SUPPLEMENTAL INFORMATION

Mediator-regulated transcription through the +1 nucleosome

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein purification

Expression and purification of recombinant core histones (H2A, H2B, H3, H4) has been described (Barrero and Malik, 2006). To express SII as a recombinant protein in E. coli, its full-length cDNA (Yoo et al., 1991) was subcloned into the pET11d-6His vector. Following expression in BL21(DE3)pLysS E. coli, the protein was purified over Ni-NTA agarose and S-Sepharose. The triple mutant (S261A,D262A,E263A) was generated by PCR-based methods. For details on purification of other factors used here, see Fig. S2 and Fig. S4.

Template Constructions

As described under Experimental Procedures in the main text, the d19B, d39B and d134B templates were assembled from modular components consisting of an arm that contained the HNF4 binding sites and the core promoter and a corresponding "601" sequence (Lowary and Widom, 1998). The appropriate arms were released from their pGEM-T (Promega) backbones by digestion with Sca I and Ava I and gel-purified (Qiagen). Corresponding biotinylated PCR products containing the 601 sequence were also digested with Ava I and gel purified.

Ligations for the arms and 601-containing fragments (chromatinized or as naked DNA) were set up as follows. Typical ligations for generation of templates sufficient for 12 transcription reactions were done in 360 μl and contained 50 mM Tris.HCl (pH 7.9 at 4 ^{o}C), 10 mM $MgCl_2$, 10 mM DTT, 1.67 mM ATP, and 4800 units T4 DNA ligase (NEB). The reactions contained 1.8 μg of the arm and 100 ng of the appropriate post-dialysis 601 fragment (see below) and were incubated for 16 hr at 15 ^{o}C.

Chromatin assembly

Mononucleosome assembly was achieved by salt-dialysis. In typical reactions, the appropriate Ava I-digested 601 fragment (600 ng) was mixed with 1.4 μg recombinant histones at 2 M NaCl in 50 μl buffer containing 10 mM Tris.HCl (pH 7.9 at 4 ^{o}C), 0.2 mM EDTA, and 0.1% NP-40; for naked DNA templates, histones were omitted. Samples were placed in Millipore Slide-a-Lyzer tubes and dialyzed to 30 mM NaCl in the same buffer. Dialysis was carried out over 20 hr using a peristaltic pump to slowly deliver the diluent (10 mM Tris.HCl [pH 7.9 at 4 ^{o}C], 0.2 mM EDTA, and 0.1% NP-40). For monitoring efficiency of mononucleosome formation, end-labeled 601 fragments were used and assessed by EMSA using previously described conditions (Malik et al., 1998).

In vitro transcription assays

For in vitro transcription, ligated templates were diluted 4-fold in transcription buffer (TB: 50 mM Hepes.KOH [pH 8.2], 12 mM Tris.HCl [pH 7.9 at 4 ^{o}C], 4 mM $MgCl_2$, 0.1 mM EDTA, 12 % glycerol, 0.2 mg/ml BSA, 0. 5 mM DTT, 0.05 % NP-40) and immobilized to M280 Dynabeads. After washing with TB, in vitro transcription reactions with these templates were done using pure components (see also Fig. S2 and Fig. S4) essentially as described (Malik and Roeder, 2003). Basic reactions were reconstituted with all the GTFs, Pol II and PC4; Mediator, SII and other chromatin cofactors were added during PIC formation as specified in figure legends. For standard reactions, PIC formation was first allowed to take place (45 min, 30^{o}C) and transcription was initiated by NTP addition (100 μM CpA, 100 μM dATP, 25 μM each of ATP, GTP, UTP, and 5 μM α^{32}P-CTP) for variable times, as specified. Note that because concentrations of NTP, including ATP, had to be reduced to minimize background in the system, dATP was included as a supplementary energy source for reactions dependent on TFIIH and PBAF (see Fig. S7).

