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CHD8 Associates with Human Staf and Contributes to Efficient U6 RNA Polymerase III Transcription▽

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Chromatin remodeling and histone modification are essential for eukaryotic transcription regulation, but little is known about chromatin-modifying activities acting on RNA polymerase III (Pol III)-transcribed genes. The human U6 small nuclear RNA promoter, located 5′ of the transcription start site, consists of a core region directing basal transcription and an activating region that recruits the transcription factors Oct-1 and Staf (ZNF143). Oct-1 activates transcription in part by helping recruit core binding factors, but nothing is known about the mechanisms of transcription activation by Staf. We show that Staf activates U6 transcription from a preassembled chromatin template in vitro and associates with several proteins linked to chromatin modification, among them chromodomain-helicase-DNA binding protein 8 (CHD8). CHD8 binds to histone H3 di- and trimethylated on lysine 4. It resides on the human U6 promoter as well as the mRNA IRF3 promoter in vivo and contributes to efficient transcription from both these promoters. Thus, Pol III transcription from type 3 promoters uses some of the same factors used for chromatin remodeling at Pol II promoters.

The state of chromatin packaging defines in large part the transcriptional competency of genes transcribed by RNA polymerases (Pol) I and II. In this process, it is clear that the packaging of DNA into certain chromatin states has a repressive effect on transcription, in particular on the initiation and elongation steps, as histone octamers within nucleosomes can block the binding of transcription initiation factors and hamper the progress of RNA Pol along a gene (24, 40, 48, 79). For transcription to take place, the chromatin template needs to be modified such that the accessibility of transcription factors to their promoter targets is increased and transcription elongation is facilitated (36, 74).

Chromatin modification at Pol II genes is accomplished by a large number of factors (47) that are recruited to chromatin through contacts with (i) histones with various modifications in their N-terminal regions, (ii) the DNA, and (iii) certain activators. Such activators can bind to chromatin templates and recruit chromatin-modifying factors, which then remodel nucleosomes or modify histones (11, 23). Successive waves of chromatin modifications are thought to allow for the regulated assembly of transcription initiation complexes, leading to active transcription.

Much less is known about the requirements for chromatin modification at Pol III-transcribed genes. Pol III, like Sp6 Pol, is capable of transcribing through a nucleosome on a mononucleosomal template, causing an intranucleosomal loop followed by transfer of the histone octamer to a different position of the same template (62, 63). This shows that Pol III can transcribe through a single nucleosome without the help of chromatin-modifying activities. However, a large number of observations suggests that the initiation step of Pol III transcription, as well as elongation through more than a single nucleosome, is influenced by chromatin-modifying activities. For example, transcription of an Alu element in vitro is profoundly repressed by packaging of the template into nucleosomes (10), and histone acetylation dramatically enhances transcription by Pol III through dinucleosomal templates as well as nucleosomal arrays (68, 69). Moreover, nucleosome depletion in vivo activates Pol III transcription at certain normally repressed Pol III promoters in the *Saccharomyces cerevisiae* (yeast) genome (16), and in the case of the yeast U6 snRNA gene, binding of the transcription factors IIIC and IIIB is accompanied by changes in nucleosome position (56). Such observations are consistent with the proposal that chromatin modification, either histone modification or chromatin remodeling or both, is indeed required. However, which factors are involved and what role, if any, is played by chromatin-remodeling factors acting on Pol III-transcribed genes are so far largely undocumented.

The human U6 small nuclear RNA (snRNA) promoter is a type 3 Pol III promoter, i.e., it is located in the 5′ flanking sequence of the gene and contains two elements required for basal transcription in vitro, a TATA box and a proximal sequence element (PSE), as well as a distal sequence element (DSE) that enhances transcription from the basal promoter (17). The U6 promoter and all type 3 Pol III promoters are very similar to the Pol II promoters of snRNA genes, which also contain a DSE and a PSE but lack the TATA box (17).

The DSEs of both type 3 Pol III promoters and Pol II

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snRNA promoters generally contain an octamer sequence, which recruits Oct-1, and an SPH (for *Sph1 p*ostoctamer *h*omology) sequence, which recruits a transcription factor called selenocysteine tRNA-activating factor (Staf) (52) or SPH binding factor (17, 45). Oct-1 activates transcription both through its POU (*P*it-1, *o*ct-1, *u*nc86) DNA binding domain, which, thanks to a positioned nucleosome, contacts the PSE binding factor $SNAP_c$ and helps its recruitment, and through its activation domains (78). Staf was cloned first from *Xenopus laevis*, but two human proteins, ZNF143 and, to a lesser extent, ZNF76, are similar to *Xenopus* Staf, share similar DNA binding specificities, and can activate Pol II and III snRNA gene transcription (35). How the activation domains of Oct-1 and Staf activate transcription from DSE-containing promoters is unknown, but by analogy to activators of mRNA-encoding Pol II genes, it seems probable that the mechanism involves the modification of chromatin.

Among the large number of chromatin-binding proteins identified in recent years are the *c*hromodomain-*h*elicase-*D*NA binding, or CHD, proteins. These highly conserved but poorly understood proteins are characterized by two tandem chromodomains in their N terminus followed by an SNF2-like helicase domain. In mammalian cells, there are nine CHD proteins, which can be divided into three subfamilies according to sequence similarities (53). Subfamily I contains CHD1 and CHD2, subfamily II contains CHD3 to CHD5, and subfamily III contains CHD6 to CHD9. To date, the best-characterized CHDs are CHD1 and CHD3/CHD4. CHD1 and CHD2 contain a DNA binding domain with an AT hook (2). CHD1 can assemble chromatin in vitro (29, 46) and remodel nucleosomes (67). It associates with NCoR and histone deacetylases as well as with RNA splicing proteins, linking it to transcription repression and pre-mRNA splicing, respectively (65). CHD1 also associates with the transcription elongation machinery (25, 58) and is concentrated in actively transcribed areas, suggesting that it is involved in Pol II transcription activation, probably at the transcription elongation step. Indeed, the double chromodomain of human CHD1 interacts with histone H3 di- or trimethylated at lysine 4, a marker of active chromatin (13, 59).

The subfamily II CHD3 (Mi2a) and CHD4 (Mi2b) proteins are closely related and characterized by two PHD (plant homeodomain) zinc fingers (3, 15) located N terminal of the double chromodomain. They are in complexes containing the histone deacetylases HDAC1/HDAC2 and referred to as the Mi2 (71), NuRD (nucleosome-remodeling histone deacetylase) (76, 77), and NRD (nucleosome remodeling and deacetylating) (66) complexes. These complexes can remodel chromatin to facilitate access of deacetylases and repress transcription (72). CHD4 is, however, also implicated in transcription activation because it associates with BRG1, a subunit of the SWI/SNF complex, as well as with a complex of nucleolar proteins containing UBF, MCRS1 (microspherule protein 1), and RFP (RET finger protein) implicated in activation of rRNA gene transcription by Pol I (55). Mouse CHD5 has recently been shown to correspond to a tumor suppressor controlling cell proliferation and apoptosis (4).

The subfamily III members contain three conserved motifs called CR1 through CR3, as well as a SANT domain (1), two BRK domains, and a slightly divergent AT hook DNA binding domain, all located C terminal of the helicase domain. Human chromodomain helicase DNA binding protein 8 (CHD8) copurifies with the MLL1-WDR5 complex (8), and mouse CHD8 has recently been shown to interact with CTCF through its BRK domains and to be involved in CTCF-dependent insulator function, as its down-regulation by RNA interference (RNAi) reduced the insulator activity of the H19 DMR (differentially methylated region) insulator (19).

Here, we have explored the role of human Staf (hStaf; ZNF143) in transcription from the human U6 snRNA gene. We found that hStaf activates transcription from a U6 gene template assembled into chromatin and further identified CHD8 as an hStaf-associated protein required for efficient U6 transcription in vivo, as well as for transcription of the IRF3 gene, a Pol II-transcribed gene. These results show that a function of CHD8 is in activation of transcription from type 3 Pol III promoters. This in turn suggests that transcription of Pol III genes requires chromatin modification and that at least in the case of type 3 Pol III promoters, some of the factors used are also involved in chromatin remodeling of Pol II promoters.

MATERIALS AND METHODS

GST-hStaf expression and purification. Glutathione *S*-transferase (GST)-hStaf was expressed in *Escherichia coli* BL21 with the T7 expression system (61) from a pSBet vector expressing hStaf tagged with GST followed by a TEV site at the N terminus and six histidines at the C terminus.

