by immunoblotting with antibodies for SSRP1 or hSpt16 (lanes 1 and 3, respectively) and compared with controls (lanes 2 and 4). Actin was used as a control for equal loading of the cell extracts. The levels of 47S pre-rRNA in SSRP1 (grey bar) and hSpt16 (white bar) RNAi-treated cells were determined by qRT–PCR from three independent experiments. The results were normalized to 18S rRNA levels of the respective samples, and the mean and standard deviation values have been plotted. (**F**) The levels of tRNA^{Tyr} and 7SL RNA from two independent experiments were determined by qRT–PCR in cells treated with SSRP1-RNAi (grey bar) or control RNAi (black bar). The levels were normalized to GAPDH mRNA levels and expressed as a percentage of those detected in control RNAi-treated cells. The data of duplicate samples are presented as the average and range.

cofactor highlights the importance of chromatin dynamics in ribosomal DNA transcription. Indeed, various chromatin remodellers 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 remodeller (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 (Angelov *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 Zomerdijk, 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 (Pavri *et al*, 2006; Zhou *et al*, 2008). Furthermore, the chromatin remodeller 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; Shivaswamy and Bhargava, 2006; Yuan *et al*, 2007a; Arimbasseri and Bhargava, 2008). Furthermore, TFIIIC, required at type 1 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 5S 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 remodellers, 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 rDNA 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 remodellers, 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 5S 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 5 U of *Ava*II (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 BS^3 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 Iα 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 Iα purification and mass spectrometry

Pol Iα was purified from HeLa nuclear extracts through a series of chromatographic steps, including Superose 6, DEAE Sepharose, SP

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Sepharose, 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 Iα 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 digested 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), Spt16 (sc-28734), PAF53 (Pol I subunit; BD Transduction Laboratories; P95220), hRPA19 (Pol I and III subunits; Miller *et al*, 2001) or hRRN3 (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 RPC5 (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 ^{32}P end-labelled oligonucleotide complementary to the 5′-end of the pre-rRNA; 81–125 relative to the transcription start site at +1; human rDNA sequence U13369) and S1 nuclease protection as described (James and Zomerdijk, 2004). Levels of the precursors for $tRNA^{Tyr}$ and 7SL RNA were determined by quantitative RT–PCR (with primers identical to those used in the ChIP assay, see below) and normalized to GAPDH mRNA levels as described in the Supplementary data. BrUTP incorporation and microscopy are detailed in the Supplementary data.

ChIP

ChIP assays were performed essentially as described (Tan *et al*, 2006). Briefly, exponentially growing HeLa cells were crosslinked with 1% formaldehyde for 10 min at 37°C. The nuclei were isolated and sonicated to yield chromatin fragments of ~500–600 bp in length. The sheared chromatin was immunoprecipitated overnight with protein G-agarose previously bound with the SSRP1 monoclonal antibody (10D1) or control IgG antibody. After extensive washes, the immunoprecipitates were subjected to deproteination and crosslinking reversal. The following primer sets in the human rDNA repeat were used for the quantitative real-time PCR analyses (numbering according to the human rDNA repeat sequence U13369; 1 is the transcription start site): rDNA promoter (42787–42811 and 9–33), 5′-ETS (1477–1496 and 1555–1572), 18S rRNA gene (3990–4010 and 4072–4092), 28S rRNA gene (9546–9565 and 9869–9889) and the IGS (35176–35200 and 35231–35346). For the Pol II-transcribed γ-actin gene (990–1009 and 1102–1123) we used: 5′-GCTGTTCCAGGCTCTGTTCC-3′ (sense), 5′-ATGCTCACACGCCA-CAACATGC-3′ (antisense). The primers for the Pol III-transcribed genes were as follows: 5S rRNA gene (6–24 and 84–103), 5′-CGG CCATACCACCCTGAAC-3′ (sense), 5′-GCGGTCTCCCATCCAAGTAC-3′ (antisense); U6 snRNA gene (7–31 and 69–89), 5′-GCTTCGGCAG-CACATATACTAAAAT-3′ (sense), 5′-ACGAATTTGCGTGTCATCCTT-3′ (antisense); $tRNA^{Tyr}$ gene (1–30 and 55–84, relative to the precursor transcript; intron: 38–58), 5′-CCTTCGATAGCTCAGCTGGTAGAGCG-GAGG-3′ (sense), 5′-CGGAATTGAACCAGCGACCTAAGGATGTCC-3′ (antisense); 7SL RNA gene (107–137 and 226–255), 5′-GTGTCCGCA CTAAGTTCGGCATCAATATGG-3′ (sense), 5′-TATTCACAGGCGC-GATCCCACTACTGATC-3′ (antisense). The primer sequences for the Tyr-tRNA 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_t) for each PCR product (Livak and Schmittgen, 2001). Data were quantitatively analysed according to the formula $2^{-\Delta[C_t(\mathrm{IP})-C_t(\mathrm{input})]} - 2^{-\Delta[C_t(\mathrm{control\ IgG})-C_t(\mathrm{input})]}$, 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.

Supplementary data

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

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