Combinatorial Control of DNase I-hypersensitive Site Formation and Erasure by Immunoglobulin Heavy Chain Enhancer-binding Proteins*

Received for publication, August 13, 2003, and in revised form, November 21, 2003
Published, JBC Papers in Press, December 1, 2003, DOI 10.1074/jbc.M308973200

Haruhioko Ishii‡, Ranjan Sen¶, and Michael J. Pazin¶¶

From the §Graduate Program in Biophysics and Structural Biology, the ¶Rosenstiel Basic Medical Sciences Research Center and Department of Biology, Brandeis University, Waltham, Massachusetts 02454 and the ¶¶Cutaneous Biology Research Center, Harvard Medical School, Massachusetts General Hospital East, Charlestown, Massachusetts 02129

DNase I-hypersensitive sites in cellular chromatin are usually believed to be nucleosome-free regions generated by transcription factor binding. Using a cell-free system we show that hypersensitivity does not simply correlate with the number of DNA-bound proteins. Specifically, the leucine zipper containing basic helix-loop-helix protein TFE3 was sufficient to induce a DNase I-hypersensitive site at the immunoglobulin heavy chain $\mu$ enhancer in vitro. TFE3 enhanced binding of an ETS protein PU.1 to the enhancer. However, PU.1 binding erased the DNase I-hypersensitive site without abolishing TFE3 binding. Furthermore, TFE3 binding enhanced transcription in the presence and absence of a hypersensitive site, whereas endonuclease accessibility correlated strictly with DNase I hypersensitivity. We infer that chromatin constraints for transcription and nucleosome sensitivity can differ.

Immunoglobulin heavy chain (IGH) gene expression is regulated by the $\mu$ enhancer that is located in the intron between the VDJ$\mu$ and C$\mu$ exons (1, 2). This enhancer is necessary for expression of rearranged IGH genes in transgenic mice (3), and sufficient to confer B lymphocyte-specific transcription in transfection and transgenic assays (4–7). In addition to its transcription activation potential, the enhancer also activates VDJ recombination. This activity was first demonstrated in artificial recombination substrates (8–10), and has recently been confirmed by deleting the element from the endogenous locus (11–13). Multiple DNA-binding proteins determine the properties of the enhancer. At least 15 different $\mu$ enhancer-binding proteins have been identified, most of which appear to contribute to enhancer activity to varying degrees (14, 15).

$\mu$ enhancer-binding proteins can be broadly classified into two categories: those whose expression is tissue restricted and those that are widely expressed. Even among the tissue-restricted proteins, there are none whose expression profile coincides precisely with those cells in which the IGH gene is expressed. For example, PU.1, which is the most tissue-restricted $\mu$ enhancer-binding protein that interacts with the $\mu$B site of the enhancer, is expressed in both B cells and macrophages (16). Yet, the IGH gene is not expressed in macrophages. The sequence CANNTGG is the most repeated element in the $\mu$ enhancer. This motif binds the basic helix-loop-helix family of transcription factors that are typically expressed in most cell types. E2A proteins bind to two of five such elements (17, 18) and TFE3/USF (basic helix-loop-helix-zip) proteins bind to another (19, 20). Interestingly, in one of the earliest examples of in vivo footprinting these were the only elements shown to be protein-bound in B cells (21, 22). The basis for tissue-specific DNA binding by apparently ubiquitously expressed proteins remains an enigma. It is clear that none of these elements by themselves generate enhancer activity in transient transfection assays into B cells, or non-B cells. Rather, the function of the $\mu$ enhancer is determined by combinatorial control: the combined action of tissue-restricted and ubiquitously expressed proteins, which cannot be predicted from the individual activities.

The smallest functional domain of the enhancer, $\mu$70, contains three protein binding sites: $\mu$A, $\mu$B, and $\mu$E3. These sites bind the proteins Ets-1, PU.1, and TFE3, respectively (23). The $\mu$70 enhancer mimics several important properties of the full enhancer (23–26). First, this tripartite enhancer activates transcription in B cells, but not in several non-lymphoid cells or T cells. Second, the three proteins that bind to this enhancer exemplify tissue-restricted as well as ubiquitously distributed proteins. Third, enhancer activity in transient, or stable, transfection assays require all three sites to be intact, indicating that its function is the result of combinatorial mechanisms. Transcription activity generated by endogenous factors in B cells can be mimicked in non-lymphoid cells by ectopic co-expression of the tissue-restricted Ets-1 and PU.1 proteins. In such assays, mutation of the third ($\mu$E3) site abolishes transcriptional activity suggesting that the enhancer recruits a cellular factor to the $\mu$E3 site (23). Unlike the full enhancer, however, the tripartite enhancer activates transcription in macrophages (27). The distinction in $\mu$ enhancer activity between B cells and macrophages, both of which express PU.1, is an interesting example of the acquisition of greater tissue specificity with increased numbers of enhancer-binding proteins.

