

# Mediator–Nucleosome Interaction

Yahli Lorch,\* Jenny Beve,<sup>†</sup>  
Claes M. Gustafsson,<sup>†</sup> Lawrence C. Myers,<sup>‡</sup>  
and Roger D. Kornberg\*<sup>§</sup>

\*Department of Structural Biology  
Stanford School of Medicine  
Stanford, California 94305

<sup>†</sup>Department of Medical Nutrition  
Karolinska Institute

NOVUM  
S-141 86 Huddinge  
Sweden

<sup>‡</sup>Department of Biochemistry  
Dartmouth Medical School  
Hanover, New Hampshire 03755

## Summary

Mediator, a multiprotein complex involved in the regulation of RNA polymerase II transcription, binds to nucleosomes and acetylates histones. Three lines of evidence identify the Nut1 subunit of Mediator as responsible for the histone acetyltransferase (HAT) activity. An “in-gel” HAT assay reveals a single band of the appropriate size. Sequence alignment shows significant similarity of Nut1 to the GCN5-related N-acetyltransferase superfamily. Finally, recombinant Nut1 exhibits HAT activity in an in-gel assay.

## Introduction

Mediator forms an interface between activator proteins and the RNA polymerase II transcriptional machinery (reviewed in Bjorklund et al., 1999). It is required for transcriptional activation in reconstituted systems and plays an important role in transcriptional regulation in vivo. Originally isolated from yeast (Flanagan et al., 1991; Kim et al., 1994), Mediator complexes have since been identified in human and murine systems (Fondell et al., 1996; Jiang et al., 1998; Naar et al., 1998, 1999; Sun et al., 1998; Boyer et al., 1999; Rachez et al., 1999). Structural features and also the mode of interaction with RNA polymerase II have been conserved from yeast to humans (Asturias et al., 1999).

Yeast Mediator comprises five Srb proteins (Thompson et al., 1993), seven Med proteins (Myers et al., 1998), and eight proteins identified by various screens for mutations affecting transcription (Carlson, 1997; Gustafsson et al., 1998). Several of these screens were for loss of repression, including, most recently, repression of *HO* transcription (Tabtaing and Herskowitz, 1998). The URS2 region of the *HO* promoter represses transcription of a reporter gene on a plasmid, and mutations in several Mediator proteins, including Nut1 and Nut2, relieve this repression. *NUT1* is nonessential in yeast, and its mutant phenotypes have only been seen in combination with

*NUT2* mutations. Deletion of *NUT1* relieves repression by URS2 in a *nut2-1* background but not on its own. *NUT1* is also synthetically lethal with a temperature-sensitive allele of *NUT2*. Nut1 and Nut2 therefore appear to function cooperatively. *NUT2* is essential for yeast cell viability, and a human homolog of the Nut2 protein has been reported as a component of two human Mediator complexes.

Besides enabling activated transcription, purified Mediator stimulates basal transcription about 10-fold and stimulates the protein kinase of basal transcription factor TFIID some 30- to 50-fold (Kim et al., 1994). None of these Mediator activities has so far been shown to reside in a particular subunit or set of subunits. Neither have targets of Mediator activity been identified, aside from RNA polymerase II. Here, we report on an enzymatic activity of the Nut1 subunit, directed toward chromatin. As Mediator has been thought to interact with RNA polymerase on otherwise naked promoter DNA, the chromatin connection points to additional, previously unsuspected, roles in the transcription process.

## Results

### Mediator Binding to Nucleosomes

Mediator–nucleosome interaction was investigated by gel electrophoretic mobility shift analysis. A single, discrete, shifted nucleosome was observed, indicative of one-to-one Mediator–nucleosome complex formation (Figure 1A, arrow). Mediator and not a contaminant in the purified Mediator preparation was responsible for the shift, since an antibody against the Med2 Mediator subunit caused a supershift. In the presence of antibody, most nucleosomes failed to enter the gel, while the intensity of the band due to the Mediator–nucleosome complex was much diminished (64% lower than in the absence of antibody). In contrast, the intensity of the band due to free nucleosomes, which provides an internal control for antibody specificity, was nearly constant (10% lower than in the absence of antibody). An approximate dissociation constant of  $5 \times 10^{-8}$  M was estimated from the concentration dependence of complex formation. The affinity was unaffected by the addition of ATP.

