Mi2β Shows Chromatin Enzyme Specificity by Erasing a DNase I-hypersensitive Site Established by ACF

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ATP-dependent chromatin-remodeling enzymes are linked to changes in gene expression; however, it is not clear how the multiple remodeling enzymes found in eukaryotes differ in function and work together. In this report, we demonstrate that the ATP-dependent remodeling enzymes ACF and Mi2β can direct consecutive, opposing chromatin-remodeling events, when recruited to chromatin by different transcription factors. In a cell-free system based on the immunoglobulin heavy chain gene enhancer, we show that TFE3 induces a DNase I-hypersensitive site in an ATP-dependent reaction that requires ACF following transcription factor binding to chromatin. In a second step, PU.1 directs Mi2β to erase an established DNase I-hypersensitive site, in an ATP-dependent reaction subsequent to PU.1 binding to chromatin, whereas ACF will not support erasure. Erasure occurred without displacing the transcription factor that initiated the site. Other tested enzymes were unable to erase the DNase I-hypersensitive site. Establishing and erasing the DNase I-hypersensitive site required transcriptional activation domains from TFE3 and PU.1, respectively. Together, these results provide important new mechanistic insight into the combinatorial control of chromatin structure.

Chromatin remodeling and recruitment of RNA polymerase to promoters coordinately regulate gene transcription in eukaryotes. One class of chromatin-remodeling enzyme, the ATP-dependent remodeling enzymes, move nucleosomes and alter histone-DNA contacts (1). These enzymes are generally multisubunit protein complexes. The core ATPase component of these complexes comes in at least three varieties, including the SWI2/SNF2, ISWI, and Mi2 subfamilies (2, 3). At present, it is not well understood how these enzymes differ functionally. One limiting case is that they may perform the same task while being recruited to different sites; alternatively, they may perform different functions. It is also possible that they have different expression patterns or post-translational regulation. Changes induced by these enzymes increase or decrease access of sequence-specific DNA binding proteins to target sites in promoters and enhancers (4). Another type of chromatin remodeling is mediated by enzymes that covalently modify histones, such as acetyltransferases or methyltransferases that are usually a part of multisubunit protein complexes (5, 6). These modifications may serve as docking sites for other proteins, and these changes may alter histone-DNA contacts thereby enabling access of the transcription machinery to the DNA. Chromatin remodeling is also mediated by changes in DNA methylation (7). These three chromatin-remodeling systems interact to define the chromatin state of a locus at a given time in a given cell type (4, 7). Finally, a complex set of protein-protein interactions allows DNA-bound transcription factors to recruit RNA polymerase to initiate gene transcription. Thereafter, RNA polymerases must continually overcome nucleosomal barriers to elongate and produce the primary transcript.

Transcription activation (TA) domains have been identified on many DNA-binding transcription factors using either cotransfection assays or fusions to heterologous DNA-binding domains. It is perhaps not surprising that several well characterized TA domains have been shown to positively influence chromatin remodeling and RNA polymerase recruitment. For example, the VP16 transactivation domain interacts with components of the basal transcription machinery such as TBP and TFII B (8), recruits the ATP-dependent remodeling SWI/SNF complex (9) as well as associates with the histone acetyltransferase-containing Spt-Ada-Gcn5 acetyltransferase complex (10). However, the question as to which function, if any, predominates for a factor in the normal context remains open, and they may vary at different loci. Moreover, transcription factors can direct chromatin remodeling in the absence of activation.

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6 The abbreviations used are: TA, transcription activation; HS, hypersensitive site; DHS, DNase I-hypersensitive site; TSA, trichostatin A; TF, transcription factor.
domains (11, 12). An additional level of complexity in most regulatory sequences is that they contain binding sites for multiple transcription factors. At these promoters or enhancers, each factor can participate in more than one function or, alternatively, preferentially recruit chromatin-remodeling enzymes, the basal transcription machinery, or other transcription factors. The mechanisms of combinatorial transcription activation are relatively unexplored.