Like most characterized regulatory sequences, the $\mu$ enhancer is marked by a tissue-specific DNase I-hypersensitive site in cellular chromatin (28, 29). The $\mu$ enhancer-hypersensitive site is re-created in IGH transgenic mice and is strongest when neighboring matrix attachment regions are included in the transgene (30, 31). In model systems transcription factor binding is important in initiating chromatin structure changes (32–34), as well as maintaining chromatin structure (35). Using Xenopus egg extracts to assemble chromatin we previously
showed that the μβ-binding protein PU.1 increases restriction enzyme accessibility to sites close to the enhancer (36). In addition, expression of PU.1 induces sterile μ transcripts and restriction enzyme access to the endogenous IGH locus in pro-T cells. Overall, these studies show that the μ enhancer is sufficient to alter chromatin structure, a feature that is likely to form the basis of its function as a transcription and recombination enhancer.

We present an analysis of chromatin remodeling mediated by the μ70 domain of the IGH enhancer. Of the three proteins that bind to this enhancer, TFE3 alone was sufficient to induce a DNase I-hypersensitive site, endonuclease accessibility, positioning of nucleosomes adjacent to the enhancer, and transcriptional activation on chromatin templates in vitro. PU.1, an ETS protein, was recruited to chromatin by TFE3. Surprisingly, PU.1 binding abolished TFE3-induced DNase I hypersensitivity and endonuclease accessibility without affecting TFE3 binding or TFE3-induced nucleosome positioning. In contrast to its effects on chromatin structure, PU.1 had little if any effect on TFE3-activated transcription in vitro, suggesting that the requirements for nuclelease accessibility and transcription are different. These observations suggest that occupancy of multiple sites and nucleosome positioning are not sufficient to generate DNase I hypersensitivity. Thus, important regulatory events may be mediated by DNA-bound proteins that are not marked by hypersensitivity to DNase I.

**EXPERIMENTAL PROCEDURES**

*Plasmodiophora myctera* dimer constructs (wt, μβ, μE3−) have been described (23). The μA μB construct that contains mutations in both the μA site (GCAGGAAGACA → GCATCGAGCA) and μB site (TTTGGGGGAA → CCCCGGGGAA) was made as follows. The μA fragment containing the μB mutation (23) was amplified by PCR with oligonucleotide primers with sequences 5′-CAAACACTTCTCAACACCCAC-3′ and 5′-GTTAAACTTGTAGCTGGCATCGAGCAGGTC-3′ to introduce the μA mutation. The PCR product was digested with AluI and inserted into the SalI site of the β26 chloroamphenicol acetyltransferase plasmid (37) as a dimer. The GST−Ets-1 expression vector was constructed by inserting the BamHI-BamHI fragment from pEVR-Ets-1 (24) into the BamHI site of pGEX-2T. The His-PU.1 expression vector was constructed by inserting the BamHI-EcoRI fragment from the GST-PU-expression vector (25) into the pProEx HTb (Invitrogen) plasmid digested with BamHI and EcoRI. The His-TFE3 expression vector was constructed by inserting the BamHI-KpnI fragment from the GST-TFE3 expression vector (26) into pProEx HTb (Invitrogen) digested with BamHI and KpnI.

Resins—Ets Proteins—Ets-1 was expressed in bacteria as a GST fusion protein. The GST-Ets-1 protein was expressed in bacteria and purified using glutathione affinity resin following the manufacturer’s specifications. Purified GST-Ets-1 was dialyzed against buffer D (20 mM Hepes, pH 7.6, 0.2 mM EDTA, 0.5 mM dithiothreitol, 100 mM KCl).