The Mediator preparation altered the electrophoretic mobility of naked DNA only slightly and failed to generate a discrete, shifted species (Figure 1B). The affinity was at least 5-fold less than that for Mediator–nucleosome interaction. There was little effect of anti-Med2 antibody (data not shown), also arguing against the formation of a defined Mediator–DNA complex.

### Mediator-Associated Histone-Acetyltransferase Activity

Mediator–nucleosome interaction could have functional consequences, such as histone modification. Purified Mediator proved to possess HAT activity, directed toward histone H3, and to a much lesser extent toward H4. This activity cochromatographed with Mediator protein in the last step of purification (Figures 2A and 2B).

<sup>§</sup> To whom correspondence should be addressed (e-mail: kornberg@stanford.edu).

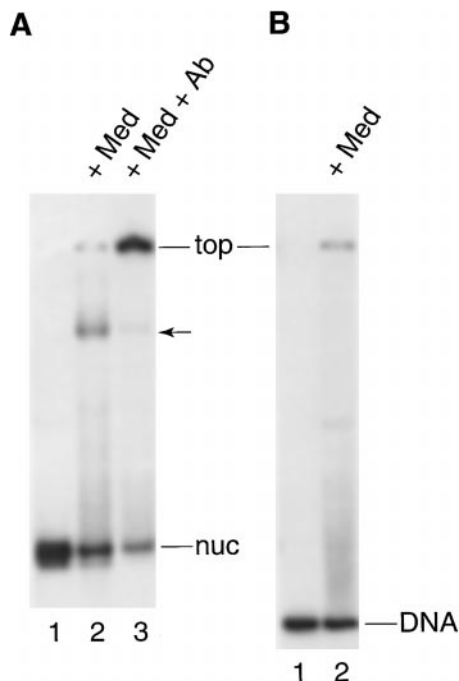


Figure 1. Mediator Binding to Nucleosomes

(A) Gel shift analysis of Mediator-nucleosome interaction.  $^{32}$ P-labeled nucleosomes (0.75 ng) were incubated alone (lane 1), with Mediator (TSK-Heparin fraction 43, 250 ng; lane 2), or with Mediator and anti-Med2 antibodies (800 ng; lane 3) in a total volume of 5  $\mu$ l, containing 15 mM HEPES (pH 7.5), 3 mM  $MgCl_2$ , and 25 mM potassium acetate, for 5 min at 30°C. Electrophoresis was in a 3% polyacrylamide gel in 10 mM Tris (pH 7.5) and 1 mM EDTA at 180V. An autoradiograph of the gel is shown. Top of gel, position of band due to nucleosomes ("nuc"), and band due to Mediator-nucleosome complex (arrow) are indicated.

(B) Gel shift analysis of Mediator-DNA interaction. The same  $^{32}$ P-labeled DNA as was incorporated in nucleosomes in (A) was incubated alone (0.75 ng; lane 1) or with Mediator (TSK-Heparin fraction 43, 250 ng; lane 2) followed by electrophoresis as in (A). Top of gel and position of band due to naked DNA ("DNA") are indicated.

Activity was comparable with either chromatin (nucleosome core particles) or free histones (purified histone octamer) as substrate (Figure 2C).

The specificity of Mediator-associated HAT activity was investigated with a variety of chromatin and transcription proteins as possible alternative substrates. A low level of acetylation, comparable to that observed for H4, was found for histone H1, HMG14, and HMG17 (data not shown). No acetylation was detected with yeast TATA-binding protein, TFIIB, TFIIE, TFIIIF, TFIIH, RNA polymerase II, Gcn4, human Jun, lysozyme, cytochrome c, or BSA (data not shown).

#### Mediator-Associated HAT Activity Resides in the Nut1 Subunit

The subunit responsible for the Mediator-associated HAT activity was investigated by an "in-gel" assay (Mizzen et al., 1999). Resolution by SDS-PAGE in the presence of histones and incubation with  $^3$ H-labeled acetyl-CoA yielded a single labeled protein band with an apparent molecular weight slightly greater than that of a marker of 126 kDa (Figure 2D). Mediator contains three