The immunoglobulin μ heavy chain gene enhancer (μ enhancer), a transcription and recombination enhancer (13, 14), is an archetype of combinatorial control (15). Most proteins that bind to the enhancer are expressed in a wide variety of cell types, though enhancer activity is restricted to lymphocytes. The tissue distribution of even the most restricted subset of enhancer-binding proteins does not coincide precisely with cells in which the enhancer is active. We have proposed that tissue specificity of transcriptional activity is dictated by a combinatorial mechanism (16). To simplify the problem we previously defined a three-part μ enhancer fragment (μ70) (17–20) that exhibits three of the most important characteristics of the full enhancer. First, μ70 is tissue-restricted, in that it is active in B cells, but not in T or non-lymphoid cells. Second, the three proteins that are required for μ70 activity fall into the two broad categories of being ubiquitously (widely) expressed and having more limited tissue distribution. Third, multimerized versions of each binding site do not activate transcription in B cells indicating that μ70 is activated by the combined action of the three proteins. Conversely, mutation in any one of the three binding sites eliminates enhancer activity in B cells. μ70 is composed of the DNA elements μB, μE3, and μA (Fig. 1A) that bind the proteins PU.1, TFE3, and Ets-1, respectively. μ70 activity in B cells is recapitulated in non-lymphoid cells by ectopic expression of PU.1 and Ets-1, with endogenous μE3 binding protein(s) being recruited to the enhancer. In the combinatorial transactivation assay we have also shown that activity of one of two TA domains on TFE3 and a TA domain on Ets-1 is essential to activate the μ70 enhancer, whereas a TA domain on PU.1 is not required (17, 19). How the three proteins cooperate to activate transcription remains unclear.

To begin to address the mechanism of combinatorial control we investigated the function of μ70 binding proteins in vitro in the context of chromatin. Of the three proteins, only TFE3 binds stably to chromatin in vitro, resulting in the generation of a DNase I-hypersensitive site, positioned nucleosomes, enhanced restriction enzyme access to proximal sites and transcriptional activity (21). Binding of PU.1 and Ets-1 is enhanced by TFE3. PU.1 in combination with TFE3 retains the TFE3-positioned nucleosomes and transcriptional activity without the TFE3-induced DNase I hypersensitivity. Ets-1 binding does not affect the TFE3-induced DNase I-hypersensitive site. These observations indicate that DNase I hypersensitivity is not necessarily proportional to the number of DNA-bound transcription factors, because occupancy of two TFE3 binding sites is sufficient to generate a hypersensitive site, whereas occupancy of six sites (two TFE3, two PU.1, and two Ets-1) does not generate a hypersensitive site. Thus, chromatin structure mediated by the μ enhancer-binding proteins in vitro is determined by the combinatorial control of transcription factors.

DNase I-hypersensitive sites frequently mark cis-regulatory regions, and as a result have been a useful tool for identifying regulatory regions in organisms with complex genomes. Techniques for genome-wide analysis of DNase I sites have augmented the usefulness of DNase I mapping (22, 23). However, DNase I-hypersensitive sites are not well understood at the molecular level, likely because they are heterogeneous. One way to generate a DHS is by creating a nucleosome free region; a nucleosome-free region results if the DNA-bound factors prevent nucleosome assembly over the bound sequence (11, 12, 24). Nucleosome disruption can be ATP-dependent and may involve nucleosome translocation, displacement, or unfolding. A second route to a DNase I HS is utilized by “pioneer” factors of which the best characterized belongs to the Fox A family (25). These factors induce DNase I HS in pre-assembled nucleosomal arrays by disrupting histone H1-induced chromatin compaction in vitro. ATP hydrolysis is not required for this process, which is proposed to occur by direct interaction of the transcription factor with histones H3 and H4 (26). Histone modifications and DNA folding may also produce DNase I-hypersensitive sites in chromatin.

In this report we investigate the mechanism by which these enhancer-binding proteins alter chromatin structure. We demonstrate that TFE3-induced generation of a DNase I-hypersensitive site requires ATP-dependent chromatin remodeling and an activation domain in TFE3. The ISWI-containing ACF-remodeling enzyme is able to mediate this alteration of structure. However, once created the hypersensitive site is maintained in the absence of ATP. We found that PU.1 erases a pre-formed DNase I-hypersensitive site in a reaction that requires ATP and the N-terminal domain of PU.1. Of five purified remodeling activities tested, only recombinant Mi2β directed by PU.1 disrupted TFE3-induced hypersensitivity. Thus, we find that ATP-dependent remodeling enzymes can have opposing functions on the same template, we identify Mi2β as an enzyme that can erase an established DNase I-hypersensitive site, and we find remodeling enzymes can function subsequent to transcription factor binding, rather than only facilitating transcription factor binding.