Chromatin assembly, MNase footprinting, and in vitro transcription from the chromatin template. The S-190 extract was prepared and the template assembled into chromatin as described previously (78). Recombinant hStaf was added to a concentration of 90 nM at the end of the chromatin assembly reaction. The quality of the chromatin template was checked by micrococcal nuclease (MNase) digestion. MNase footprinting was performed by linear PCR as described in reference 78. For transcription assays 10 μl of chromatin template with or without added hStaf was mixed with 15 μl of HeLa whole-cell extract from which endogenous hStaf had been previously depleted by incubation with anti-hStaf antibody beads in a final volume of 40 μl containing 25 mM HEPES-KOH, pH 7.9, 60 mM KCl, 10 ng/ml of poly(dG-dC) · poly(dG-dC) (GE Healthcare), 5 mM $MgCl_2$, 1 mM EGTA, 5 mM spermidine, 4 mM dithiothreitol (DTT), 2 mM each of ATP, GTP, CTP, and UTP, and 2 ng of recombinant TATA box-binding protein. The reaction mixture was incubated at 30°C for 1 h, and RNA was analyzed as described previously (78).

ChIPs. The chromatin immunoprecipitation (ChIP) protocol was adapted from that described in reference 78. HeLa cells were fixed with 1% formaldehyde. After nucleus lysis, chromatin was sonicated for 8 min with a Bioruptor (Diagenode) with intervals of 30 seconds on and 30 seconds off. For each ChIP sample, 600 μl of chromatin and 1 μg of antibody were mixed and incubated overnight at 4°C. Six microliters of protein G magnetic beads was then added to capture DNA/protein/antibody complexes. After being extensively washed, the DNA/protein/antibody complexes were eluted twice with 150 μl of elution buffer (50 mM $NaHCO_3$, 1% sodium dodecyl sulfate [SDS]). The supernatant was collected and incubated at 67°C for 5 h to reverse the formaldehyde cross-linking, and the nucleic acids were precipitated and analyzed by PCR. The primers used for PCR were located in the proximal (U6-4U [5′TTCTTGGGTAGTTTGCAG 3′] and U6-2L [5′ GTTTCGTCCTTTCCACAAG 3′]) region of the U6-1 active promoter, in the promoter region (U64-1 and U64-120 [see reference 7]) of the inactive U6-4 promoter, or in the U1 promoter region (U1-4UP [5′CACGAA GGAGTTCCCGTG3′] and U1-2L [5′CCCTGCCAGGTAAGTATG3′]) as indicated in the figure legends. For the PCRs, serial dilutions were performed in test experiments to establish conditions giving rise to linear response.

Identification of hStaf-associated proteins. The GST pull-down experiment was carried out according to the procedure described in reference 27. Briefly, 200 μg of GST fusion protein-coupled glutathione beads prepared as described above were equilibrated with buffer D200 (50 mM HEPES-KOH, pH 7.9, 200 mM KCl, 20% glycerol, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT). Nuclear HeLa cell extracts (6) from 1×10^9 HeLa cells were diluted 1:2 with D200 buffer and incubated with glutathione beads coupled to fusion proteins for 3 h at 4°C. After the incubation, the beads were washed extensively with K200 buffer (D200 buffer without glycerol) before elution with 2 column volumes of T300 buffer (50 mM HEPES-KOH, pH 7.9, 300 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM

DTT, 0.05% Tween 20) followed by 2 column volumes of T600 buffer (50 mM HEPES-KOH, pH 7.9, 600 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.05% Tween 20). The eluted proteins were trichloroacetic acid (TCA) precipitated for MudPIT analysis. TCA-precipitated proteins were denatured with urea, reduced, alkylated, and digested with endoproteinase Lys-C (Roche) followed by modified trypsin (Roche) as described in reference 73. Peptide mixtures were pressure loaded onto 100 μm fused silica microcapillary columns packed with 5 μm C_{18} reverse phase (Aqua; Phenomenex), strong cation-exchange particles (Partisphere SCX; Whatman), and reverse phase (30). Loaded microcapillary columns were placed in-line with a Quaternary Agilent 1100 series high-performance liquid chromatography pump and a Deca-XP ion trap mass spectrometer equipped with a nano-liquid chromatography electrospray ionization source (ThermoFinnigan). Fully automated six-step MudPIT runs were carried out, as described in reference 14. Tandem mass spectra were searched with SEQUEST (9) against a database of 60,238 amino acid sequences, consisting of 34,180 human proteins (NCBI 2006-09-05 release), 177 usual contaminants (such as human keratins, immunoglobulin G, and proteolytic enzymes), 126 epitope-tagged proteins, and, to estimate false-discovery rates, 30,119 randomized amino acid sequences derived from each nonredundant protein entry. Spectrum/peptide matches were retained only if they had a normalized difference in cross-correlation scores of at least 0.08, and minimum cross-correlation score of 1.8 for singly charged spectra, 2.5 for doubly charged spectra, and 3.5 for triply charged spectra. In addition, peptides had to be fully tryptic and at least seven amino acids long. Peptide hits from multiple runs were compared using CONTRAST (64) and contrast-report (14). A further criterion for consideration was that proteins had to be detected by at least two such peptides in all combined runs. Under this set of criteria, no false-positive "shuffled" proteins were detected (false-discovery rate = 0). Spectral counts were normalized as described in reference 80 to calculate normalized spectral abundance factors, parameters used to estimate relative protein levels.

Co-IPs. Antibodies against hStaf and CHD8 were cross-linked to protein G agarose beads (Roche) with dimethyl pimelimidate (Sigma). The antibody against hStaf (antibody 19164) was raised against peptide RIASRIQQGETPG LDD, which is present in ZNF143 but not in ZNF76. The antibodies against CHD8 were raised against peptide DSLTDDSFNQVTQDPIEE (antibody 19224) or peptide SQGYDSSERDFSLIDDPM (antibody 19225). For coimmunoprecipitations (co-IPs), HeLa nuclear extracts (200 μg protein) were incubated with 100 μl of protein G beads with cross-linked antibody for 3 h at 4°C. The beads were washed extensively with K300 buffer, and the immunoprecipitated proteins were released by the addition of 100 μl of 2× Laemmli buffer and boiling for 5 min. The eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by immunoblotting.

Reporter cell line construction. To establish the IMR90-C2.5 clonal cell line expressing a U6 promoter-directed unstable RNA, IMR-90Tert cells were transfected by the calcium phosphate method with 5 μg of pU6/RA.2+U6end-Dsred, a derivative of pDsRed-Express-DR (Clontech) with an insert consisting of the human U6 promoter followed by a piece of β-globin gene cloned in the reverse orientation as in pU6/Hae/RA.2 (28), followed by the 3′ end of the U6 gene and 3′ flanking sequences. The cells were split 48 h later and kept under G418 selection (500 μg/ml) for 21 days. Individual clones were expanded and tested for expression of the U6 construct.

Protein knock-down with RNAi and RT-PCR. RNAi oligonucleotides were transfected into IMR90-C2.5 cells with Hi-Perfect transfection reagent (QIAGEN). The cells were collected 24 h after the last of three rounds of transfections performed at 24-h intervals. Total RNA was isolated with miRNeasy mini kit (QIAGEN). One microgram of total RNA was used for first-strand DNA synthesis with TaqMan reverse transcription (RT) reagents (Applied Biosystems), and the resulting cDNA was used as the starting material for PCRs. For the PCRs, we first diluted the PCR templates 1:10, 1:50, and 1:250 to determine, for each primer pair, the highest template concentration that still gave a signal in the linear range. These concentrations were used for subsequent experiments. The intensity of the PCR bands was measured with an Alpha imager (Alphainnotech).

CHD8 interaction with histone peptides. The protocol of CHD8 interaction with histone peptides was adapted from reference 43. hStaf-associated proteins were eluted with 600 mM KCl as described above, dialyzed against buffer D150T (10% glycerol, 20 mM HEPES-KOH, pH 7.9, 150 mM KCl, 0.5 mM EDTA, 0.05% Tween 20), and incubated with 2 μg of different biotinylated histone H3 peptides for 2 h at 4°C. The peptides were purchased either from Upstate Biotechnology or from the Tufts University peptide synthesis facility. At the end of the incubation, 20 μl of streptavidin beads was added to each sample, and the sample was incubated at 4°C for 2 h. The beads were washed and the bound proteins eluted by Laemmli buffer and boiling. The samples were analyzed by SDS-PAGE and immunoblotting with anti-CHD8 and anti-hIno80 antibodies. Antibodies for hStaf, CHD8, and hIno80 were produced by Custom Hybridoma.

RESULTS

hStaf activates U6 transcription from a chromatin template. To explore the role of hStaf in transcription from the human U6 gene, we first tested for the presence of this protein on the human U6 promoter by ChIP. Exponentially growing HeLa cells were treated with formaldehyde to cross-link protein complexes to DNA, and chromatin was extracted, sonicated, and used for IPs with an anti-hStaf antibody. Anti-Brf2 and anti-TFIIB antibodies were used as positive and negative controls, respectively, and a sample mock immunoprecipitated without antibody was also included. The PCR was carried out with two sets of primers, one specific for the promoter region of the U6 gene we worked with (test primers; U6-1 gene) and the other specific for the 5′ flanking region of one of the pseudo-U6 genes (control primers; U6-4 gene) (7). The top panel of Fig. 1A shows the linearity of the PCR with a titration of the input material, and the bottom panel shows the results obtained with the various antibodies. hStaf was present on the human U6 gene but not the pseudo-U6 gene (Fig. 1A, bottom panel, lanes 5 and 6). As expected, so was Brf2, whereas TFIIB was absent from both the gene and pseudogene (lanes 7 to 10).