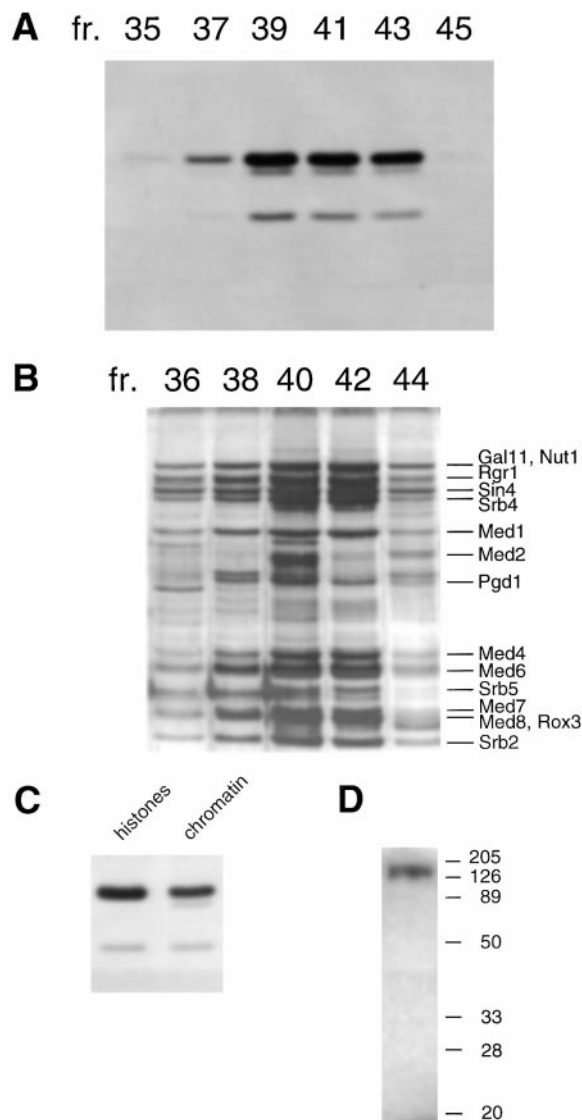


Figure 2. Mediator-Associated HAT Activity

(A) Profile of Mediator-associated HAT activity on TSK-Heparin. Mediator was purified from commercial yeast as described. Fractions (fr.) from the TSK-Heparin column (0.5  $\mu$ l) were incubated with nucleosome core particles (2.4 ng) and  $^3$ H-acetyl CoA (4  $\mu$ l) in 50 mM Tris (pH 8), 10% glycerol, 1 mM DTT, and protease inhibitors in a total volume of 20  $\mu$ l for 25 min at 30°C. SDS (5  $\mu$ l of 10% SDS, 250 mM Tris [pH 6.8], 100 mM DTT, 0.1% bromphenol blue, and 10% glycerol) was added, followed by heating for 4 min at 100°C and electrophoresis in an SDS-PAGE polyacrylamide gel as described. An autoradiograph of the gel is shown.

(B) SDS-PAGE of Mediator fractions from TSK-Heparin. Fractions (fr.) from the TSK-Heparin column were subjected to SDS-PAGE and silver staining. Bands due to Mediator polypeptides are identified as in Myers et al. (1998).

(C) Mediator-associated HAT activity with histones and chromatin as substrates. HAT assays were performed as in (A) with Mediator (TSK-Heparin fraction 41) and histone octamers ("histones," 1.3 ng) or nucleosome core particles ("chromatin," 2.4 ng) as substrates.

(D) In-gel assay of Mediator-associated HAT activity. Mediator (7.5  $\mu$ l TSK-Heparin fraction 41) was analyzed by in-gel HAT assay as described. Positions of molecular weight markers, revealed by silver staining, are indicated.

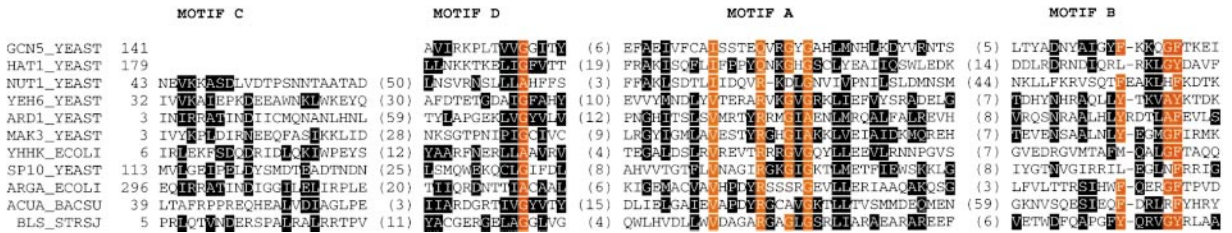


Figure 3. Sequence Alignment of Nut1 with Members of the GNAT Superfamily

Sequences of conserved regions of Nut1 and of representative members of the GNAT superfamily are depicted as in Neuwald and Landsman (1997). Moderately conserved residues are highlighted in black and highly conserved residues in red. Numbers in parentheses are spacer lengths between conserved motifs.

subunits of about this size, Nut1 (129 kDa), Rgr1 (123 kDa), and Gal11 (111 kDa). Identification of the labeled band with Nut1 was supported by a lack of HAT activity of Mediator isolated from a *nut1* deletion strain (data not shown) and by the following additional lines of evidence.