TFE3 and PU.1 were expressed in bacteria as Histon-tagged proteins and purified by Ni2+ affinity chromatography followed by DNA affinity chromatography. DNA affinity columns were made following the method described in Ref. 38. For the TFE3 affinity columns, oligonucleotides with sequences 5′-pCCTTGAGGCACATGGCCTGCGACATG-3′ and 5′-C6dGTGTGAGGTGCGAGGGCA-3′ (where d6 is O2-phenyl-L-2-deoxyinosine) were synthesized and processed essentially as described in Ref. 38 and after an amelioration and ligation were coupled to N-hydroxy-succinimide-activated Sepharose 4 Fast Flow (Amersham Biosciences). For the PU.1 affinity columns, oligonucleotides with sequences 5′-pCCTCTCCAGTTTCCCTTTCCTCCCAC-3′ and 5′-pG6dAATGGGGGAAAGGGGAAGAATCAGGGA-3′ were synthesized and processed in the same way.

Bacterial cells expressing His-TFE3 were re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM imidazole, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and lysed by incubation with lysozyme followed by sonication. The lysate was centrifuged at 15,000 rpm for 20 min. The supernatant was incubated with nickel-nitrilotriacetic acid-agarose resin (Qiagen) and eluted stepwise by imidazole. The purified protein was dialyzed against 25 mM Hepes, pH 7.6, 12.5 mM MgCl2, 100 mM KCI, 10% glycerol, 1 mM dithiothreitol, 0.1% Nonidet P-40, loaded onto a DNA affinity column, and was eluted stepwise with the same buffer containing variable KCl concentrations. The purified TFE3 was dialyzed against buffer D.

His-PU.1 was initially purified by a nickel affinity column under denaturing condition. Bacterial cells expressing His-PU.1 were re-suspended in 8 mM urea, 10 mM Tris-HCl, pH 8.0, 100 mM sodium phosphate, pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and centrifuged at 15,000 rpm for 20 min. The supernatant was incubated with nickel-nitrilotriacetic acid-agarose resin and eluted stepwise with imidazole. The fractions containing PU.1 were diluted into 8 mM urea, 25 mM Hepes, pH 7.9, 12.5 mM MgCl2, 0.5 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and the renatured protein was renatured by stepwise dialysis against the same buffer containing 6, 3, 2, 1, 0.5, and 0% urea. The renatured protein was passed through a filter with a pore size of 0.45 μm and was applied to a DNA affinity column. The protein was eluted stepwise by variable KCl. The purified PU.1 was dialyzed against 25 mM Hepes, pH 7.9, 12.5 mM MgCl2, 200 mM KCI, 10% glycerol, 1 mM dithiothreitol, 0.1% Nonidet P-40.

**Chromatin Assembly—**Chromatin was assembled with plasmid DNA, *Drosophila* core histones, and S-190 assembly extract derived from *Drosophila* embryos, essentially as described previously (39). The ratio of core histones to DNA was varied between 0.7 and 1.1 (w/w) to attain complete assembly.

**Proteinase K Digestion—**Micrococcal nuclease digestion of chromatin was done essentially as described (39). Micrococcal nuclease was diluted in buffer R (10 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM EDTA, 10% glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) supplemented with 30 mM CaCl2 or to 5, 1.3, or 0.4 units/ml. 25 μl of chromatin aliquots (containing 125 ng of DNA) were digested with 2.5 μl of the diluted micrococcal nuclease for 10 min at room temperature and the reaction was terminated by addition of 2.5 μl of stop solution (6 mM Tris, pH 8.0, 200 mM EDTA, 100 μg/ml RNase A). The samples were de-proteinized by proteinase K digestion, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with ethanol. DNA from chromatin treated with 2.5 μl of micrococcal nuclease for 5 min on a 5% acrylamide gel electrophoresis through a 1% agarose gel, and visualized by ethidium bromide staining. Fractionated DNA was transferred to a nylon membrane and hybridized to a 32P-labeled oligonucleotide probe specific to an enhancer proximal region (5′-TGATGCTGGTTGGACTTGAATG-CCG-3′). The same membrane was stripped and re-hybridized to a 32P-labeled primer (5′-TGCACTGGCTGAACTTCACTGCAGCC-3′). DNase I digestion of chromatin was done essentially as described in Ref. 39. 25-μl Chromatin aliquots (125 ng of DNA) were digested with 2.5 μl of (80 μg/ml) DNase I for 1 min at room temperature and the reaction was terminated by addition of 2.5 μl of stop solution. The samples were de-proteinized by proteinase K digestion, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with ethanol. 50 ng of DNA was used for footprinting assay by primer extension with Vent (exo-) polymerase and a 32P-labeled primer (5′-TGCGCTGGTCAGTTGGATTGC-3′). The samples were then analyzed by electrophoresis through 6% denaturing gels followed by autoradiography.