Next, we tested the binding of hStaf to a U6 template preassembled into chromatin in vitro. The U6 template was preassembled with *Drosophila melanogaster* S190 extract, as described before (78). hStaf was then added to the chromatin template, and binding was visualized by partial micrococcal nuclease digestion followed by linear PCR with an end-labeled primer, as described before (see reference 78). As shown in Fig. 1B, the pattern obtained with naked DNA was different from that obtained with chromatin-assembled DNA, as expected (compare lanes 1 and 2). Upon the addition of hStaf, a clear footprint over the hStaf binding site (SPH sequence) was obtained (lane 3). To confirm that the footprint corresponded to binding to preassembled chromatin rather than to naked DNA, we tested the effect of apyrase addition. As shown in Fig. 1B, right panel, the addition of apyrase obliterated the footprint, indicating that it is ATP dependent (compare lanes 5 and 7). Since hStaf is not known to require ATP to bind to naked DNA (see, for example, reference 45), the ATP requirement suggests that hStaf is indeed binding to preassembled chromatin and requires the help of an ATP-dependent chromatin-modifying activity to do so.

We then tested the transcriptional activity of such a template. The addition of recombinant hStaf to the preassembled chromatin template before the start of the transcription assay activated U6 transcription (Fig. 1C, lanes 1 and 2), and this was dependent on an intact SPH sequence (compare lanes 2 and 4; note that lanes 1, 2, 3, and 4 are from the same gel). As observed before (45), the addition of purified recombinant hStaf to a HeLa cell extract also activated U6 transcription from a naked DNA template in an SPH sequence-dependent manner, although in our hands, this activation was rather weak (lanes 5 to 8). Thus, like many transcription factors, including chimeric proteins containing the VP16 activation domain (see, for example, reference 37), hStaf can activate transcription from both naked (45) and chromatinized template. Together,

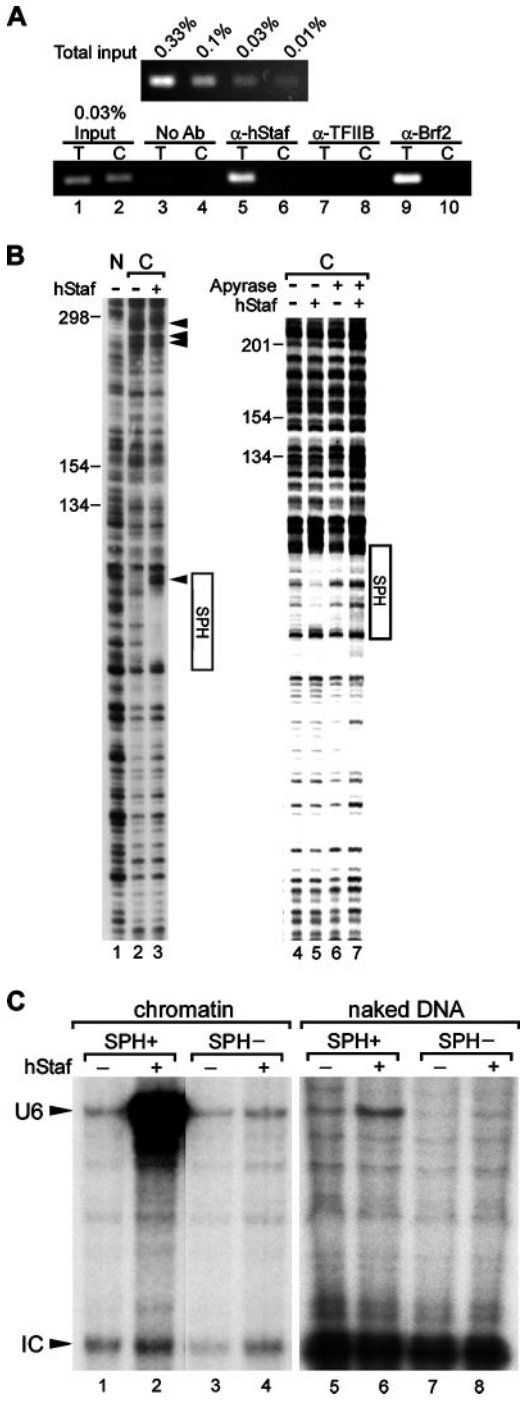


FIG. 1. hStaf activates U6 transcription from a chromatin template. (A) ChIP of hStaf on the U6-1 promoter. Rapidly growing HeLa cells were treated with formaldehyde, and cross-linked chromatin was extracted, sonicated, and used as starting material for IPs with the antibodies indicated above the lanes or with no antibody (No Ab). The upper panel shows a titration of input material analyzed by PCR with test primers (T) U6-4U and U6-2L specific for the U6-1 promoter. The lower panel shows immunoprecipitated material analyzed by PCR with test primers U6-4U and U6-2L as described above or control primers (C) U64-1 and U64-120 hybridizing to an inactive U6-4 gene. (B) Either naked (N; lane 1) or chromatinized (C; lanes 2 to 7) U6 template was incubated with hStaf and/or apyrase as indicated above the lanes, treated with micrococcal nuclease, and then analyzed by linear PCR as described previously (73). The location of the SPH sequence is indicated. Arrowheads mark the differences in the digestion patterns of chromatinized and naked DNA. Numbers to the left of the lanes indicate sizes of DNA markers in nucleotides. (C) hStaf activates U6 transcription from a chromatin template. A U6 template with wild-type (lanes 1, 2, 5, and 6) or mutated (lanes 3, 4, 7, and 8) SPH motif (SPH motif TTCCCATGATTCCTTCAT mutated to TT*aaa*ATGATTCCT TCAT [lowercase italic letters indicate mutations]) was either assembled (lanes 1 to 4) or not assembled (lanes 5 to 8) into chromatin with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) hStaf and incubated with an hStaf-depleted HeLa cell extract for transcription. The signal corresponding to U6 promoter-directed transcription (U6), as well as an internal control (IC) corresponding to a radiolabeled G-less RNA fragment added at the beginning of the transcription reaction to control for RNA handling, is indicated. Lanes 1 through 4 are from one gel, and lanes 5 through 8 are from another.

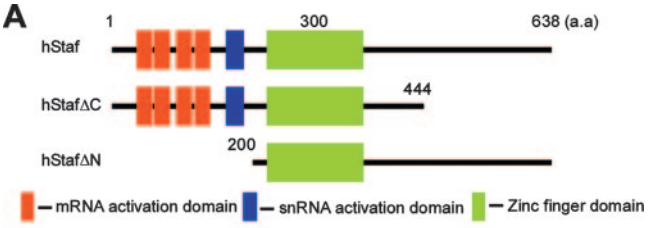
the results indicate that hStaf is capable of binding to its target site in a chromatinized U6 template and may activate transcription at least in part by recruiting histone-modifying or chromatin-remodeling factors.

Identification of hStaf-associating proteins. *Xenopus* Staf can be divided into three major regions: a transcription activation domain, a zinc finger domain, and a C-terminal domain with no known function (51). The activation domain contains a region required for activation of snRNA-type genes and another region composed of four imperfect tandem repeats required for the activation of mRNA-encoding Pol II genes (51). The zinc finger domain contains seven zinc fingers of the C_2-H_2 type, different sets of which can be used to bind to different DNA targets (49). These regions are conserved in human Staf, as illustrated in Fig. 2A.

To understand how hStaf activates U6 transcription, we set out to identify hStaf-associated proteins by conducting pull-down experiments with full-length hStaf as well as versions of hStaf lacking C-terminal (hStafΔC) or N-terminal (hStafΔN) sequences, all fused to GST (Fig. 2A). We used Nef, a human immunodeficiency virus type 1 protein localized in the cytoplasm, fused to GST, as a negative control. The GST fusion proteins expressed in *E. coli* and coupled to glutathione agarose beads were incubated with HeLa nuclear extracts, and proteins retained on the beads were sequentially eluted with 300 and 600 mM KCl and precipitated with TCA for MudPIT analysis.