**Experimental Procedures**

**Plasmids**—His-PU.1ΔN expression vector was constructed as follows. DNA encoding the C-terminal region of PU.1 from His-PU.1 expression vector was amplified by PCR with oligonucleotide primers with sequences 5′-GGA ATT CGA AGC TGA TGG CTT GGA GCC TG-3′ and 5′-GCT CTA GAT CAT GGG TTG GGA GGG ACT TGA TGG GCC GGG AGG CGC C-3′, and with sequences 5′-GGA ATT CGA AGC TGA TGG CTT GGA GCC TG-3′ and 5′-GCT CTA GAT CAT GGG TTG GGA GGG ACT TGA TGG GCC GGG AGG CGC C-3′. The PCR product was digested with EcoRI and XbaI and was inserted into pProEx HTa (Invitrogen) plasmid digested with EcoRI and XbaI. The PCR product was digested with EcoRI and XbaI and was inserted into pProEx HTa (Invitrogen) plasmid digested with EcoRI and XbaI.

Expression vectors for His-TFE3ΔN and His-TFE3ΔNΔC were constructed as follows. His-TFE3 expression vector was used as a template for PCR with pairs of oligonucleotide primers with sequences 5′-CGG GAT CAT CGG AGG CAA AGG CCC TTT TGA AGG-3′ and 5′-GCT CTA GAT CAG TTG GCC GGG AGG CGC C-3′. The PCR product was digested with EcoRI and XbaI and was inserted into pProEx HTa (Invitrogen) plasmid digested with EcoRI and XbaI.

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BamHI and XbaI and were inserted into pProEx HTb (Invitrogen) plasmid digested with BamHI and XbaI.

**Protein Purification**—Purification of 6-histidine-tagged full-length TFE3 and PU.1 was described previously (21).

PU.1ΔN, TFE3ΔN, and TFE3ΔNΔC were expressed in bacteria as 6-histidine-tagged proteins and purified by Ni²⁺ affinity chromatography under native condition, essentially as described for TFE3 (20), but without DNA affinity chromatography. TFE3ΔN was dialyzed against 50 mM Tris-HCl, pH 7.6, and was further purified with a Uno Q column with a linear 0 to 1 M NaCl gradient. The purified proteins were dialyzed against buffer D (20 mM Hepes, pH 7.6, 0.2 mM EDTA, 0.5 mM dithiothreitol, 100 mM KCl). ACF, NAP1, and topoisomerase I fragment (ND423) were purified as described (27).

NURF (NURF301 and ISWI) was provided by Hua Xiao and Carl Wu (28). SWI/SNF was prepared as described (29). BRG1 was prepared as described (30). N-terminal FLAG-tagged human Mi2β was expressed in the baculovirus system and purified as described previously for other remodeling ATPases (29–31). SF9 cells were grown in 1l spinner flasks to log phase (10⁶ cells/ml) and infected with Mi2 baculovirus at a ratio of 10 colony-forming units/cell (multiplicity of infection = 10). Infected cells were incubated for 42 h at 27 °C, and nuclear extracts were made following standard techniques. Mi2-containing nuclear extracts were then incubated with M2-agarose and incubated at 4 °C for 12 h before being poured onto a column and washed with BC-100, BC-300, BC-500, BC-800, BC-1000, and BC-100. (BC-100 is 20 mM HEPES, pH 7.9, 0.2 mM EDTA, 10% glycerol, 100 mM KCl, 1 mM dithiothreitol; BC-300 contains 300 mM KCl, etc.) The protein was then eluted by adding BC-100 with 0.5 mg/ml FLAG peptide and incubating for 1 h at 4 °C. Fractions were quantified by Bradford assay against bovine serum albumin standards, frozen in liquid nitrogen, and stored at −80 °C until use.

**Chromatin Assembly**—Chromatin assembly using *Drosophila* embryo S-190 extract was done as described previously (21, 32).