**Indirect End Labeling Analysis—**The chromatin samples digested with DNase I or micrococcal nuclease (2.5 μl of 0.4 units/ml) were also analyzed by indirect end labeling analysis. 50 ng of DNA from the DNase I- or micrococcal nuclease-treated samples was linearized by treatment with AlwNI. The DNA was separated in a 1.2% agarose gel, blotted to a nylon membrane, which was hybridized to a 32P-labeled oligonucleotide probe (5′-TGCGACTGGCTGAACTTCACTGCAGCC-3′). The samples were then analyzed by electrophoresis through 6% denaturing gels followed by autoradiography.
Fig. 1. A plasmid containing the μ70 dimer is efficiently assembled into chromatin. A, schematic diagram of the μ70 dimer plasmid. μA and μB sites are indicated by ovals; μE3 and TATA are indicated by rectangles. The transcription factors Ets-1, TFE3, and PU.1 have been shown to bind to μA, μE3, and μB sites, respectively. B, experimental design. Chromatin templates were assembled using the Drosophila embryo-derived S190 extract. S190 extract and core histones were preincubated for 30 min and chromatin assembly was initiated by addition of the plasmid and ATP regeneration system as described under “Experimental Procedures.” Different combinations of transcription factors Ets-1, PU.1, and TFE3 were added after 4.5 h of chromatin assembly, when assembly was complete. The samples were further incubated for 30 min, and aliquots were removed for DNase I, micrococcal nuclease, or restriction endonuclease digestion, or transcription. C, micrococcal nuclease analysis of assembled chromatin visualized by ethidium bromide staining. Wild-type μ70 dimer plasmid assembled into chromatin was incubated with different combinations of Ets-1 (200 nM), PU.1 (100 nM), and TFE3 (57 nM) as indicated. Aliquots of the chromatin samples were digested with two different concentrations of micrococcal nuclease. After digestion, DNA was isolated and resolved by agarose gel electrophoresis. DNA was visualized by staining with ethidium bromide (Fig. 1, A). No gross nucleosomal perturbation was evident when the nucleosomal plasmids were further incubated with transcription factors prior to micrococcal nuclease digestion (Fig. 1C, lanes 3–16). Furthermore, Southern hybridization with an enhancer-proximal probe also did not reveal local changes in micrococcal nuclease digestion by the transcription factors (Fig. 1D).

**RESULTS**

The template used in these studies contains two copies of the μ70 domain of the immunoglobulin heavy chain μ enhancer cloned 5' to a TATA-containing c-fos gene promoter and a chloroamphenicol acetyltransferase gene (Fig. 1A). This plasmid is transcriptionally active in transiently transfected B cells and macrophages, but not in several other cell types (23). Transcription activity requires all three binding sites in μ70, indicating that its tissue-restricted activity is the result of combinatorial mechanisms. We assembled this plasmid into chromatin using Drosophila embryo extracts (39, 40), followed by incubation with μ70-binding proteins, PU.1, Ets-1, and TFE3, in various combinations. Thereafter, aliquots were removed for digestion with DNase I, micrococcal nuclease, or a restriction endonuclease for structural studies, or further incubated with nuclear extracts to assay transcription (Fig. 1B). Efficient chromatin assembly was evident from agarose gel analysis of micrococcal nuclease-treated DNA followed by staining with ethidium bromide (Fig. 1C). No gross nucleosomal perturbation was evident when the nucleosomal plasmids were further incubated with transcription factors prior to micrococcal nuclease digestion (Fig. 1C, lanes 3–16). Furthermore, Southern hybridization with an enhancer-proximal probe also did not reveal local changes in micrococcal nuclease digestion by the transcription factors (Fig. 1D).