A total of 289 nonredundant proteins were identified by at least two peptide hits. After elimination of the proteins also pulled down by GST-Nef and the usual contaminants, 230 remained. Since the N-terminal domain of hStaf contains the transcription activation domains (Fig. 2A), we further eliminated 190 proteins that were pulled down by hStafΔN, which lacks these N-terminal sequences, as well as the proteins pulled down by full-length hStaf but not by hStafΔC. Twenty-five proteins that were pulled down either by both full-length hStaf and hStafΔC (13 proteins) or just by hStafΔC (12 proteins) remained. The merged list of these 25 proteins, which are likely to associate with the hStaf sequences uniquely present in hStafΔC, i.e., sequences located N terminal of the zinc finger domain (Fig. 2A), is shown in Fig. 2B, with the proteins identified only in the hStafΔC pull-down experiment indicated. Many of these proteins are involved or likely to be involved in chromatin modification (see Discussion). The protein with the

A

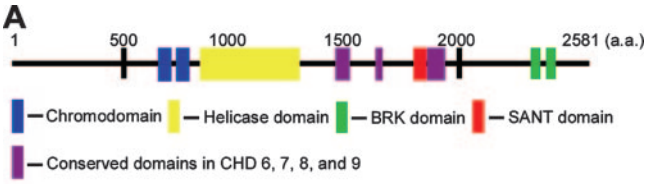


B

Acession number	Description	Number of peptides	Sequence Coverage (%)
XP_370738.1	Chromodomain helicase DNA binding protein 8	23	13.3
NP_005325.1	Host cell factor 1 (VP16-accessory protein)	18	12.1
NP_003715.1	Jerky homolog*	15	35.9
NP_005095.1	Bromodomain containing protein 2	13	23.8
NP_031397.1	Bromodomain containing protein 3	13	22.3
NP_597709.1	RAVER1	10	15.5
NP_003698.1	RUVB-like 1*	9	25.4
NP_060924.4	Nucleolar and spindle associated protein 1	9	21.8
NP_002678.2	Pinin, desmosome associated protein	8	11
NP_036377.1	SKI-interacting protein*	7	19.2
NP_006156.2	Nuclear factor related to kappa B binding protein*	7	7.9
NP_734466.1	Histone H2A	5	35.1
NP_036244.2	TPX2, microtubule associated protein homolog*	5	10
NP_003394.1	YY-1 transcription factor	5	9.7
NP_149072.1	Death inducer-obliterator 1 isoform c*	5	3.5
XP_002257.1	Importin alpha-2 subunit*	4	12.9
NP_036473.2	G-protein binding protein CRFG	4	7.6
NP_005861.1	Sin3 associated protein, 18kDa	3	28.1
NP_060023.1	INO80 complex homolog 1*	3	4.8
NP_075064.1	Vasculin*	2	8
NP_444271.1	Proline rich 8*	2	4.3
NP_055871.1	Hypothetical protein LOC23076	2	4.1
NP_056235.3	WD repeat and SOF1 containing protein*	2	3.9
NP_003971.1	Microtubule associated protein 7*	2	3.6
NP_005349.3	LIM domain only 7	2	2.2

FIG. 2. MudPIT analysis of hStaf-associated proteins. (A) hStaf constructs used in the GST pull-down experiments. a.a, amino acid. (B) Proteins associated with full-length hStaf and hStafΔC or with hStafΔC only that were identified in the MudPIT analysis. Proteins identified only in the hStafΔC pull-down experiment are marked with an asterisk. Entries for proteins known or suspected to be involved in chromatin modification are shaded blue. The proteins are arranged by the number of peptides identified and then by sequence coverage.

A



B

FIG. 3. Structure of human CHD8. (A) Diagram of human CHD8. (B) Amino acid sequence of CHD8 according to the XP_370738 entry. The amino acids marked in red are the additional amino acids missing in the current human CHD8 protein sequence, NP_065971. The underlined sequences correspond to the peptide matches identified by MudPIT analysis. In some cases, the sequence underlined corresponds to several peptides of different ionization states or to two or more adjacent peptides. The two peptide sequences in uppercase letters were used to produce anti-CHD8 antibodies.

most peptide hits was CHD8, with 23 peptide hits and a 13.3% sequence coverage in the hStafΔC analysis, and we therefore focused on it as a possible hStaf binding partner and transcription activator.

CHD8 primary structure. CHD8 belongs to subfamily III of CHD proteins. As shown in Fig. 3A, it contains two chromodomains, a helicase domain, three regions conserved in CHD6 through CHD9, a SANT domain, and two BRK domains. The peptide hits from our MudPIT results were matched to an old version of the CHD8 sequence, XP_370738, which was later replaced with a newer version, NP_065971. The older version contained an N-terminal 279-amino-acid extension (Fig. 3B, red) in place of the first two amino acids (methionine and lysine) of the newer version. Our data are consistent with a CHD8 protein matching the older sequence. Indeed, some of the CHD8 peptides identified by MudPIT were matched to the sequence unique to the large form, and one peptide spanned the junction of the large and small forms of CHD8 (Fig. 3B). Moreover, we raised two antibodies directed against peptides in the N- and C-terminal regions of CHD8 (Fig. 3B), which both recognize the same major band greater than 250 kDa in nuclear extracts (data not shown). Finally, BLAST searches of the NCBI protein database identified mouse (XP_619244) and rat (XP_573762) CHD8 homologs, as well as a partial CHD8 sequence from a chimpanzee (XP_509818). These three proteins contain N-terminal sequences that are 94, 95, and 99% identical, respectively, to the N-terminal extension of the human protein. These data strongly suggest that the large form of CHD8, XP_37038, exists in cells and was pulled down by GST-hStaf. They do not, however, exclude the possibility that the short form also associates with hStaf.

The long and short forms are likely to arise by alternative

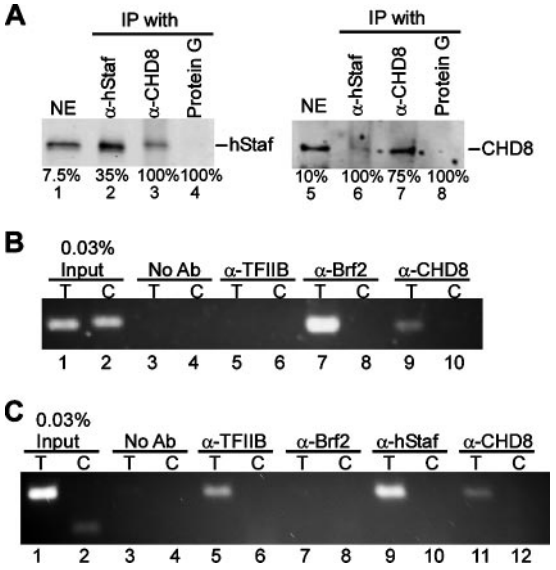


FIG. 4. CHD8 interacts with hStaf and is localized on the U6 promoter. T, test primers; C, control primers; Ab, antibody. (A) Co-IP of hStaf and CHD8. A HeLa cell nuclear extract (NE) was used as the starting material for nondenaturing IPs with antibodies indicated above the lanes or, as a control, protein G beads. The immunoprecipitates were fractionated by SDS-PAGE, transferred to a membrane, and probed by immunoblotting with antibodies directed against hStaf (left panel) or CHD8 (right panel). The percentages below the lanes indicate the fractions of the total samples loaded in the lane. (B) CHD8 is localized on the U6-1 promoter. ChIPs were performed as described for the lower panel of Fig. 1A, with the antibodies indicated above the lanes. (C) CHD8 and hStaf are localized on the Pol II snRNA U1 promoter. ChIPs were performed with the antibodies indicated above the lanes as described for the lower panel of Fig. 1A except that the precipitated material was analyzed by PCR with primers hybridizing to the human U1 promoter (see Materials and Methods for the primer sequences).

splicing, because the first two amino acids of the short form (absent in the long one) are encoded by the end of the second exon displayed in the University of California—Santa Cruz human genome viewer. We identified a stretch of genomic sequence about 150 bp downstream of the second exon that is able to encode the entire N-terminal unique sequence of the large form. Thus, the short and long forms may arise by differential inclusion of either exon 2 or another exon located about 150 bp downstream.

The CHD8 protein coimmunoprecipitates with hStaf and is localized on the U6 and U1 promoters in vivo. To confirm the interaction between CHD8 and hStaf, we tested whether the two proteins coimmunoprecipitated. As shown in Fig. 4A, hStaf was easily detected in anti-CHD8 immunoprecipitates, and CHD8 was detected, albeit weakly, in anti-hStaf immunoprecipitates (lanes 3 and 6), suggesting that CHD8 and hStaf interact inside cells. However, since hStaf is involved in transcription activation of several Pol II and Pol III genes (34, 50), it was possible that hStaf interacted with CHD8 but did not recruit CHD8 to the U6 promoter. We therefore tested whether CHD8 is localized on the U6 promoter by ChIP as described above. We also tested the Pol II snRNA promoter U1, as hStaf can also activate Pol II snRNA promoters (35). As shown in Fig. 4B, CHD8 was detected on the U6-1 promoter but not on the promoter of the inactive U6-4 gene (compare lanes 9 and 10). Moreover, both hStaf and CHD8 were detected on the Pol II U1 snRNA promoter, as shown in Fig. 4C (lanes 9 to 12). The antibody used in the ChIP specifically recognizes the large isoform of CHD8, indicating that this isoform resides on the U6 and U1 promoters. This does not exclude the possibility that the small isoform is also present.