Chromatin assembly with recombinant ACF, NAP-1, topoisomerase I fragment, and native, purified *Drosophila* core histones was done as described by Fyodorov and Levenstein (27) with core histones to DNA ratio of 1:1. The assembled chromatin was analyzed by micrococcal nuclease digestion essentially as described (27). When indicated, ATP was depleted with the adduction of Apyrase (Sigma) to a final concentration of 2 units/ml.

**DNase I Analyses**—DNase I digestion of chromatin was done essentially as described (32). 25 μl of chromatin aliquots (125 ng of DNA) were digested by addition of 2.5 μl of DNase I (80 μg/ml for chromatin assembled with S190 extract, 0.5 μg/ml for chromatin assembled with purified proteins) diluted in Buffer Rc (10 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 30 mM CaCl₂) followed by incubation at room temperature for 1 min. The reactions were terminated, and the samples were deproteinized by proteinase K digestion, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with ethanol.

For indirect end-labeling analysis, about 50 ng of DNA from the DNase I-treated samples was linearized by digestion with AlwNI. The DNA was separated in a 1.1% agarose gel, transferred to a nylon membrane, which was hybridized to a 32P-labeled oligonucleotide probe (5′-TGC CAG TGG CGA TAA GTC CAG TGG CGA TAA GTG TCT TAC CG-3′).

For DNase I footprinting analysis, ~50 ng of DNA from the DNase I-treated samples was used for primer extension with Vent (exo-) polymerase and a 32P-labeled primer (5′-TGC AGT CGC GGT TGG AGT AGT AGG CGC-3′). The samples were then analyzed by electrophoresis through 6% denaturing gels followed by autoradiography.

**RESULTS**

**DNA Binding Domains Are Not Sufficient for Establishing or Erasing of Hypersensitive Sites**—TFE3 is a leucine zipper-containing basic helix-loop-helix protein that has been previously shown to contain two transcription trans-activation domains...
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A, schematic representation of full-length PU.1 and PU.1ΔN. PU.1ΔN lacks residues 1–145 of the full-length protein. The gray box indicates the ETS domain, and the black box indicates the N-terminal transcription activation, PEST, and interferon regulatory factor-interaction domains of PU.1. B, indirect end-labeling analysis. Aliquots of μg dimer plasmid assembled into chromatin were incubated as follows: lane 1, no proteins; lane 2, 100 nM full-length PU.1; lanes 3–5, 50 nM, 100 nM, and 200 nM PU.1ΔN, respectively; lane 6, 57 nM TFE3; lane 7, 57 nM TFE3 and 100 nM full-length PU.1; lanes 8 and 9, 57 nM TFE3, and 50 nM, 100 nM, and 200 nM PU.1ΔN, respectively. The lane order is the same as in B.

(33, 34). These are located on either side of a central DNA-binding domain (Fig. 1B). We first investigated whether these domains were essential to establish a DNase I-hypersensitive site in a cell-free system, to make a chromatin template containing a dimerized fragment of the IgH enhancer. The enhancer fragment recapitulates several features of IgH regulation in cultured cells (Fig. 1A). Initially we used *Drosophila* embryo extracts as a source of factors for chromatin assembly and remodeling. Two truncation mutants (Fig. 1B) were purified as hexa-histidine-tagged proteins and used in chromatin-remodeling reactions. Transcription factors were added after chromatin assembly was complete, followed by DNase I treatment and analysis of chromatin structure (DNase I hypersensitivity by indirect end-labeling, measuring double-stranded cuts), or protein binding (primer extension footprinting, measuring nicks, and double-stranded cuts).

A deletion mutant lacking the N-terminal trans-activation domain (TFE3ΔN) established hypersensitivity (marked by arrow) at the enhancer, which contains all of the transcription factor binding sites. The deletion mutant also positioned nucleosomes up to 500 bp away from the transcription factor binding sites (boundaries marked by asterisks), as evidenced by additional lower intensity bands flanking protected regions of nucleosome size (Fig. 1C, lanes 3–5). Previously, we found that both micrococcal nuclease and DNase I gave similar patterns of protection and cutting in this region, allowing us to infer nucleosome positioning. Full-length TFE3 established a DNase I-hypersensitive site and positioned nucleosomes, as reported previously (Fig. 1C, lane 2; arrow and asterisks). However, removal of both the N- and C-terminal domains reduced the intensity of the hypersensitive site (Fig. 1C, lanes 6–8).