In abortive initiation and n-mer formation reactions, NTP compositions (including ATP/dATP) varied and are specified in figure legends. Except for Fig. S6, where acetyl CoA dependence was specifically assessed, all reactions in an individual experiment in which p300 effects were analyzed contained acetyl CoA. Reactions were stopped by concentrating the beads, removing the supernatant and resuspending the beads in 10 mM EDTA, 0.1 % SDS, and 0.25 mg/ml proteinase K. After digestion, supernatants were processed for electrophoresis (typically on 8% PAG containing 7 M urea). To visualize oligomeric RNAs, supernatants from the transcription reactions were cipped and analyzed directly by electrophoresis on 25% PAG containing 7 M urea (Malik et al., 1998). Gels were autoradiographed; selected experiments were quantified (see Main Text).

PIC recruitment assay

Binding reactions were set up exactly as described for the transcription assays except that they were scaled up 5 times. After binding (but without exposing to NTPs), the beads were washed with TB (4 X 400 μl) and eluted in SDS-PAGE sample buffer. After electrophoresis, filters were immunoblotted with specified antibodies.

SUPPLEMENTAL FIGURES

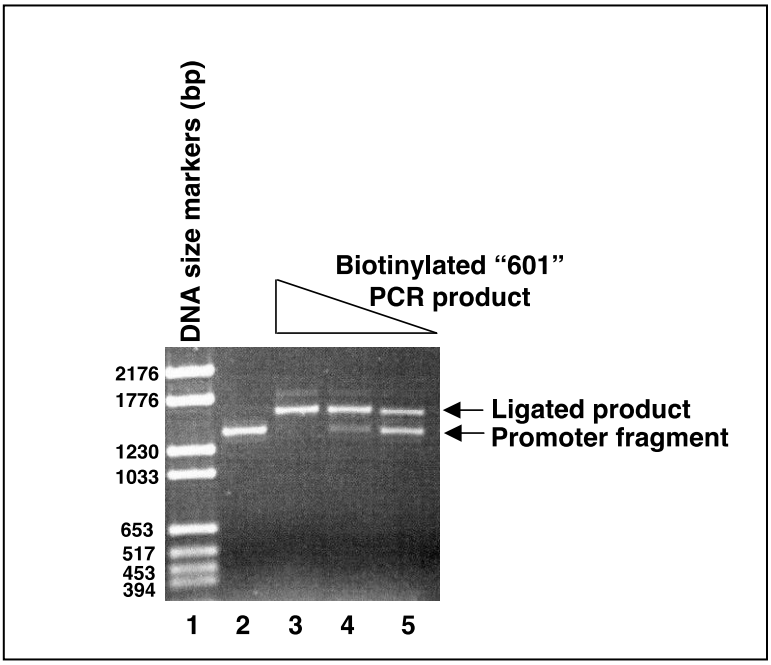

Figure S1 (related to main Figure 1) Ligation efficiency of the d39B template

A gel-purified Sca1-Ava1 fragment containing the Ad ML core promoter and HNF4 binding sites (lane 2) was ligated with varying amounts of Ava I-digested biotinylated PCR product bearing the 601 sequence (lanes 3-5) as described in Experimental Procedures. After ligation the products were extracted with phenol-chloroform and precipitated with ethanol. Products were analyzed by electrophoresis on a 1.0% agarose gel and visualized by ethidium bromide staining. The ligated product and parent fragment are highlighted. DNA size markers were run in lane 1.