CHD8 interacts specifically with histone H3 methylated at lysine 4. Chromodomains recognize and interact with methylated lysine residues of histones (21, 31, 39). In particular, the double chromodomain of human CHD1 interacts with di- and trimethylated lysine 4 of histone H3 (13, 59). Since CHD8 contains the double chromodomain characteristic of CHD proteins, we tested whether CHD8 interacts with lysine-methylated histone H3. Material partially purified by affinity chromatography over a GST-hStaf column, containing CHD8 as well as human Ino80 (and other hStaf-associated proteins) was incubated with biotinylated peptides corresponding to the first 21 amino acids of histone H3. First, unmodified, dimethylated-K4 (K4M2), acetylated-K9 (K9Ac), and phosphorylated-S10 (S10P) peptides were tested. After incubation with the partially purified material, the biotinylated peptides were recovered with streptavidin beads, and the presence of CHD8 and, as a control, human Ino80 was tested by immunoblotting. As shown in Fig. 5A, the largest amount of CHD8 was recovered with the H3 K4M2 peptide followed by the unmodified H3 peptide, whereas little or no detectable CHD8 was recovered with the H3 K9Ac and H3 S10P peptides. In contrast, human Ino80 bound equally to all peptides, suggesting that the differential binding of CHD8 is not due to different peptide amounts. We then compared CHD8 interactions with biotinylated H3 peptides (amino acids 1 to 21 or 21 to 44) dimethylated at K4, K9, or K36. As shown in Fig. 5B, CHD8 bound efficiently to dimethylated-K4 peptide and weakly to dimethylated-K9 and -K36 peptides, whereas hIno80 bound equally well to all peptides.

It has been shown that different methylation levels at lysines 4 and 9 of histone H3 are enriched in different regions of a transcribed gene (38, 41, 42, 44) and may thus bear different information. We therefore tested the affinities of CHD8 for H3 peptides carrying different numbers of methyl groups on the K4 residue. As shown in Fig. 5C, CHD8, unlike the hIno80 control, bound more efficiently to dimethylated and especially trimethylated H3K4 peptides than to monomethylated and unmodified peptides. The strong CHD8 interaction with trimethylated H3K4 peptide was not prevented by treatment with RNase A, suggesting that it is not mediated by an RNA molecule (Fig. 5D). Thus, CHD8 interacts preferentially with histone H3 di- and trimethylated on lysine 4.

Since CHD8 localizes to the U6-1 promoter and since it associates with peptides corresponding to the N terminus of histone H3 di- and trimethylated on lysine 4, we checked whether histone H3 carrying these modifications is enriched in the vicinity of the U6 promoter. Indeed, as shown in Fig. 5E, ChIPs with antibodies specific for histone H3 di- and trimethylated on lysine 4 revealed the presence of these modified H3 histones in the U6 promoter region. These results suggest that Pol III genes, like Pol II genes, can be modified by methylation of histone H3 on lysine 4, and they are consistent with the U6 promoter being a CHD8 target.

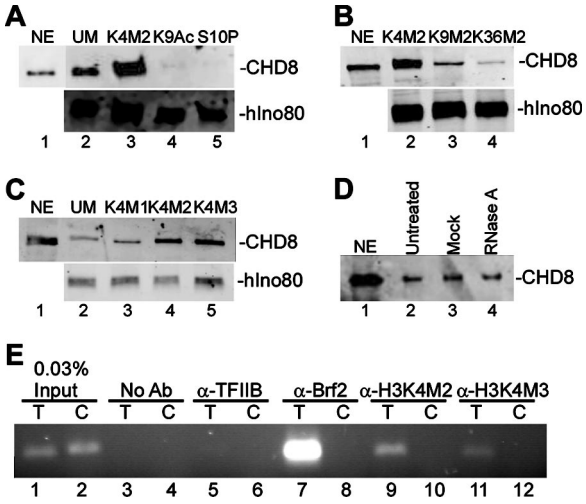


FIG. 5. CHD8 interacts with histone H3 peptides. NE, nuclear HeLa cell extract. (A) CHD8 interacts specifically with an unmodified histone H3 N-terminal peptide and a histone H3 N-terminal peptide dimethylated at lysine 4. CHD8 partially purified over the GST-hStaf affinity column was incubated with biotinylated histone H3 peptides carrying various modifications, as indicated above the lanes. The protein-peptide complexes were recovered with streptavidin beads, fractionated by SDS-PAGE, transferred to a membrane, and probed by immunoblotting with an anti-CHD8 antibody as indicated to the right of the panels. For a control, the membrane was also probed with an anti-hIno80 antibody as indicated to the right of the panels. (B) CHD8 preferentially interacts with a histone H3 peptide dimethylated at lysine 4. The experiment was performed as described for panel A but with the H3 peptides indicated above the lanes. (C) CHD8 preferentially interacts with histone H3 peptides di- and trimethylated at lysine 4. The experiment was performed as described for panel A but with the H3 peptides indicated above the lanes. In panels A, B, and C, the H3 peptides corresponded to the first 21 amino acids of histone H3 unmodified (UM), dimethylated at lysine 4 (K4M2), acetylated at lysine 9 (K9Ac), phosphorylated at serine 10 (S10P), dimethylated at lysine 9 (K9M2), monomethylated at lysine 4 (K4M1), trimethylated at lysine 4 (K4M3), or corresponded to amino acids 21 to 44 of histone H3 dimethylated at lysine 36 (K36M2). (D) The interactions between CHD8 and the H3 K4M3 peptide are not prevented by treatment with RNase A. The experiment was performed as described for panel A, but the protein samples were left untreated or treated with water (Mock) or RNase A before incubation with biotinylated H3 K4M3 peptide. (E) The U6-1 promoter region contains histone H3 di- and trimethylated at lysine 4. ChIPs were performed as described for the lower panel of Fig. 1A. Ab, antibody; T, test primers; C, control primers.

CHD8 is involved in U6 transcription. The interaction between CHD8 and hStaf as well as the localization of CHD8 on an active, but not an inactive, human U6 promoter suggests that CHD8 may be involved in U6 transcription in vivo. To address this possibility, we knocked down CHD8 in an IMR90/hTert U6 reporter cell line (IMR90-C2.5) containing an integrated hU6/Hae/RA.2 construct derivative in which the human U6-1 promoter directs the synthesis of unstable transcripts (28; see Materials and Methods). The reporter line was used because it is difficult to detect transcriptional changes by measuring the cellular U6 snRNA content. The reasons for this are not clear but could be due to the existence of different populations of U6 snRNA (for example, assembled or not assembled with snRNPs) with different stabilities. The reporter cell line was transfected with two different anti-CHD8 double-stranded silencing RNA oligonucleotides (small interfering RNAs [siRNAs]) and CHD8 mRNA and protein levels were measured with RT-PCR and Western blot analysis, respectively. RNA levels derived from the U6 reporter were tested by RT-PCR. As shown in Fig. 6A, both CHD8 protein and mRNA levels went down significantly after anti-CHD8 siRNA transfection (oligonucleotides C8-1 and C8-2) but not after control siRNA transfection (oligonucleotide C) (see the three upper panels). hStaf levels were unaffected (data not shown). In independent CHD8 knock-down experiments, we observed decreases in U6 reporter transcription ranging from 4-fold