This functional difference was not due to DNA binding, as all three TFE3 derivatives bound to chromatin templates comparably as shown by footprinting the same chromatin templates at base pair resolution (Fig. 1D). (Note that the full lane of the footprinting panels is ~150 bp; this region corresponds to 1/25 of a lane in the indirect end labeling experiments, the region marked E in the ~5.4-kb region analyzed in the indirect end-labeling experiments, and furthermore that the indirect end-labeling experiment only detects double-stranded cuts, whereas the footprinting experiment detects nicks and double-stranded cuts.) We conclude that the DNA binding by TFE3 is not sufficient to program a DNase I-hypersensitive site. At least one other domain is required in a step that occurs after binding to chromatin, perhaps to recruit remodeling enzymes.

PU.1 contains its DNA binding ETS-domain at the C terminus (Fig. 2A). The N-terminal 140 amino acids have been previously shown to contain a weak transcription activation domain (35, 36), and a PEST domain that is essential to interact with members of the interferon regulatory factor family (37, 38). Previously, we reported that PU.1 added with TFE3 bound chromatin but antagonized formation of a DNase I-hypersensitive site. An N-terminal truncation mutant lacking the activation and PEST regions (PU.1ΔN, Fig. 2A) failed to antagonize the TFE3-induced hypersensitive site (Fig. 2B, compare lanes 8–10 to lane 6). Full-length PU.1 antagonized TFE3-mediated DNase I HS formation, as shown previously (Fig. 2B, compare lane 7 to 6). In the presence of TFE3, the full-length and deletion mutant bound comparably to chromatin (Fig. 2C, lanes 1–5, respectively). The lane order is the same as in B.
to chromatin did not require ATP (Fig. 3B compare lanes 2 and 5). However, establishing a DNase I-hypersensitive site required ATP, as the site was not established if chromatin was treated with apyrase prior to TFE3 binding (Fig. 3A, compare lanes 2 and 5). Maintenance of the site did not require remodeling, because apyrase treatment did not affect the DNase I-hypersensitive site or the positioned nucleosomes flanking the enhancer when added after the site was established (Fig. 3A, lane 6). We conclude that TFE3 and ATP are required to establish a DNase I-hypersensitive site, suggesting the involvement of an ATP-dependent enzyme. By contrast, ATP-dependent remodeling is not required for TFE3 binding to chromatin or to maintain the hypersensitive site.

Erasing Hypersensitivity Requires ATP—We investigated the mechanism used by PU.1 to antagonize DNase I-hypersensitive site formation. In our previous report, and in the experiments shown in Figs. 1 and 2, PU.1 and TFE3 were simultaneously added to chromatin templates. These conditions did not distinguish between antagonizing the establishment of the hypersensitive site versus erasure of an established TFE3-induced hypersensitive site. Therefore, we first tested the hypothesis that PU.1 could disrupt an established hypersensitive site. Chromatin was first incubated with TFE3 for 30 min to induce hypersensitivity, followed by incubation with PU.1 for an additional hour. PU.1 binding was similar when added during or after TFE3 binding and did not displace TFE3 (Fig. 3B, lanes 3 and 4). Indirect end-labeling analysis showed erasure of the hypersensitive site (Fig. 3A, lanes 3 and 4). The flanking positioned nucleosomes were present (Fig. 3A, lane 4), as seen before when TFE3 and PU.1 were added simultaneously to chromatin templates. Thus, PU.1 recruitment to an established hypersensitive site leads to its erasure, apparently without affecting nucleosome positioning or TFE3 binding.