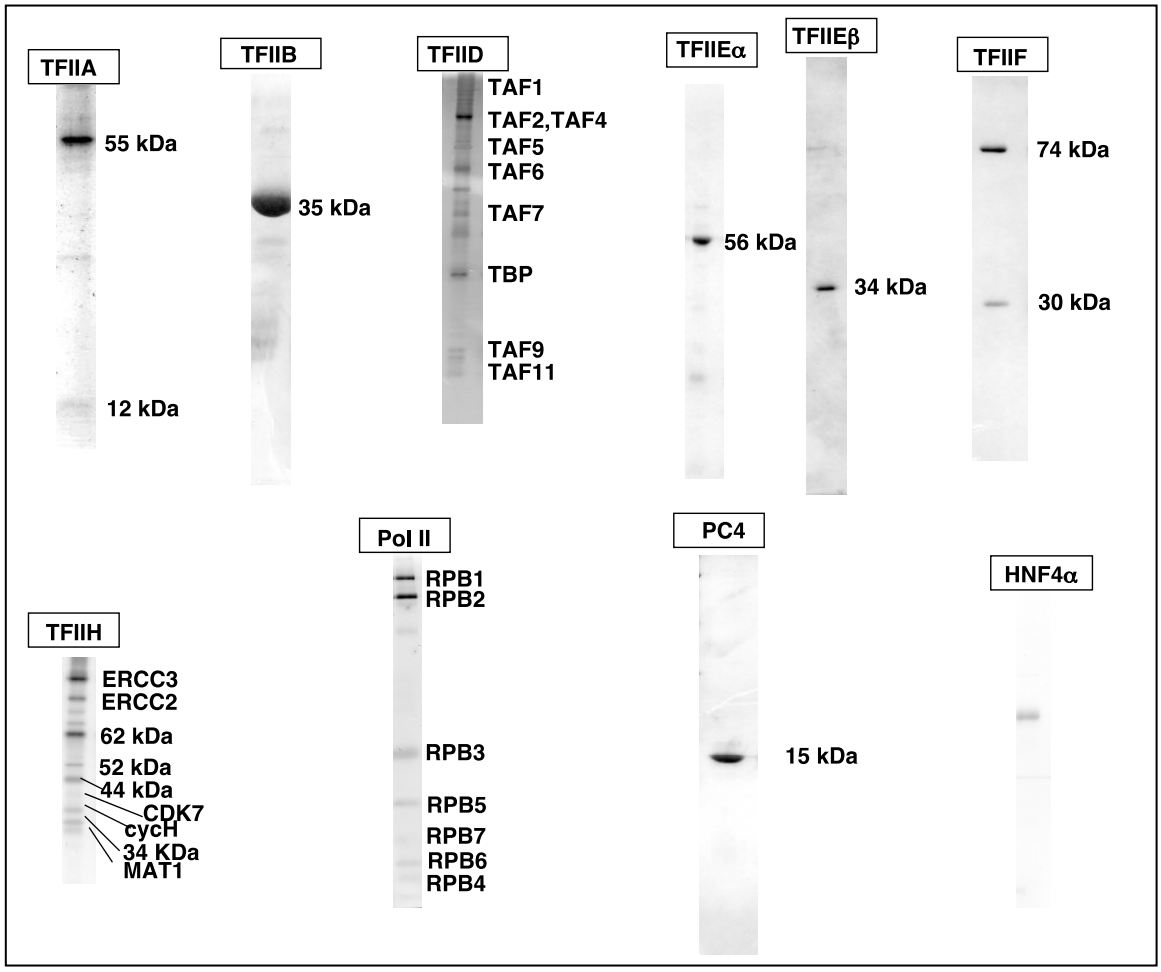

Figure S2 (related to main Figure 2) Basic components of the reconstituted in vitro transcription system

Basic transcription reactions were reconstituted from the above components. GTFs (TFIIB, TFIID, TFIIE [consisting of separately purified TFIIEα and TFIIEβ subunits], TFIIF, and TFIIH), PC4 and Pol II were purified as described (Malik and Roeder, 2003). TFIIA was expressed in Sf9 through a baculovirus vector that expressed both p55 and p12. Nuclear extract from infected cells was purified over Ni-NTA and DE52. HNF4α purification has also been described (Barrero and Malik, 2006). TFIID, TFIIH and Pol II preparations were stained with silver following SDS-PAGE; the rest with Coomassie.