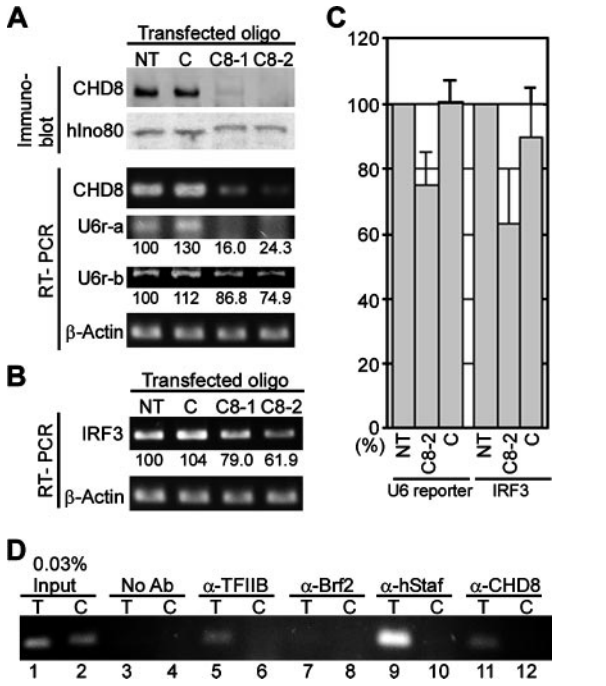


FIG. 6. CHD8 is required for expression of the U6 reporter. (A) The IMR90-C2.5 reporter cell line was either not transfected (NT) or transfected with the siRNAs indicated above the lanes. After three rounds of transfection, the CHD8 and hIno80 contents were analyzed by immunoblotting (two upper panels) and the CHD8 mRNA, U6 reporter transcript, and β-actin mRNA contents were analyzed by RT-PCR (four lower panels). U6r-a and U6r-b, expression from the U6 reporter plasmid, experiments a and b, respectively. (B) CHD8 is involved in transcription of the IRF3 gene. The IRF3 and β-actin mRNA contents were analyzed by RT-PCR. The numbers below the lanes indicate the intensity of the RT-PCR signal normalized to the β-actin signal. NT, cells not transfected. (C) The relative amount (nontransfected cells [NT] = 100) of U6 reporter transcript or IRF3 mRNA from control oligonucleotide (C)- or C8-2 oligonucleotide-transfected cells. The averages from six (for U6 reporter) or four (for IRF3 mRNA) independent RNAi experiments were plotted and the standard deviations (error bars) are shown. Wilcoxon rank sum tests were used to test the significance of the RNAi knock-down effect. For the U6 reporter ($n = 6$), the P value for C8-2-transfected cells in comparison to nontransfected cells is 0.0022 and the P value for the control oligonucleotide-transfected cells in comparison to nontransfected cells is 1. For the IRF3 mRNA ($n = 4$), the P value for C8-2-transfected cells in comparison to nontransfected cells is 0.0286 and the P value for the control oligonucleotide-transfected cells in comparison to nontransfected cells is 0.31. (D) CHD8 is localized on the IRF3 promoter. ChIPs were performed as described for the lower panel of Fig. 1A except that the PCR was performed with test primers specific for the IRF3 promoter (T) or control (C) primers hybridizing to an inactive U6-4 gene. Ab, antibody.

(Fig. 6A, panel U6r-a) to 0.6-fold (panel U6r-b). However, even though the decrease in the U6 transcript was moderate, it was significant ($n = 6$, $P = 0.0022$ [Wilcoxon rank sum test]; see the legend to Fig. 6C). Thus, CHD8 contributes to efficient transcription from the human U6 promoter in vivo. The modest effect of CHD8 knock-down on ongoing U6 transcription is, perhaps, not unexpected, as CHD8 may be part of an extensive network of chromatin modifying proteins at the U6 promoter, which may have partially redundant functions.

CHD8 is involved in IRF3 expression. In addition to regulating transcription of Pol III genes, hStaf has been reported to regulate the transcription of seven Pol II mRNA genes (34). We tested mRNA levels for three of them, AKR1A1, IRF3, and TCP1, after decreasing cellular CHD8 levels by RNAi as described before. As shown in Fig. 6B, IRF3 mRNA levels went down after transfection of two different siRNAs directed against CHD8 (C8-1 and C8-2) but not after transfection of a control siRNA. The effect was again modest but statistically significant ($n = 4$, $P = 0.0286$ [Wilcoxon rank sum test]; see the legend to Fig. 6C) and suggested that CHD8 could be involved in transcription regulation of the IRF3 gene. We did not observe, however, a significant reduction in mRNA levels for the AKR1A1 and TCP1 genes (data not shown), suggesting either that CHD8 is not involved in transcription of these genes or that these mRNAs are stable, preventing the detection of subtle changes over a relatively short period of time.

If CHD8 is indeed involved in IRF3 expression, one should be able to detect it at the IRF3 promoter. We performed a ChIP with anti-CHD8 and other antibodies and used a primer set specific for the IRF3 promoter to amplify the immunoprecipitated DNA fragments. As shown in Fig. 6D, the ChIP results indicated the presence of TFIIB, but not the Pol III transcription factor Brf2, on the IRF3 promoter (lanes labeled T) but not on the 5′ flanking region of the inactive U6-4 gene (lanes labeled C). Importantly, hStaf as well as CHD8 was also specifically detected on the IRF3 promoter (lanes 9 to 12). The CHD8 signal was weak but comparable to that of the positive TFIIB control (lane 5). These results show that hStaf and CHD8 reside on a Pol II mRNA promoter.

DISCUSSION

Most chromatin-modifying activities have been studied in the context of Pol II transcription. Little is known about chromatin-modifying activities required for transcription of the few hundred Pol III genes interspersed in the genome. In particular, it is not clear whether these activities are dedicated to Pol III genes. The identification of CHD8 as an hStaf-associated polypeptide involved in U6 transcription strongly suggests that Pol III transcription from type 3 promoters involves chromatin remodeling. Moreover, the observations that the DSEs in type 3 Pol III promoters and in Pol II snRNA promoters are interchangeable (17), that CHD8 is localized on both the Pol III U6 and Pol II U1 promoters, and that CHD8 is involved in transcription from the IRF3 mRNA promoter suggest that type 3 Pol III promoters, Pol II snRNA promoters, and Pol II mRNA promoters share common chromatin-modifying activities. Thus, the DSE-binding factor hStaf and perhaps Oct-1, which are both also used by some Pol II mRNA promoters, may recruit similar chromatin-modifying activities in these three contexts.

If type 3 Pol III promoters recruit at least some of the chromatin-modifying activities used by Pol II genes, they may use different chromatin-remodeling activities than type 1 and 2 Pol III promoters. Indeed, the WICH complex, composed of the Williams syndrome transcription factor WSTF, SNF2h, and other factors, was recently shown to associate with the type 1 5S and the mixed type 2 and 3 7SL genes and to be involved in their transcription, as down-regulation of WSTF by RNAi resulted in reduced 5S and 7SL transcription (5). However, no function of WICH in transcription from type 3 promoters could be demonstrated. How WICH is recruited to 5S rRNA and 7SL promoters is unknown, but a key player may well turn out to be the TFIIIC complex. This complex, which is recruited by type 1 and 2 promoters but not by type 3 promoters, is essential for chromatin remodeling of the yeast U6 promoter (which, unlike the human U6 promoter, is a type 2 Pol III promoter with a TATA box) (56, 57), suggesting that the complex recruits chromatin-remodeling activities. Moreover, two of the human TFIIIC subunits display histone acetyltransferase activity (18, 26). Thus, TFIIIC may be central to chromatin modification at type 1 and 2 Pol III promoters, both modifying histone tails and recruiting chromatin-remodeling activities.

The mechanism by which hStaf and CHD8 activate transcription from the U6 promoter and how the action of hStaf combines with that of Oct-1 remain to be elucidated. In previous experiments, we showed that the Oct-1 POU domain could activate U6 transcription from a chromatin template when added during chromatin assembly (78). hStaf is capable of activating U6 transcription even when added after chromatin assembly, suggesting that it might bind to the U6 promoter before Oct-1. Its interaction with CHD8 suggests that the mechanism by which it activates U6 transcription involves recruitment of CHD8, which in turn leads to chromatin remodeling. However, it is also possible that CHD8 binds DNA before hStaf. Indeed, we showed that, like CHD1, CHD8 binds preferentially to histone H3 carrying a di- or trimethyl group at lysine 4 and that the U6 promoter region is enriched in histone H3 carrying these modifications. It is conceivable that in vivo, these modifications occur before CHD8 binding and then allow binding of CHD8 through contacts with lysine 4-methylated histone H3 and perhaps through direct contacts with the DNA through the CHD8 putative C-terminal DNA binding domain. DNA-bound CHD8 could then help recruit hStaf to its DNA binding site by protein-protein interaction.

The recent discovery that CHD8 associates with CTCF (19) together with our observation that CHD8 can be localized around the U6 promoter region open the possibility that CTCF is bound to insulator sites at or close to the U6 promoter. The U6-1 gene resides in a relatively barren region in the human genome, with very few expressed sequence tags encoded in the upstream 215 kb (http://genome.ucsc.edu/cgi-bin/hgGateway). In the downstream region, the first known gene is located 5 kb away and corresponds to the human homolog of mouse Corl1, a transcription corepressor expressed only in the brain and testis (32). The U6-1 gene may, therefore, require an active insulator to separate itself from its probably largely transcription-silent environment. Although no exact matches were identified, several sequences diverging from the human and mouse

β-globin and H19 CTCF binding sites (12, 20) at only a few positions can be found around the U6-1 gene.