TFE3 binds chromatin and recruits PU.1—Factor binding to DNA templates was assayed by DNase I footprinting using primer extension (39). Full-length Ets-1, TFE3, and PU.1 were expressed in bacteria and purified by affinity chromatography. Each protein bound to naked DNA and produced a characteristic pattern of protections and enhancements. Ets-1 binding to μA, although weak because of autoinhibition (41), was evident from the protection of several bands (Fig. 2, lanes 4, 6, 8, and 10, indicated by open arrows). PU.1 binding to μB induced prominent enhancement of DNase I cleavage (Fig. 2, lanes 3, 7, and 8, indicated by solid arrows), and TFE3 produced a footprint over the μE3 site that extended into the Ets-1 binding site (Fig. 2, lanes 5–7, indicated by solid lines). There was no significant difference in the footprints whether each protein was added alone, or in combination with other proteins.

We also examined factor binding to chromatin. Of the three proteins, only TFE3 bound to chromatin by itself to produce a clear footprint (Fig. 2, lane 13). In addition to the protection pattern seen with naked DNA, TFE3 binding to chromatin also generated two regions of enhanced DNase I cleavage (indicated by asterisks in Fig. 2, lane 13). PU.1 bound weakly to chromatin plasmid as shown by a slight increase in the intensity of the bands indicated by the solid arrows (Fig. 2, compare lanes 11 and 12 to 9 and 10). At the concentration used, Ets-1 binding was not evident by itself (Fig. 2, lane 10).

TFE3 significantly increased PU.1 binding, which was apparent by prominent enhancement of the bands marked by the
Naked DNA (lanes 1–8) or chromatin (lanes 9–16) were incubated with different combinations of Ets-1 (200 nM), PU.1 (100 nM), and TFE3 (57 nM) as indicated. Aliquots of each sample were digested with DNase I. The concentration of DNase I used to digest chromatin was 1,000 times higher than that used for naked DNA. After DNase I treatment, DNA was isolated and analyzed by primer extension followed by denaturing polyacrylamide gel electrophoresis. Positions of μA, μE3, and μB sites are indicated by brackets. DNase I digestion patterns of naked DNA and chromatin in the absence of the transcription factors are shown in lanes 1 and 9, respectively. Enhanced DNase I cleavage sites induced by PU.1 are marked by solid arrows. The solid bars indicate regions protected from DNase I digestion by TFE3. Open arrows mark the position of prominent bands that are protected by Ets-1 protein binding. Ets-1 binding in the presence of TFE3 is seen by protection of the lower band. Asterisks indicate TFE3-induced enhanced DNase I cleavage, which is suppressed by PU.1 binding. Representative data from one of five experiments is shown.

In addition, enhanced DNase I cleavage induced by TFE3 alone (marked by asterisks) was reduced because of PU.1 binding. When Ets-1 was added in addition to TFE3, some protection of bands around the μA site (marked by open arrows) was observed, indicating weak Ets-1 binding (Fig. 2, compare lanes 14 and 16 to 11 and 12). Because these bands are weak to begin with in the absence of TFE3, it is not clear whether Ets-1 binding is by the presence of TFE3, or Ets-1 binding was simply not detectable in the absence of TFE3. Note also that similar protection around the μA site was observed when PU.1 was added together with TFE3, suggesting weak binding of PU.1 to μA site (Fig. 2, compare lane 15 to 13) (25). Enhanced PU.1, or Ets-1, binding in the presence of TFE3 was also observed when the template DNA was preincubated with these proteins prior to chromatin assembly (data not shown), suggesting that these ETS proteins do not bind stably to the nucleosomal μ enhancer without help; we surmise that prebound ETS proteins in these studies were displaced by subsequent nucleosome assembly. We have previously shown that Ets-1 binding to naked DNA is enhanced by TFE3, probably because of direct interactions between the two proteins that reduces the effect of the autoinhibitory domain of Ets-1 (26). However, PU.1 and TFE3 do not bind cooperatively to naked DNA. Thus, the enhancement of PU.1 binding seen here is chromatin-specific, perhaps caused by alterations in chromatin structure induced by TFE3. It has been observed that binding of transcription factors within a nucleosome can be cooperative, even if binding of the same transcription factors to naked DNA is not inherently cooperative (42–44).