Next, we investigated ATP requirements for PU.1-mediated antagonism of DNase I-hypersensitive site formation. Following chromatin assembly and TFE3 binding, ATP was depleted using apyrase, and the reaction was further incubated with PU.1. PU.1 did not erase DNase I hypersensitivity after apyrase treatment (Fig. 3A, lane 7), although PU.1 binding was unaffected (or perhaps enhanced) by ATP-depletion (Fig. 3B, lane 7). These observations indicated that an ATP-dependent step was required after PU.1 binding to erase the DNase I site, and PU.1 binding was not sufficient to erase TFE3-induced hypersensitivity. Note that erasure of the site is not the result of displacement of TFE3 (Fig. 3B, compare lane 4 to lane 2), or nucleosomes returning to the pre-TFE3 state (Fig. 3A, compare lanes 3, 4, and 8 to lane 1 in the asterisk-marked region). However, ATP was not required to maintain the erased state (Fig. 3A, lane 8). Thus, both establishment and erasure of the DNase I-hypersensitive site require energy, suggesting the involvement of one (or more) ATP-dependent enzymes.

Finally we tested whether histone acetylation might play a role in the observed changes in chromatin structure using the histone deacetylase inhibitor trichostatin A (TSA). Although our cell-free reactions are not supplemented with cofactors for histone-modifying enzymes, some enzymes copurify with cofactors and can execute a single round of chemistry. If histone deacetylation were, in part, responsible for erasure of the site, then TSA might prevent
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Erasure. Erasure was not affected by TSA added either before or after PU.1 (Fig. 3C, compare lanes 7 and 8 to 3). TSA alone did not alter hypersensitivity, nor did it enhance establishment by TFE3 (Fig. 3C, lanes 5 and 6). Taken together, these studies suggest a role for ATP-dependent enzymes, perhaps without histone deacetylation, in these remodeling reactions.

Different Chromatin-remodeling Activities Establish and Disrupt a DNase I-Hypersensitive Site—Involvement of protein domains other than the DNA binding domains of both transcription factors and the requirement for ATP strongly suggested that ATP-dependent chromatin-remodeling enzymes may be recruited to establish and erase hypersensitivity. We hypothesized the Drosophila embryo extract used for chromatin assembly was presumably the source of these activities. To identify remodeling enzymes that may be involved we used a purified system to assemble chromatin. Chromatin was assembled using recombinant ACF (an ATP-dependent remodeling enzyme composed of ISW1 and ACF1), NAP-1, and topoisomerase I fragment with native Drosophila core histones. Assembly was followed by the addition of TFE3, or PU.1, or both proteins together. Micrococcal nuclease digestion assays confirmed equivalent chromatin assembly in the presence, or absence, of the transcription factors (Fig. 4A). As with the embryo extracts, TFE3 bound well (Fig. 4D, lane 2), PU.1 alone bound poorly, but PU.1 binding was significantly enhanced in the presence of TFE3 (Fig. 4D, lanes 3 and 4). Analysis of DNase I-treated chromatin by indirect end labeling showed that TFE3 binding induced a DNase I-hypersensitive site and positioned nucleosomes (Fig. 4B, lanes 1 and 2). Establishment of the site was ATP-dependent, and in this system the only known ATPase was ISW1, which can change nucleosome positions and establish DNase I sites (Fig. 4C). However, unlike the results with chromatin assembled in embryo extracts, PU.1 binding did not fully antagonize the hypersensitive site in the purified system (Fig. 4B, lane 4). Supplementing the reactions with fresh ACF had no effect (data not shown). We conclude that the TFE3-dependent hypersensitivity can be catalyzed by ACF, but that PU.1-mediated disruption requires a different chromatin-remodeling enzyme.