Our analysis of hStaf-associated proteins has revealed several proteins known, or likely, to be involved in chromatin modification. For example, HCF-1 is a heterodimeric protein generated from its 2,035-amino-acid precursor by site-specific proteolysis, whose N-terminal subunit associates with the Sin3 histone deacetylase and the Set1/Ash2 histone methyltransferase complexes (75). It might bring the histone methyltransferases needed to give rise to the di- and trimethylated histone H3 present around the U6 promoter. Brd2 and Brd3 are bromodomain proteins known to interact with acetylated histones and, at least in the case of Brd2, to activate transcription (60). As well, hIno80, nuclear factor related to kappa B binding protein, and RUVB-like 1 protein are members of the hIno80 complex (22), whose yeast counterpart is involved in transcription activation as well as in other processes, such as DNA repair (33, 54, 70). Although we cannot exclude the possibility that these factors are involved only in Pol II transcription of mRNA genes activated by hStaf, they are all potential chromatin-modifying factors involved in Pol III transcription from the U6 promoter.

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REFERENCES

1. **Aasland, R., A. F. Stewart, and T. Gibson.** 1996. The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB. Trends Biochem. Sci. **21:** 87–88.
2. **Aravind, L., and D. Landsman.** 1998. AT-hook motifs identified in a wide variety of DNA-binding proteins. Nucleic Acids Res. **26:**4413–4421.
3. **Aubry, F., M. G. Mattei, and F. Galibert.** 1998. Identification of a human 17p-located cDNA encoding a protein of the Snf2-like helicase family. Eur. J. Biochem. **254:**558–564.
4. **Bagchi, A., C. Papazoglu, Y. Wu, D. Capurso, M. Brodt, D. Francis, M. Bredel, H. Vogel, and A. A. Mills.** 2007. CHD5 is a tumor suppressor at human 1p36. Cell **128:**459–475.
5. **Cavellán, E., P. Asp, P. Percipalle, and A. K. Farrants.** 2006. The WSTF-SNF2h chromatin remodeling complex interacts with several nuclear proteins in transcription. J. Biol. Chem. **281:**16264–16271.
6. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
7. **Domitrovich, A. M., and G. R. Kunkel.** 2003. Multiple, dispersed human U6 small nuclear RNA genes with varied transcriptional efficiencies. Nucleic Acids Res. **31:**2344–2352.
8. **Dou, Y., T. A. Milne, A. J. Tackett, E. R. Smith, A. Fukuda, J. Wysocka, C. D. Allis, B. T. Chait, J. L. Hess, and R. G. Roeder.** 2005. Physical association and coordinate function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase MOF. Cell **121:**873–885.
9. **Eng, J. K., A. L. McCormack, and J. R. Yates III.** 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. **5:**976–989.
10. **Englander, E. W., A. P. Wolffe, and B. H. Howard.** 1993. Nucleosome interactions with a human Alu element. Transcriptional repression and effects of template methylation. J. Biol. Chem. **268:**19565–19573.
11. **Erkine, A. M.** 2004. Activation domains of gene-specific transcription factors: are histones among their targets? Biochem. Cell Biol. **82:**453–459.
12. **Farrell, C. M., A. G. West, and G. Felsenfeld.** 2002. Conserved CTCF insulator elements flank the mouse and human beta-globin loci. Mol. Cell. Biol. **22:**3820–3831.
13. **Flanagan, J. F., L. Z. Mi, M. Chruszcz, M. Cymborowski, K. L. Clines, Y. Kim, W. Minor, F. Rastinejad, and S. Khorasanizadeh.** 2005. Double chromodomains cooperate to recognize the methylated histone H3 tail. Nature **438:**1181–1185.
14. **Florens, L., and M. P. Washburn.** 2006. Proteomic analysis by multidimensional protein identification technology. Methods Mol. Biol. **328:**159–175.
15. **Ge, Q., D. S. Nilasena, C. A. O'Brien, M. B. Frank, and I. N. Targoff.** 1995. Molecular analysis of a major antigenic region of the 240-kD protein of Mi-2 autoantigen. J. Clin. Investig. **96:**1730–1737.
16. **Guffanti, E., R. Percudani, O. Harismendy, J. Soutourina, M. Werner, M. G. Iacovella, R. Negri, and G. Dieci.** 2006. Nucleosome depletion activates poised RNA polymerase III at unconventional transcription sites in Saccharomyces cerevisiae. J. Biol. Chem. **281:**29155–29164.
17. **Hernandez, N.** 2001. Small nuclear RNA genes: a model system to study fundamental mechanisms of transcription. J. Biol. Chem. **276:**26733–26736.
18. **Hsieh, Y. J., T. K. Kundu, Z. Wang, R. Kovelman, and R. G. Roeder.** 1999. The TFIIIC90 subunit of TFIIIC interacts with multiple components of the RNA polymerase III machinery and contains a histone-specific acetyltransferase activity. Mol. Cell. Biol. **19:**7697–7704.
19. **Ishihara, K., M. Oshimura, and M. Nakao.** 2006. CTCF-dependent chromatin insulator is linked to epigenetic remodeling. Mol. Cell **23:**733–742.
20. **Ishihara, K., and H. Sasaki.** 2002. An evolutionarily conserved putative insulator element near the 3′ boundary of the imprinted Igf2/H19 domain. Hum. Mol. Genet. **11:**1627–1636.
21. **Jacobs, S. A., and S. Khorasanizadeh.** 2002. Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. Science **295:**2080–2083.
22. **Jin, J., Y. Cai, T. Yao, A. J. Gottschalk, L. Florens, S. K. Swanson, J. L. Gutierrez, M. K. Coleman, J. L. Workman, A. Mushegian, M. P. Washburn, R. C. Conaway, and J. W. Conaway.** 2005. A mammalian chromatin remodeling complex with similarities to the yeast INO80 complex. J. Biol. Chem. **280:**41207–41212.
23. **Kingston, R. E., and G. J. Narlikar.** 1999. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev. **13:**2339–2352.
24. **Kornberg, R. D., and Y. Lorch.** 1999. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell **98:**285–294.
25. **Krogan, N. J., M. Kim, S. H. Ahn, G. Zhong, M. S. Kobor, G. Cagney, A. Emili, A. Shilatifard, S. Buratowski, and J. F. Greenblatt.** 2002. RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach. Mol. Cell. Biol. **22:**6979–6992.
26. **Kundu, T. K., Z. Wang, and R. G. Roeder.** 1999. Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. Mol. Cell. Biol. **19:** 1605–1615.
27. **Li, M., D. Chen, A. Shiloh, J. Luo, A. Y. Nikolaev, J. Qin, and W. Gu.** 2002. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature **416:**648–653.
28. **Lobo, S. M., and N. Hernandez.** 1989. A 7 bp mutation converts a human RNA polymerase II snRNA promoter into an RNA polymerase III promoter. Cell **58:**55–67.
29. **Lusser, A., D. L. Urwin, and J. T. Kadonaga.** 2005. Distinct activities of CHD1 and ACF in ATP-dependent chromatin assembly. Nat. Struct. Mol. Biol. **12:**160–166.
30. **McDonald, W. H., R. Ohi, D. T. Miyamoto, T. J. Mitchison, and J. R. Yates III.** 2002. Comparison of three directly coupled HPLC MS/MS strategies for identification of proteins from complex mixtures: single-dimension LC-MS/MS, 2-phase MudPIT, and 3-phase MudPIT. Int. J. Mass Spectrom. **219:** 245–251.
31. **Min, J., Y. Zhang, and R. M. Xu.** 2003. Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. Genes Dev. **17:**1823–1828.
32. **Mizuhara, E., T. Nakatani, Y. Minaki, Y. Sakamoto, and Y. Ono.** 2005. Corl1, a novel neuronal lineage-specific transcriptional corepressor for the homeodomain transcription factor Lbx1. J. Biol. Chem. **280:**3645–3655.
33. **Morrison, A. J., J. Highland, N. J. Krogan, A. Arbel-Eden, J. F. Greenblatt, J. E. Haber, and X. Shen.** 2004. INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. Cell **119:** 767–775.
34. **Myslinski, E., M. A. Gerard, A. Krol, and P. Carbon.** 2006. A genome scale location analysis of human Staf/ZNF143-binding sites suggests a widespread role for human Staf/ZNF143 in mammalian promoters. J. Biol. Chem. **281:** 39953–39962.
35. **Myslinski, E., A. Krol, and P. Carbon.** 1998. ZNF76 and ZNF143 are two human homologs of the transcriptional activator Staf. J. Biol. Chem. **273:** 21998–22006.
36. **Narlikar, G. J., H. Y. Fan, and R. E. Kingston.** 2002. Cooperation between complexes that regulate chromatin structure and transcription. Cell **108:**475–487.
37. **Neely, K. E., A. H. Hassan, A. E. Wallberg, D. J. Steger, B. R. Cairns, A. P. Wright, and J. L. Workman.** 1999. Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays. Mol. Cell **4:**649–655.
38. **Ng, H. H., F. Robert, R. A. Young, and K. Struhl.** 2003. Targeted recruitment