Nut1 was identified by sequence alignment as a member of the GCN5-related N-acetyltransferase (GNAT) superfamily (Figure 3; Neuwald and Landsman, 1997). Residues 1–243 of Nut1 contain all four motifs characteristic of the superfamily, with similar spacings of the motifs to those in other family members. The only significant deviation is a one-residue gap in motif A of Nut1, not seen for the other family members. Insertion of this gap results in perfect alignment of all highly conserved residues and, for example, an overall sequence similarity of 43% between Nut1 and Hat1 in motif A.

To test whether the region of sequence similarity between Nut1 and members of the GNAT superfamily indeed possesses HAT activity, residues 1–256 of Nut1 were expressed in tagged form in bacteria and purified by affinity chromatography. An in-gel assay revealed a peak of HAT activity eluting from the affinity column coincident with that of the recombinant protein (Figures 4A and 4B). Similar results were obtained with a recombinant fragment of Nut1 containing residues 1–440 (data not shown).

### Discussion

Nut1 was previously identified as a component of Mediator by the occurrence of a major band of the expected molecular weight in the most highly purified Mediator preparations (Gustafsson et al., 1998). All tryptic peptides from this band contained amino acid sequences corresponding to Nut1 protein. Moreover, affinity purification of tagged Nut1 from yeast cell extract yields a complex of Nut1 with Mediator proteins (T. Borggreffe and R. D. K., unpublished data).

The evidence presented here for gel mobility shift of nucleosomes by Mediator, copurification of HAT activity with the Mediator complex, and detection of a high molecular weight Mediator protein by an in-gel HAT assay may all be attributed to the HAT activity of Nut1. The mobility shift and HAT activity could, however, be due to distinct Mediator components. For example, the mobility shift could reflect the previously reported affinity of Med8 for naked DNA (Chaves et al., 1999). It remains to be determined whether Med8 interacts with nucleosomes as well. It also remains to rule out any contribution to the HAT activity from other Mediator-associated

proteins(s). Additional HATs could be present but not survive the conditions of the in-gel assay.

The sequence alignment of Nut1 with members of the GNAT superfamily required the introduction of a gap in motif A. The residue at this position is not conserved and lies in a loop connecting  $\beta$  strand 4 and  $\alpha$  helix 3 of the GCN5 crystal structure (Rojas et al., 1999; Trievel et al., 1999). While no such gap was found in other members of the superfamily, a shortened loop could well be accommodated without gross perturbation of the protein structure.

Mediator–nucleosome interaction may underlie the genetics of *NUT1* and *NUT2*. Phenotypes of *NUT1* and *NUT2* mutations have been observed only when promoters are transferred to plasmids, disrupting the normal chromosomal environment (Tabtaing and Herskowitz, 1998). The repressive effect of *NUT1* and *NUT2* may reflect the stabilization or reinforcement of chromatin structure, for example by the maintenance of acetylation at an important site in a histone tail or the acetylation of a nonhistone protein.

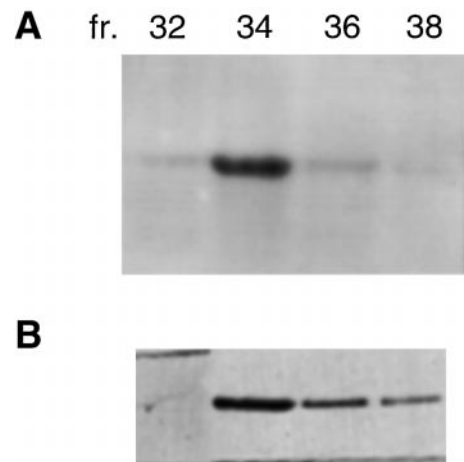


Figure 4. HAT Activity of Recombinant Nut1 Fragment

(A) Profile of HAT activity on Mono S. Equal amounts of Mono S fractions (fr.) of recombinant Nut1 fragment (residues 1–256) were analyzed by an in-gel assay as described. The size of the protein in the labeled band, based on comparison with molecular weight markers (data not shown), was consistent with that expected for the Nut1 fragment.

