SUPPORTING ONLINE MATERIAL

Materials and Methods

Plasmids for expression of recombinant FACT in Sf9 cells.

The original cloning of hSSRP1 was described in (1), and isolation of hSPT16 cDNA was described in (2). The open reading frame (ORF) sequence of SSRP1 was amplified using the polymerase chain reaction (PCR) and cloned into the pET28b vector (Novagen) to generate N-terminally 6His-tagged SSRP1. 6His-SSRP1 was subcloned into the pVL1392 baculovirus transfer vector (PharMingen) at the NotI and SmaI sites. The hSPT16 sequence was assembled in the pET28a vector from several fragments of the ORF. A Flag-tag was added at the N-terminus of the hSTP16 ORF by PCR amplification of the N-terminal fragment using an oligonucleotide primer containing Flag sequence. The Flag-hSPT16 sequence was excised from the pET28 vector using NotI and BamHI and subcloned into the pVL1393 baculovirus transfer vector at the NotI and BglII sites. Flag-hSPT16ΔC was generated as follows: the pET28a-Flag-hSPT16 plasmid was digested with NotI and SpeI, which produced a Flag-hSPT16 fragment that lacked the last 684 base pairs. The resulting fragment was subcloned into the pVL1392 baculovirus transfer vector at the NotI and Xbal sites.

Expression and purification of recombinant FACT and its subunits.

Sf9 cells were infected at a multiplicity of infection of 10 with baculovirus containing either 6His-hSSRP1, or Flag-hSTP16, or Flag-hSPT16ΔC and incubated at 27°C for 72 h. FACT subunits were either expressed separately or co-expressed. The infected cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in FLAG-600 buffer (600 mM NaCl, 20 mM HEPES, pH7.6, 0.1% NP-40, 15% glycerol, 1mM PMSF). The cell suspension was subject to 3
freeze-thaw cycles in liquid N$_2$ and a warm water bath. To reduce the viscosity of the whole-cell extract, the DNA was sheared by brief sonication. The recombinant FACT complex was purified in two steps. First, after incubation of the cell extract with anti-Flag M2-agarose (Sigma) for 4 h to overnight at 4°C, the resin was washed extensively with FLAG-600 buffer and equilibrated with FLAG-100 buffer. Bound proteins were eluted in the presence of 0.2 mg/ml Flag peptide (Sigma). In the second purification step, peak FACT fractions that had been eluted from the M2-agarose beads were incubated with Ni-NTA agarose (Quiagen) for 2 h at 4°C. The resin was washed extensively with a buffer containing 10 mM imidazole, and the bound proteins were eluted with 250 mM imidazole. Peak fractions containing the FACT complex were dialyzed against BC-100 buffer (100 mM KCl, 10 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 20% glycerol, 10mM β-2-mercaptoethanol, 0.2 mM PMSF) and stored at –80 °C.

**In vitro transcription assays.**

The transcription assays presented in Figs. 2, 3 and S2 were performed in a highly defined Pol II-dependent transcription system (3). Chromatin templates were assembled using RSF (4) and native core histones isolated from HeLa cells. Native FACT was purified from HeLa cells as described (5). Native and recombinant FACT was titrated into the transcription reactions, as indicated in the figures.

The strategy used in the experiments shown in Fig. 3, B-D, was as described in (6). In brief, nucleosomes were reconstituted on the 204-bp DNA fragment by octamer exchange method (7) using chicken erythrocyte donor chromatin. The nucleoprotein complexes were resolved by native polyacrylamide gel electrophoresis (8). Yeast Pol II was purified from yeast extracts, and authentic elongation complexes containing His-tag-
immobilized Pol II and the 9-nt RNA (EC9) were assembled on the 50-bp DNA fragment, washed, and transcribed as described in (6). After assembly, the elongation complex containing the 9-nt RNA (EC9) was labeled with $\gamma^{-32}P$ ATP (if the RNA was to be labeled) and directly ligated to the 204-bp DNA or nucleosomal templates, incubated in the presence of either 200 $\mu$M each of ATP, CTP, and GTP only or with 0.5 $\mu$M $\alpha$-32P GTP (3000Ci/mmol) to obtain pulse-labeled EC45. EC45 was either first eluted into solution with imidazole or transcribed in the immobilized state. Transcription was conducted in the presence of all NTPs at various KCl concentrations (6), with or without 1 $\mu$g of human FACT. Pulse-labeled EC64 was obtained by incubation of EC45 with 10 $\mu$M each of GTP and UTP, and then with 100 $\mu$M each of ATP, CTP, 0.5 $\mu$M $\alpha$-32P GTP (3000Ci/mmol) for 10 min. Each extension step was followed by washing the resin three times with 1 ml of TB300 and two times with 1 ml of TB40. Transcription was conducted in the presence of all NTPs at various KCl concentrations, with or without 0.6 $\mu$g of human rFACT or rFACT$\Delta$C. The products of the transcription reaction were analyzed in two assays: first, labeled RNA was resolved on an 8 % denaturing polyacrylamide gel, and second, the resin was collected by centrifugation, and the supernatant, which contained the fully transcribed templates, was analyzed by electrophoresis on a native polyacrylamide gel. Reconstitutes in the gels were quantified using a PhosphorImager.