of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol. Cell **11:**709–719.
39. **Nielsen, P. R., D. Nietlispach, H. R. Mott, J. Callaghan, A. Bannister, T. Kouzarides, A. G. Murzin, N. V. Murzina, and E. D. Laue.** 2002. Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. Nature **416:**103–107.
40. **Orphanides, G., and D. Reinberg.** 2000. RNA polymerase II elongation through chromatin. Nature **407:**471–475.
41. **Peters, A. H., S. Kubicek, K. Mechtler, R. J. O'Sullivan, A. A. Derijck, L. Perez-Burgos, A. Kohlmaier, S. Opravil, M. Tachibana, Y. Shinkai, J. H. Martens, and T. Jenuwein.** 2003. Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol. Cell **12:**1577–1589.
42. **Pokholok, D. K., C. T. Harbison, S. Levine, M. Cole, N. M. Hannett, T. I. Lee, G. W. Bell, K. Walker, P. A. Rolfe, E. Herbolsheimer, J. Zeitlinger, F. Lewitter, D. K. Gifford, and R. A. Young.** 2005. Genome-wide map of nucleosome acetylation and methylation in yeast. Cell **122:**517–527.
43. **Pray-Grant, M. G., J. A. Daniel, D. Schieltz, J. R. Yates III, and P. A. Grant.** 2005. Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. Nature **433:**434–438.
44. **Rice, J. C., S. D. Briggs, B. Ueberheide, C. M. Barber, J. Shabanowitz, D. F. Hunt, Y. Shinkai, and C. D. Allis.** 2003. Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol. Cell **12:**1591–1598.
45. **Rincon, J. C., S. K. Engler, B. W. Hargrove, and G. R. Kunkel.** 1998. Molecular cloning of a cDNA encoding human SPH-binding factor, a conserved protein that binds to the enhancer-like region of the U6 small nuclear RNA gene promoter. Nucleic Acids Res. **26:**4846–4852.
46. **Robinson, K. M., and M. C. Schultz.** 2003. Replication-independent assembly of nucleosome arrays in a novel yeast chromatin reconstitution system involves antisilencing factor Asf1p and chromodomain protein Chd1p. Mol. Cell. Biol. **23:**7937–7946.
47. **Saha, A., J. Wittmeyer, and B. R. Cairns.** 2006. Chromatin remodelling: the industrial revolution of DNA around histones. Nat. Rev. Mol. Cell Biol. **7:**437–447.
48. **Santoro, R., J. Li, and I. Grummt.** 2002. The nucleolar remodeling complex NoRC mediates heterochromatin formation and silencing of ribosomal gene transcription. Nat. Genet. **32:**393–396.
49. **Schaub, M., A. Krol, and P. Carbon.** 1999. Flexible zinc finger requirement for binding of the transcriptional activator staf to U6 small nuclear RNA and tRNA(Sec) promoters. J. Biol. Chem. **274:**24241–24249.
50. **Schaub, M., E. Myslinski, C. Schuster, A. Krol, and P. Carbon.** 1997. Staf, a promiscuous activator for enhanced transcription by RNA polymerases II and III. EMBO J. **16:**173–181.
51. **Schuster, C., A. Krol, and P. Carbon.** 1998. Two distinct domains in Staf to selectively activate small nuclear RNA-type and mRNA promoters. Mol. Cell. Biol. **18:**2650–2658.
52. **Schuster, C., E. Myslinski, A. Krol, and P. Carbon.** 1995. Staf, a novel zinc finger protein that activates the RNA polymerase III promoter of the selenocysteine tRNA gene. EMBO J. **14:**3777–3787.
53. **Schuster, E. F., and R. Stoger.** 2002. CHD5 defines a new subfamily of chromodomain-SWI2/SNF2-like helicases. Mamm. Genome **13:**117–119.
54. **Shen, X., G. Mizuguchi, A. Hamiche, and C. Wu.** 2000. A chromatin remodelling complex involved in transcription and DNA processing. Nature **406:**541–544.
55. **Shimono, K., Y. Shimono, K. Shimokata, N. Ishiguro, and M. Takahashi.** 2005. Microspherule protein 1, Mi-2beta, and RET finger protein associate in the nucleolus and up-regulate ribosomal gene transcription. J. Biol. Chem. **280:**39436–39447.
56. **Shivaswamy, S., and P. Bhargava.** 2006. Positioned nucleosomes due to sequential remodeling of the yeast U6 small nuclear RNA chromatin are essential for its transcriptional activation. J. Biol. Chem. **281:**10461–10472.
57. **Shivaswamy, S., G. A. Kassavetis, and P. Bhargava.** 2004. High-level activation of transcription of the yeast U6 snRNA gene in chromatin by the basal RNA polymerase III transcription factor TFIIIC. Mol. Cell. Biol. **24:**3596–3606.
58. **Simic, R., D. L. Lindstrom, H. G. Tran, K. L. Roinick, P. J. Costa, A. D. Johnson, G. A. Hartzog, and K. M. Arndt.** 2003. Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. EMBO J. **22:**1846–1856.
59. **Sims, R. J., III, C. F. Chen, H. Santos-Rosa, T. Kouzarides, S. S. Patel, and D. Reinberg.** 2005. Human but not yeast CHD1 binds directly and selectively to histone H3 methylated at lysine 4 via its tandem chromodomains. J. Biol. Chem. **280:**41789–41792.
60. **Sinha, A., D. V. Faller, and G. V. Denis.** 2005. Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A. Biochem. J. **387:**257–269.
61. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
62. **Studitsky, V. M., D. J. Clark, and G. Felsenfeld.** 1995. Overcoming a nucleosomal barrier to transcription. Cell **83:**19–27.
63. **Studitsky, V. M., G. A. Kassavetis, E. P. Geiduschek, and G. Felsenfeld.** 1997. Mechanism of transcription through the nucleosome by eukaryotic RNA polymerase. Science **278:**1960–1963.
64. **Tabb, D. L., W. H. McDonald, and J. R. Yates III.** 2002. DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. J. Proteome Res. **1:**21–26.
65. **Tai, H. H., M. Geisterfer, J. C. Bell, M. Moniwa, J. R. Davie, L. Boucher, and M. W. McBurney.** 2003. CHD1 associates with NCoR and histone deacetylase as well as with RNA splicing proteins. Biochem. Biophys. Res. Commun. **308:**170–176.
66. **Tong, J. K., C. A. Hassig, G. R. Schnitzler, R. E. Kingston, and S. L. Schreiber.** 1998. Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature **395:**917–921.
67. **Tran, H. G., D. J. Steger, V. R. Iyer, and A. D. Johnson.** 2000. The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. EMBO J. **19:**2323–2331.
68. **Tse, C., T. Sera, A. P. Wolffe, and J. C. Hansen.** 1998. Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III. Mol. Cell. Biol. **18:**4629–4638.
69. **Ura, K., H. Kurumizaka, S. Dimitrov, G. Almouzni, and A. P. Wolffe.** 1997. Histone acetylation: influence on transcription, nucleosome mobility and positioning, and linker histone-dependent transcriptional repression. EMBO J. **16:**2096–2107.
70. **van Attikum, H., O. Fritsch, B. Hohn, and S. M. Gasser.** 2004. Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair. Cell **119:**777–788.
71. **Wade, P. A., P. L. Jones, D. Vermaak, and A. P. Wolffe.** 1998. A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. Curr. Biol. **8:**843–846.
72. **Wang, H. B., and Y. Zhang.** 2001. Mi2, an auto-antigen for dermatomyositis, is an ATP-dependent nucleosome remodeling factor. Nucleic Acids Res. **29:**2517–2521.
73. **Washburn, M. P., D. Wolters, and J. R. Yates III.** 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. **19:**242–247.
74. **Workman, J. L.** 2006. Nucleosome displacement in transcription. Genes Dev. **20:**2009–2017.
75. **Wysocka, J., M. P. Myers, C. D. Laherty, R. N. Eisenman, and W. Herr.** 2003. Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3–K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. Genes Dev. **17:**896–911.
76. **Xue, Y., J. Wong, G. T. Moreno, M. K. Young, J. Cote, and W. Wang.** 1998. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol. Cell **2:**851–861.
77. **Zhang, Y., G. LeRoy, H. P. Seelig, W. S. Lane, and D. Reinberg.** 1998. The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell **95:**279–289.
78. **Zhao, X., P. S. Pendergrast, and N. Hernandez.** 2001. A positioned nucleosome on the human U6 promoter allows recruitment of SNAPc by the Oct-1 POU domain. Mol. Cell **7:**539–549.
79. **Zhou, Y., R. Santoro, and I. Grummt.** 2002. The chromatin remodeling complex NoRC targets HDAC1 to the ribosomal gene promoter and represses RNA polymerase I transcription. EMBO J. **21:**4632–4640.
80. **Zybailov, B., A. L. Mosley, M. E. Sardiu, M. K. Coleman, L. Florens, and M. P. Washburn.** 2006. Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae. J. Proteome Res. **5:**2339–2347.