TFE3 Induces DNase I Hypersensitivity and Nucleosome Positioning—To investigate the effects of these proteins on chromatin structure, nucleosome sensitivity of the same chromatin used for footprinting was analyzed using indirect end labeling. The chromatin was treated with either DNase I or micrococcal nuclease, de-proteinized, restricted, and assayed by Southern blotting. Location of the restriction sites and the probes used are shown in Fig. 3A. As expected from the footprinting studies, DNase I digestion revealed no obvious perturbations with PU.1, Ets-1, or the two together (Fig. 3B, lanes 2–4). However, TFE3 binding generated a DNase I-hypersensitive site (marked by an arrow, Fig. 3B, lane 5) whose location coincided closely with μ enhancer sequences in the plasmid. In addition,
at least two other DNase I cleavage sites were detected that flanked the \( \mu \) enhancer and its associated hypersensitive site (Fig. 3B, marked by asterisks). The spacing of these sites (~190 bp) suggested that they were because of positioned nucleosomes flanking the enhancer. Similar results were obtained with micrococcal nuclease (see below). Thus, TFE3 binding induced a DNase I-hypersensitive site and nucleosome positioning.

**Combinatorial Control of Chromatin Structure**—We next examined the ability of combinations of enhancer-binding proteins to change chromatin structure. Addition of Ets-1 did not change the DNase I-hypersensitive site induced by TFE3 alone (Fig. 3B, lane 6). Unexpectedly, DNase I hypersensitivity disappeared when PU.1 was present in addition to TFE3, regardless of whether Ets-1 was, or was not, present in the reaction (Fig. 3B, lanes 7 and 8). Our footprinting data demonstrated that TFE3 remained bound to the enhancer in the presence of PU.1, or both PU.1 and Ets-1 (Fig. 2, lanes 7 and 8). Thus, PU.1 did not erase DNase I hypersensitivity by antagonizing TFE3 binding. These observations demonstrate that DNase I hypersensitivity did not simply parallel the number of proteins bound to DNA. Specifically, maximal hypersensitivity was observed with two TFE3 sites occupied; this was not enhanced by addition of Ets-1, but abolished upon PU.1 binding. Consequently, even when four sites were protein-bound, a DNase I-hypersensitive site was not formed if PU.1 was one of the bound proteins.

**Recruitment of PU.1 Does Not Alter Nucleosome Positions**—We asked whether PU.1 might erase DNase I-hypersensitive sites by altering nucleosome positions. The DNase I cleavage sites induced by TFE3 that flanked the enhancer were maintained even in the absence of the hypersensitive site (Fig. 3, marked by asterisks, compare lanes 5–8), suggesting that positioned nucleosomes adjacent to the enhancer were not affected by PU.1 binding. We also examined nucleosome positioning with micrococcal nuclease, which preferentially digests DNA in non-nucleosomal regions of chromatin. TFE3 alone induced a site of enhanced micrococcal nuclease cleavage adjacent to the enhancer and also resulted in protection of sequences further away (Fig. 3B, lanes 13, indicated by an arrow and solid lines, respectively). The pattern was essentially unchanged with Ets-1 plus TFE3 (Fig. 3B, lane 14). However, micrococcal nuclease sensitivity adjacent to the enhancer was reduced in the presence of PU.1, or all three proteins together (Fig. 3B, lanes 15 and 16). In contrast to the strong effects of PU.1 at the enhancer, PU.1 did not affect more distal features of the chromatin. Protected regions downstream, which we infer to be because of positioned nucleosomes flanking the enhancer, were maintained even when TFE3-mediated hypersensitivity was lost (Fig. 3B, lanes 13–16, marked by vertical lines). We therefore exclude the mechanism that PU.1-induced erasure of the hypersensitive site was mediated by large changes in nucleosome positioning close to the enhancer. Furthermore, because there is no transcription under these reaction conditions, it is unlikely that the change in chromatin structure is a consequence of transcription. Inclusion of trichostatin A with TFE3, or PU.1, did not affect the changes we observed, suggesting that de-acetylation was also not involved in establishing, maintaining, or erasing the DNase I-hypersensitive site.\(^2\) Our interpretation is that PU.1 binding antagonizes formation of an altered chromatin structure that is visualized as nucleosome hypersensitivity.