(B) Immunoblot analysis of fractions from Mono S. Equal amounts (5  $\mu$ l) of Mono S fractions (fr.) of recombinant Nut1 fragment (residues 1–256) were analyzed by immunoblotting with the use of anti-His<sub>6</sub> antibodies (Roche Molecular Biochemicals).

Mediator–nucleosome interaction may be relevant to Mediator function in human cells. Synergy between two activators in a reconstituted human transcription system required human Mediator and the use of a chromatin rather than a naked DNA template (Naar et al., 1998). No strong dependence on acetyl CoA was observed, either because of a lack of requirement for Mediator HAT activity or the presence of other HATs in the system. The human Mediator involved, termed “ARC,” was apparently identical with the independently isolated “DRIP” complex (Rachez et al., 1999). Affinity-purified DRIP exhibited HAT activity, although not tightly associated, as it could be removed by further fractionation.

#### Experimental Procedures

##### Mediator and Nucleosomes

Mediator was purified from yeast whole cell extract by chromatography on Bio-Rex 70, DEAE Sephacel, hydroxyapatite, Mono Q, and TSK-Heparin as described (Myers et al., 1998). Nucleosomes were assembled from a 217 base pair EcoRI-NdeI fragment of pUC19 labeled on the EcoRI end (by filling in with <sup>32</sup>P-dATP) and rat liver histone octamers as described (Lorch et al., 1987). Unlabeled core particles were prepared from rat liver chromatin as described (Kornberg et al., 1989). Anti-Med2 antibodies (Myers et al., 1998) were purified on protein A-Sepharose as described (Maniatis et al., 1982).

##### Recombinant Nut1 Fragments

For expression of Nut1 (residues 1–256), PCR was performed with the primers 5'-GGCCGGCATATGGAAAAGAATCAGTATAC-3' and 5'-GGCCGGCTCGAGACCTACGAATTCGTTAGT-3'. For expression of Nut1 (residues 1–440), PCR was performed with the primers 5'-GGCCGGCATATGGAAAAGAATCAGTATAC-3' and 5'-GGCCGGCTCGAGTCTTATATCCAAGCTTGT-3'. The PCR products were cleaved with NdeI and XhoI and inserted into pET-21b (Novagen), giving rise to pHisNut1(1–256) and pHisNut1(1–440). Recombinant His-Nut1(1–256) and His-Nut1(1–440) were overproduced in *Escherichia coli* BL21(DE3) pLysS cells. His-Nut1(1–256) was purified on a 7 ml Ni-NTA superflow column (Qiagen), developed with a linear gradient (35 ml) of buffer N (0.01) to N (0.3) (phosphate-buffered saline, 10% glycerol, and protease inhibitors; the number in parentheses indicates the imidazole concentration in molar units). Peak fractions of Nut1(1–256), eluting at about 0.1 M imidazole, were dialyzed against buffer A (0.05) (20 mM Hepes-KOH [pH 7.6], 10% glycerol, 1 mM DTT, and protease inhibitors; the number after the hyphen indicates the potassium acetate concentration in molar units) for 4 hr and further purified on a MonoS 5/5 column (Amersham Pharmacia Biotech), developed with a linear gradient (10 ml) of buffer A (0.05) to A (0.6). His-Nut1(1–256) eluted at about 0.45 M potassium acetate. His-Nut1(1–440) was purified on 3 ml Ni-Agarose column (Qiagen), washed with 5 column volumes each of buffers N (0.01) and N (0.02), and eluted with buffer N (0.2).

##### HAT Assays

Liquid assays were performed with <sup>3</sup>H-acetyl CoA (NEN, 0.1 mCi/ml, 4.5 Ci/mm, in 0.01 M sodium acetate [pH 5.0]) and electrophoresis in an SDS-18% polyacrylamide gel as described (Thomas and Kornberg, 1978), followed by fluorography with EN<sup>3</sup>HANCE (NEN). In-gel assays were performed with the use of SDS-10% polyacrylamide gels containing 1 mg/ml bovine histones (Sigma) as described (Mizzen et al., 1999).

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