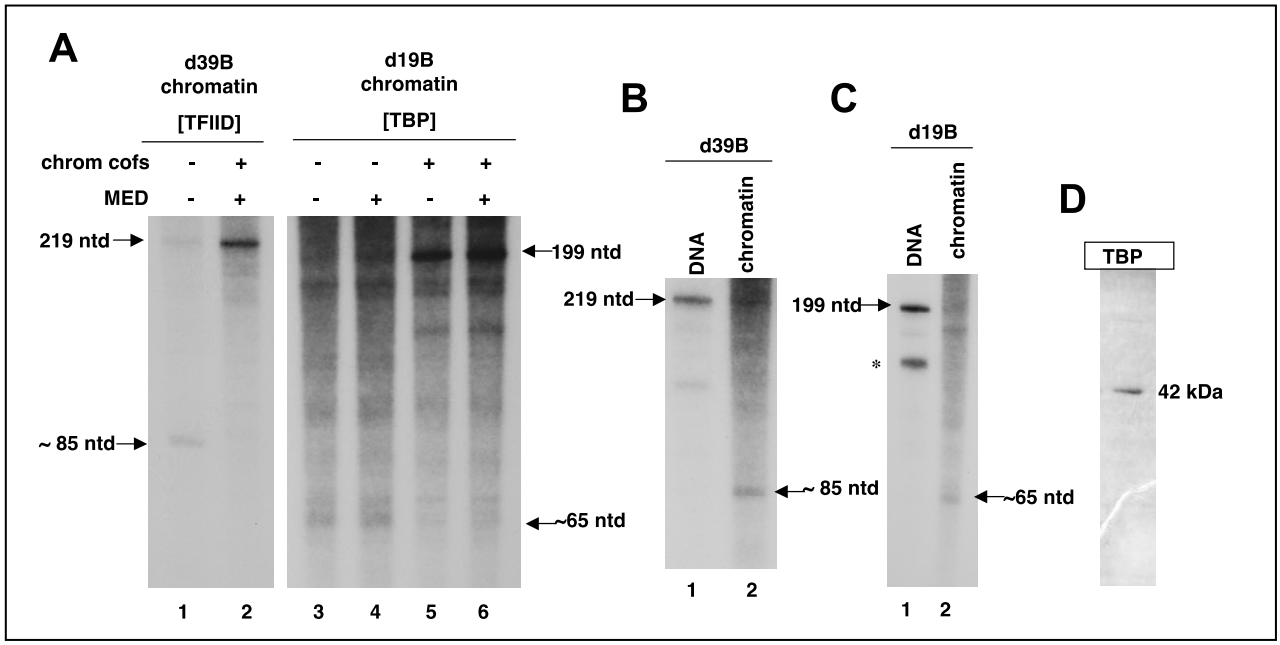

Figure S3 (related to main Figure 2) Additional in vitro transcription analyses of the d19B and d39B templates

A. Chromatinized d19B template was subjected to in vitro transcription in the presence of the indicated factors as described in the legend to Fig. 2B except that TBP was used in lieu of TFIID (lanes 3-6). Chromatin cofactors (cofs) included SII, FACT, p300, and PBAF. For reference, chromatinized d39B (lanes 1, 2) was also subjected to in vitro transcription under standard reaction conditions (containing TFIID). The full-length and paused products for each template are identified. An equivalent mononucleosomal template bearing the ligated 601 sequence in the opposite orientation (orientation "A";) did not yield the paused band (data not shown). The latter is consistent with published results (Bondarenko et al., 2006) that described a polarity associated with the nucleosome-induced blockage to Pol II movement. **B, C.** Comparison of products generated by naked DNA and chromatinized versions of d39B (panel B) and d19B (panel C). In vitro transcription reactions were performed as described in the legend to

Fig. 2B except that TBP was used in lieu of TFIID in the case of the d19B templates. Reactions contained Mediator but no chromatin cofactors were added. The full-length and paused products for each template are identified. Asterisk marks a non-specific product that is sometimes seen with short naked DNAs. **D.** Coomassie Blue stained preparation of recombinant TBP used in the transcription reactions.