**Immunoprecipitations.**

HeLa cell mononucleosomes, as well as H2A/H2B dimers and H3/H4 tetramers were prepared as described elsewhere (7). In immunoprecipitation (IP) experiments, 5 $\mu$g of FACT or Flag-Spt16 were incubated with 20 $\mu$l of M2-agarose for 1 h at 4°C in BC-
100 buffer. The unbound proteins were washed away, and 5 µg of mononucleosomes, dimers, or tetramers were added and incubated with the beads for 2 h to overnight at 4°C. The resin was washed extensively with BC-150, and bound material was eluted with 0.3 mg/ml Flag-peptide for 30 min at 4°C. A similar procedure was used for the FACT and 6His-SSRP1 IP experiments except that Ni-NTA agarose was used as the resin and elutions were performed with 300 mM imidazole.

**Fluorescent labeling of core histones and generation of immobilized dinucleosomes.**

Recombinant *Xenopus laevis* core histones were expressed in bacteria and purified as described in (4). A cysteine residue was introduced in histone H2B in place of K113 by site directed mutagenesis (H2BK113C). Wild type histone H3 contains a cysteine at position 110. H3/H4 tetramers and H2A/H2BK113C dimers were reconstituted by mixing equimolar amounts of each protein and dialyzing the mixture against 2M NaCl, 10 mM Tris-HCl pH 7.6, 1 mM EDTA (9). Reconstituted dimers and tetramers were labeled with maleimide derivatives of tetramethyl-rhodamine (TMR) and fluorescein (F) (Molecular Probes), respectively, under native conditions in the presence of TCEP (PIERCE) as described (10). Core histones were resolved by 18% SDS-PAGE. The gels were scanned on a FluorImager 595, (Molecular Dynamics) at excitation wavelengths of 488 nm for the F- and 514 nm for the TMR-labeled proteins. Emission was detected at the wavelengths of 530 nm and 610 nm for the F- and TMR-labeled proteins, respectively.

DNA templates containing two tandem repeats of the 5S rDNA nucleosome positioning sequence of *L. variegatus* (11) were biotinylated at one end in the Klenow fill-in reaction. Dinucleosomes assembled using the salt dialysis method were purified away
from free DNA and core histones by sucrose gradient sedimentation. The purified labeled dinucleosomes were immobilized on streptavidin-coated magnetic beads (Dynal).

Supplementary Figures

Fig. S1

A

Coomassie Stain

Western Blot

B

SSRP1 Spt16 SSRP1+Spt16

IP: Ni-NTA-Agarose

SSRP1 Spt16 SSRP1+Spt16

IP: M2-Agarose

WB: α-SSRP1

α-Spt16

C

Naked DNA 0 250 500 250 500 FACT (ng)
Figure S1. Reconstitution of FACT activity with recombinant proteins.

Following the original purification of FACT from HeLa cells and cloning of the respective genes, the FACT complex was reconstituted from baculovirus-expressed recombinant FLAG-hSpt16 and 6His-SSRP1 proteins (A and B). The activity of recombinant FACT was tested in an *in vitro* transcription system on chromatin templates that had been assembled either with *Drosophila* S-190 extract or with RSF (4). In both systems, recombinant FACT displayed activity comparable to that of the native complex (C and data not shown).

A. Isolation of recombinant proteins from Sf9 cells that were either co-infected with both SSRP1- and Spt16-expressing baculoviruses (lane 1) or infected with individual viruses: SSRP1 (lane 2) and Spt16 (lane 3). Purified recombinant proteins were analyzed by Coomassie Blue staining (left panel) and western blotting using anti-FACTp140 (2) and anti-SSRP1 antibodies (right panel).

B. Interaction between recombinant proteins. Recombinant FACT subunits were incubated together for 1 h at 4°C and precipitated either with anti-Flag M2 agarose (right panel) or Ni-NTA agarose (left panel). Bound proteins were eluted with SDS-PAGE sample buffer, resolved on 7.5% gel and detected by western blotting.

C. Reconstitution of FACT activity from recombinant proteins: transcription of Gal4-VP16-remodeled chromatin templates assembled using RSF. Native and recombinant FACT were added to a highly defined reconstituted Pol II-driven transcription system.
Figure S2. A schematic of full-length and C-terminally truncated hSpt16. The sequence of the Spt16 C-terminus is shown with acidic residues highlighted in red.
References