To identify a remodeling enzyme that supports erasure, we used ACF and TFE3 to assemble chromatin with a hypersensitive site, then added PU.1 together with each of four other purified remodeling enzymes. We tested enzymes from the Mi2 (Mi2β), ISW1 (NURF), and SWI2/SNF2 (hSWI/SNF) subfamilies of ATP-dependent remodeling enzymes. Mi2, ISW1, and SWI2/SNF2 ATPases are broadly expressed in mammals and present in B cells. We added ~1 molecule of remodeling ATPase per 20 nucleosomes. Each reaction was treated with DNase I, divided into two parts and analyzed by indirect end labeling and primer extension. Lanes 1–4 of the indirect end labeling analysis recapitulate the data shown in Fig. 4. By contrast to ACF alone, inclusion of recombinant Mi2β together with PU.1 reduced TFE3-induced hypersensitivity (Fig. 5A, compare lanes 3 and 4 to lanes 5 and 6). Thus, ACF and Mi2β had different activities on the same template. However, neither recombinant NURF complex (Fig. 5A, lanes 7 and 8) nor purified human SWI/SNF complex (Fig. 5A, lanes 9 and 10) strongly affected DNase I hypersensitivity. The remodeling enzymes were present at a ratio of ~1 ATPase per 20 nucleosomes. Primer extension footprinting confirmed that TFE3 and PU.1 binding was unaffected by the remodeling enzymes (Fig. 5B). We conclude that the Mi2β ATPase can mediate PU.1-depend-
As a control, we verified the enzymes were active using another assay (12, 39). Although the specific function of ACF and Mi2β indicates both are active, it was possible that hSWI/SNF, and NURF were inactive. Chromatin was assembled on a plasmid with five GAL4 sites, purified by gel filtration and remodeling in the presence of GAL4-VP16 and purified remodeling enzymes was detected by the disruption of the nucleosomal repeat. The tested enzymes remodeled this template in an ATP-dependent manner (supplemental Fig. S1). The remodeling enzymes also had similar ATPase activities (data not shown). Thus, the apparent specificity among remodeling enzymes was not caused by inactive preparations of some of the enzymes.

Finally, we tested the ability of chromatin templates from the purified system to support transcription. We found that transcription was activated in the presence of TFE3, and PU.1 did not change transcription in the presence of TFE3 (supplemental Fig. S2), consistent with results from chromatin assembled with an embryo extract. Thus, the S-190 assembly system can be replaced with a handful of defined components, including ACF and Mi2β. As expected, templates with positioned nucleosomes (TFE3 and TFE3 plus PU.1) were active for transcription. Surprisingly, a DNase I HS was not required for transcription.

**DISCUSSION**

Immunoglobulin transcription and the μ enhancer have been intensively studied by a number of laboratories, in part because of the key role of antibody production in human health. The μ enhancer is known to be under combinatorial control (15). The μ enhancer is marked by a DNase I-hypersensitive site in B lymphocytes (40, 41) and is sufficient to generate hypersensitivity in mice (40). Deletion of this enhancer in B cells reduces rearrangement and transcription at this locus (13, 14). Previously, we found that TFE3 and PU.1 controlled chromatin structure in a combinatorial manner, producing three distinct chromatin states (21). TFE3 alone induced a DNase I HS, nucleosome positioning and transcription, whereas the combination of TFE3 and PU.1 induced positioned nucleosomes and transcription without a DNase I HS. This led to the surprising conclusion that a DNase I HS is not required for transcription, although active templates contained positioned nucleosomes.

Here, we extend our previous work with three observations. First, we identify at the molecular level two remodeling enzymes that have opposing structural effects on the same template. Second, we identify Mi2β as an enzyme that can erase an established DNase I-hypersensitive site in a novel reaction without transcription factor displacement. Third, we find enzymes remodel chromatin, but are not required to facilitate transcription factor binding.

These studies confirm and challenge existing models of chromatin remodeling and DNase I HS formation. One well established mechanism for DNase I HS formation is changes in nucleosome position (11, 12, 39). However, DHS formation has also been observed in the absence of remodeling (26). Although DNA-binding proteins apparently lacking activation domains (Gal4 DBD, and NF-kB p50) can promote formation of DHS (11, 12), TFE3 DNA binding is not sufficient. Although reversing transcription factor (TF) binding can reverse nucleosome positioning changes (42), here erasure occurs without reversing either TF binding or nucleosome positioning.

We found functional roles for the ACF- and Mi2β-remodeling enzymes, which are perhaps recruited by transcription factor activation domains. We found a clear difference in the remodeling potential of ACF and Mi2β; to our knowledge, this is the first example of different ATP-dependent remodeling enzymes performing opposing functions on the same template. Specifically, PU.1 could direct Mi2β, but not ACF, to erase a DNase I-hypersensitive site established by TFE3 and ACF. Furthermore, the effect of PU.1-directed Mi2β function appears to be “dominant” over that of ACF, at least with regard to DNase I hypersensitivity. We favor a model where Mi2β performs a different remodeling reaction than ACF and other enzymes, although more complicated models are also possible.