**PU.1 Abrogates DNase I Hypersensitivity Only When Bound to the Enhancer**—It was possible that PU.1 disrupted the TFE3-induced chromatin structure by sequestering essential components in the assembly extracts. If this were so PU.1 may affect chromatin structure without binding to the \( \mu \)B site. To determine whether PU.1 binding was necessary for these effects, we repeated the structural studies with mutated templates (Fig. 4). TFE3-dependent DNase I hypersensitivity was generated on plasmids that contained a mutated \( \mu \)B site (Fig. 4, \( \mu \)B, lane 6), or one in which both \( \mu \)A and \( \mu \)B sites were mutated (Fig. 4, \( \mu \)A \( \mu \)B, lane 10), but not on a plasmid containing a mutated E3 site (Fig. 4, \( \mu \)E3, lanes 14–16). Furthermore, nucleosome ordering was evident at enhancer flanking regions on the \( \mu \)A - and \( \mu \)B-mutated templates as seen with the wild-type enhancer. Unlike the wild-type enhancer, however, PU.1 mediated disruption of the nucleosome hypersensitive site was diminished on the \( \mu \)B- template and virtually abolished on the double mutated enhancer (Fig. 4, lanes 7, 8, 11, and 12). The partial effect seen with the \( \mu \)B- template was probably because PU.1 also binds to the \( \mu \)A site, although with ~4–5-fold reduced affinity (25). We conclude that disruption of TFE3-induced chromatin structure by PU.1 requires binding of this protein to DNA.

**PU.1 Reverses TFE3-mediated Restriction Endonuclease Accessibility, but Not Transcription**—We examined structural alterations caused by TFE3 using a restriction endonuclease accessibility assay. Chromatin was lightly digested with HinIII, de-proteinated, and purified DNA was cut to completion with AlwNI prior to Southern blot analysis. Locations of all possible cutting sites relative to the probe (position marked by the asterisk) are shown in Fig. 5A. Strongly enhanced cutting was observed in the presence of TFE3 at the enhancer-proximal site 2, whereas a smaller enhancement was found at site 1 (Fig. 5B, compare lanes 9–12 with lanes 1–4, quantitated in Fig. 5, C and D). No change was observed at the distal sites 3–5 in the presence of TFE3, suggesting that TFE3 could program specific, local changes in chromatin structure. When PU.1 and TFE3 were both bound to DNA, HinIII sensitivity at site 2 was reduced to the basal level, but remained detectably higher at site 1 compared with no added proteins (Fig. 5B, lanes 13–16 and lanes 1–4). The level of enhanced sensitivity at site 1 was

\(^2\) H. Ishii, R. Sen, and M. J. Pazin, unpublished data.
similar to that seen with PU.1 alone (Fig. 5B, lanes 13–16 and 5–8), and resembles that observed in our earlier work (36). Apparently the residual enhancement of HindII cutting at site 1 is not sufficient to generate the DNase I-hypersensitive site. We conclude that PU.1 suppresses TFE3-mediated chromatin structural changes that enhance access to nucleases and restriction enzymes.

The same chromatin templates were used for in vitro transcription assays. TFE3, PU.1, or both proteins were allowed to bind to chromatin for 30 min followed by addition of nuclear extracts and rNTPs to initiate transcription. Synthesized RNA was analyzed by primer extension. A typical experiment performed in duplicate is shown in Fig. 6A. TFE3, but not PU.1, increased the amount of transcription about 10-fold (Fig. 6A, lanes 1–3, and quantitated in Fig. 6B). Unlike the results with endonuclease accessibility, no significant difference in the level of transcript was observed when PU.1 was present in addition to TFE3 (Fig. 6A, compare lanes 2 and 4). We conclude that RNA polymerase II recruitment proceeds comparably despite the absence of a closely associated DNase I-hypersensitive site. These observations suggest that nuclease accessibility and transcription activation can be mechanistically distinct.

**DISCUSSION**

Of the three proteins that bind to essential motifs of the immunoglobulin heavy chain \( \mu 70 \) enhancer fragment, we found that only TFE3 formed a stable complex with chromatin in vitro, induced DNase I and micrococcal nuclease-hypersensitive sites at the enhancer, and positioned nucleosomes flanking the enhancer. The other two \( \mu 70 \)-binding proteins, PU.1 and Ets-1, bound poorly to the same chromatin when added individually or together, but PU.1 binding was significantly enhanced by TFE3. Strikingly, binding of PU.1 abolished the TFE3-induced hypersensitive site without a loss of bound TFE3. This is in contrast to what has been observed in vitro with the HIV-1 and TCR\( \alpha \) chain enhancers, where the strength of the hypersensitive site approximately correlated with the number of proteins bound to the enhancer (34, 45). Therefore,
Transcription and Accessibility of Chromatin Templates