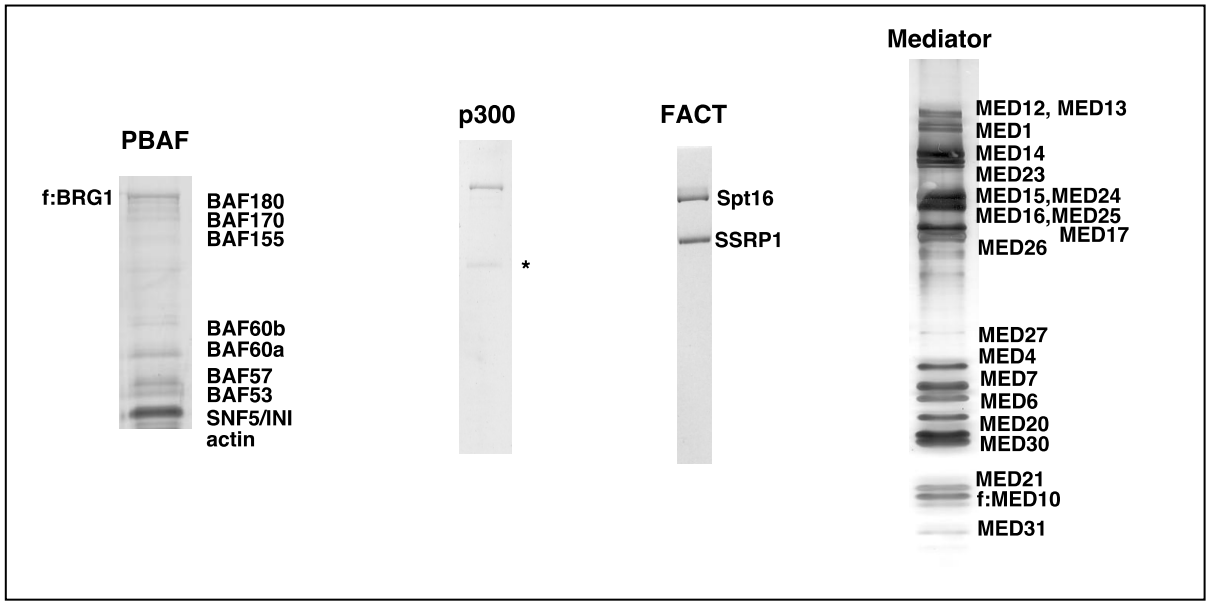

Fig. S4 (related to main Figure 3) Mediator and chromatin cofactor preparations

The purification of the chromatin coactivator p300 has been described (Barrero and Malik, 2006). PBAF was purified from nuclear extract of a f:BRG tagged HeLa cell line (Sif et al., 2001): extract was fractionated over phosphocellulose P11 and finally affinity purified over M2 agarose. FACT was purified from Sf9 cell nuclear extract after baculovirus expression of its constituent subunits, SSRP1 and Spt16 (Belotserkovskaya et al., 2003). Mediator was purified from a HeLa cell line stably expressing the FLAG-MED10/NUT2 subunit, as described in detail (Malik and Roeder, 2003). This preparation is free of detectible P-TEFb (by immunoblotting). The Mediator preparation was stained with silver following SDS-PAGE; the rest with Coomassie.