The mechanism of erasure is not fully understood, although several possibilities can be ruled out. First, there is no displacement of TFE3, the factor that established the hypersensitive site, nor loss of nucleosome positioning during the erasure. Thus, the mechanism of erasure is distinct from one where removal of transcription factor from the DNA reverses nucleo-
some positioning (42). We cannot rule out subtle alterations in nucleosome positioning during erasure, because attempts to define this at base pair resolution using primer extension analysis have not yielded clear results.7 Second, another report demonstrating combinatorial control of chromatin structure found that DNA methylation could antagonize DNase I-hypersensitive site formation and repress transcription through a deacetylase sensitive path way (43). Erasure in our system is mechanistically distinct, because trichostatin A had no effect on the generation or loss of hypersensitivity.7 Furthermore, the use of plasmid DNA and recombinant chromatin assembly system appears to preclude a role for changes in histone and DNA modifications in our assays. Taken together, we favor the idea that disruption of hypersensitivity involves a structural change in DNA nucleosome contacts.

The conservation of remodeling enzyme paralogs during evolution suggest they play distinct roles (3). Moreover, genetic evidence indicates some mammalian remodeling ATPase genes are essential (44, 45). Remodeling enzymes have also shown specificity at the level of transcriptional regulation in a limited number of cell-free reports (46–49). However, with a simple cell-free assay, remodeling ATPases in the SWI/SNF, ISWI, and Mi2 subfamilies display very similar reaction mechanisms (50).

One limiting case is that remodeling enzymes differ in their ability to be recruited by specific transcription factors. However, other cell-free studies have suggested variation in the intrinsic remodeling mechanism (51–54). Recruitment of more than one ATP-dependent remodeling enzyme to the same gene in cells has been found (55–59), again suggesting these remodeling enzymes may have distinct functions.

We identified Mi2β as a remodeling enzyme that can erase a DNase I-hypersensitive site, while beginning the characterizing the mechanism of this novel remodeling reaction. We found that established DNase I hypersensitivity can be erased upon binding of an additional transcription factor. Surprisingly, this reaction does not involve displacement of the initiating transcription factor, or loss of the nucleosome positioning induced during induction of the site. Establishment and erasure can occur in a purified system apparently lacking histone modification enzymes. These properties make this erasure reaction distinct from other reactions that reverse chromatin structure changes (42, 43, 60). We find that erasure requires the PU.1 activation/interaction domain, ATP, and the Mi2β-remodeling enzyme. We favor the models that Mi2β erases the site by changing histone-DNA contacts in the region, altering the path of the DNA or perhaps changing higher order chromatin structure, but we have not been able to test these models directly.

We find that transcription factor binding does not require ATP-dependent remodeling; remodeling enzyme function (remodeling) occurs after transcription factor binding. Related findings have been observed in a variety of systems in vitro and in vivo (12, 61–65). However, other studies have found significant barriers against transcription factor binding to chromatin (63, 65–71) or remodeling without remodeling enzymes (26). We note that at least three of these studies have found a strong barrier to one TF without a barrier to another TF, suggesting that at least some of this variability is not simply differences in experimental approach. It remains to be determined to what extent the ability of a transcription factor to bind chromatin is determined by the transcription factor, the nature of the binding site, and the particular chromatin structure.

We do not know whether DHS erasure occurs in cells. An interesting possibility is that if it does, there might be functional regulatory elements that cannot be detected by genome-wide DHS mapping. Alternatively, cells may have a mechanism that reads erased DHS as inactive regulatory elements.

Finally, it is interesting to note that PU.1 (72–74) and Mi2β (75–78) are both implicated in gene silencing. Thus, recruitment of Mi2β by promoter/enhancer-bound PU.1 may initiate histone de-acetylation or recruitment of the gene to pericentric heterochromatin (77). How the distinction is made between positive, or negative, gene activation by PU.1 remains unclear. We speculate that this may be conferred by post-translational modification of PU.1, alternative binding partners for PU.1, promoter/enhancer architecture and consequent combinatorial recruitment of remodeling activities. In this regard it is worth noting that PU.1-dependent activation of B lymphocyte enhancers does not require the N-terminal domain that we suggest recruits Mi2β (19, 79).

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