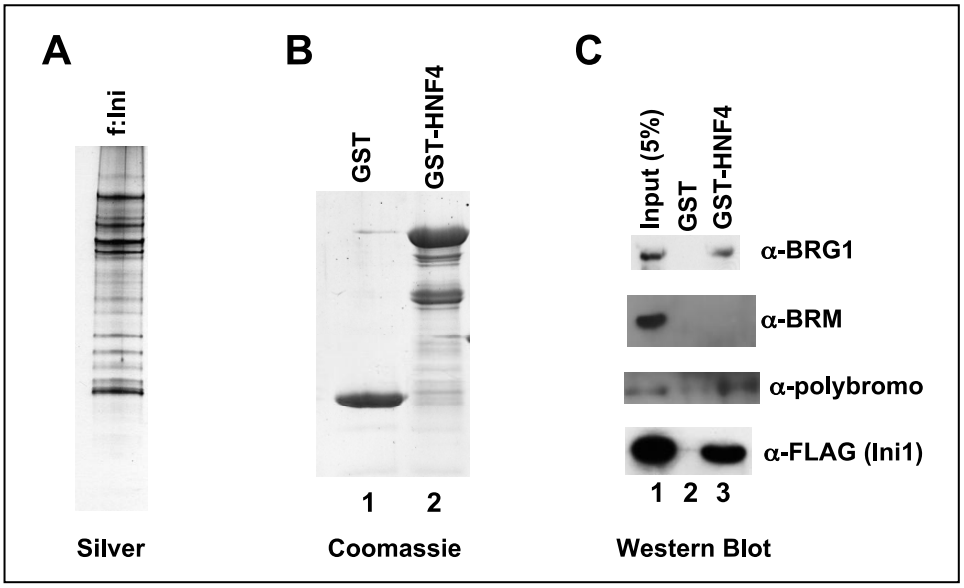

Fig. S5 (related to main Figure 3) Physical interactions between HNF4α and PBAF

A. Preparation of FLAG-Ini1-containing complexes. Nuclear extract from a HeLa cell line stably expressing FLAG-tagged Ini1 (Sif et al., 2001) were affinity purified over M2 agarose and the eluate was analyzed by SDS-PAGE and silver staining. Since Ini1 is a common subunit of three SWI/SNF-containing ATP-dependent chromatin remodeling complexes in human cells (Mohrmann and Verrijzer, 2005), this preparation is representative of all three. **B.** GST-HNF4ΔC1 (lane 2) and control GST (lane 1) proteins used in the binding assay were purified from E. coli as described (Barrero and Malik, 2006) and analyzed by SDS-PAGE and Coomassie staining. **C.** Standard GST pull-down experiments were essentially done as previously described (Barrero and Malik, 2006). Binding reactions contained 10 μg of the indicated GST proteins and were incubated with the FLAG-ini1 preparation (5 % input shown in lane 1) in buffer containing 150 mM KCl and 0.1 % NP-40. After washing in the same buffer, bound proteins were analyzed by Western blotting using the indicated antibodies. Note that HNF4α preferentially bound the PBAF complex as evidenced by specific retention of BRG1, polybromo (BAF180), and Ini1 subunits but not of BRM.

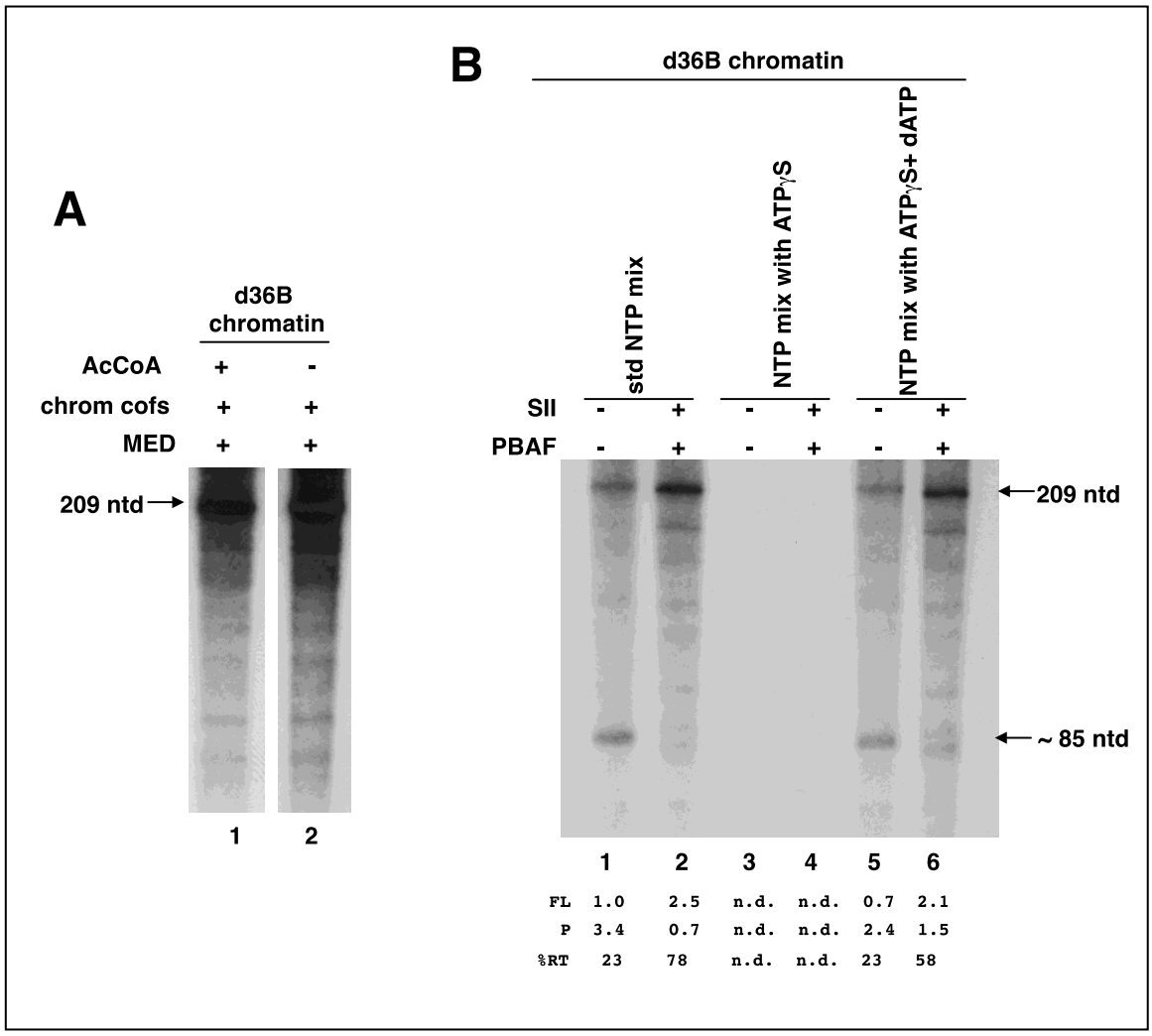

Figure S6 (related to main Figure 3) Substrate requirements for the d39B chromatin template.

A. Acetyl CoA can be dispensable in the complete assay system. Chromatinized d39B template was subjected to in vitro transcription in the presence of the indicated factors as described in the legend to Fig. 3. Chromatin cofactors (cofs) included SII, FACT, p300, and PBAF. In lane 2, acetyl CoA (Ac CoA) was omitted. The full-length product (the predominant product under these conditions) is identified. **B.** dATP can largely substitute for ATP as a source of energy for in vitro transcription of the chromatinized d39B template. Chromatinized d39B template was subjected to in vitro transcription as described in the legend to Fig. 2B. All reactions contained Mediator; SII and PBAF were

added as indicated to assess read-through resulting from energy-dependent remodeling by PBAF. Note that SII alone has minimal effect on read-through under these conditions (e.g., Fig. 3). In lanes 1 and 2, reactions were done with our standard NTP mix, which contains 100 μM dATP and 25 μM ATP (plus GTP, UTP, and CTP). In reactions shown in lanes 3 and 4, 25 μM ATPγS, which is a non-hydrolyzable analog of ATP that can be incorporated into RNA but not be utilized as a source of energy, was added in place of both ATP and dATP. In lanes 5 and 6, reactions containing ATPγS were supplemented with 100 μM dATP, which while being incapable of incorporation into RNA can fulfill the energy requirement both for transcription into the nucleosome (lane 5) and in large part also the energy requirement (PBAF) for transcription across the nucleosome (lane 6 vs. lane 2).

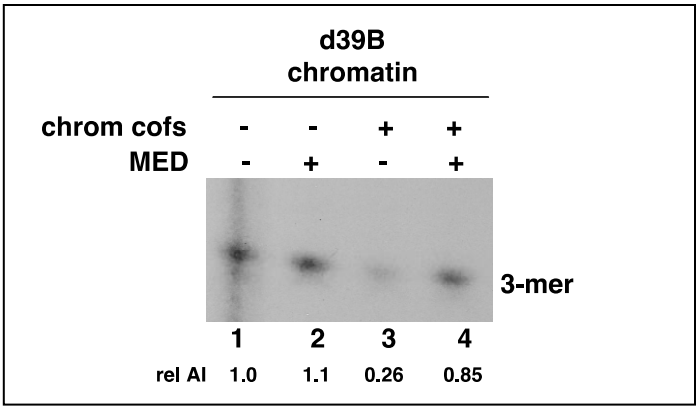

Figure S7 (related to main Figure 4) Cofactor requirements for abortive initiation from the d39B chromatin template.

Abortive initiation reactions from chromatinized d39B were performed as described in the legend to Fig. 4D. Mediator and chromatin cofactors (cofs, FACT, p300, and PBAF) were added as indicated. The abortively synthesized CpApC trimer is identified. Relative extent of abortive initiation (rel AI) normalized to the product in lane 1 was also determined. Note that concordant with their effects on production of longer transcripts, and our interpretation that Mediator stabilizes the PIC against the cofactor-mobilized nucleosome (Fig. 3B), the cofactor cocktail in fact reduces trimer synthesis by several-fold in the absence of Mediator.

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