**Fig. 6.** TFE3 activates transcription from chromatin, whereas PU.1 has no effect in vitro. A, the wild-type μ70 dimer plasmid assembled into chromatin was incubated with either no factors (lane 1), 57 nm TFE3 (lane 2), 100 nm PU.1 (lane 3), or 57 nm TFE3 and 100 nm PU.1 (lane 4) for 30 min. Two 15-μl aliquots of chromatin (containing 75 ng of DNA) were independently used in 50-μl transcription reactions. RNA was analyzed by primer extension as described under "Experimental Procedures." Representative data from one of three independent experiments is shown. B, the data from the primer extension analysis were quantitated with a phosphorimager. The level of transcript was normalized to that seen in the absence of added transcription factors (relative transcription) and the average for three independent experiments done in duplicate is presented.

Effects of DNA-binding proteins on DNase I hypersensitivity can be positive (like TFE3 in our assays), or negative (like PU.1 in our assays). Furthermore, the properties of the factors and the combinations in which they bind contribute significantly to the generation of DNase I-hypersensitive sites. Importantly, our observations suggest that some protein-bound regulatory regions in the genome may not be marked by nuclease-hypersensitive sites. Moreover, our observations suggest that the nuclease sensitivity of regulatory regions might change independently of functional status, in some cases.

TFE3 binding also enhanced access to restriction enzyme and activated transcription. In the presence of PU.1, transcriptional activation was not affected, whereas restriction enzyme accessibility was reduced to background levels. These observations suggest that local endonuclease access and RNA polymerase II access may be mechanistically distinct. One basis for the difference could be that recruitment of RNA polymerase II, which involves multiple protein-protein and protein-DNA interactions, may be less susceptible to changes in localized accessibility such as that induced by PU.1. In contrast, structural changes proposed to be the basis of hypersensitivity may expose sequences to restriction enzyme so that in the absence of a hypersensitive region restriction enzyme access is also reduced.

The μ enhancer is both a transcriptional and a recombinational enhancer. In artificial recombination substrates this enhancer is sufficient to activate V(D)J recombination (9, 46), whereas its deletion from the endogenous locus results in blockade of V<sub>H</sub> to DJ<sub>H</sub> recombination (12, 13). The distinction between transcription and restriction enzyme access in our studies may be significant to two features of V(D)J recombination. First, the early correlation between sterile transcription and V(D)J recombination has been broken down in recent studies (10, 47, 48). Our observation of transcription activation in the absence of endonuclease access parallels the observation in vivo that transcription of recombination substrates can be observed without accompanying endonuclease-mediated recombination (10). Second, from the analysis of recombination substrates it seems that recombination activating regulatory sequences are required close to both rearrangeable sequences to get efficient recombination (48). This has also been observed at endogenous antigen receptor loci; for example, deletion of the DJ<sub>1</sub> proximal promoter affects DJ<sub>1</sub>, but not DJ<sub>2</sub> rearrangements (49). Our TFE3-induced hypersensitive site has features of such a local accessibility enhancing sequence, although we recognize that the effect in vitro is much more localized than that seen in vivo.

We previously showed that restriction enzyme access to enhancer proximal sites was increased by PU.1 or Ets-1 binding to in vitro assembled chromatin. Additionally, ectopic PU.1 expression in non-B lymphoid cell lines also resulted in increased restriction enzyme access to a site within the enhancer (36). Despite differences in chromatin assembly systems, the ~2-fold enhancement of restriction enzyme access by PU.1 seen earlier was recapitulated in the present study. However, PU.1-initiated chromatin structural changes detected by restriction enzyme accessibility assay were not sufficient to induce hypersensitivity to DNase I in vitro. In contrast, TFE3 binding induced significantly higher restriction enzyme access as well as a DNase I-hypersensitive site. Although both PU.1 and TFE3 increased restriction enzyme access, albeit to different extents and at different sites, the effect of simultaneous binding of both proteins was combinatorial, rather than additive or synergistic. PU.1 binding disrupted the TFE3-induced DNase I-hypersensitive site and reduced restriction enzyme accessibility to that seen with PU.1 alone. Based on these assays, the nuclease accessibility with only PU.1 bound was indistinguishable from that with both PU.1 and TFE3 bound. Thus, the localized restriction enzyme accessibility induced by PU.1 in vivo could be an enhancer bound to ectopically expressed PU.1 alone or to PU.1 plus endogenous TFE3.

A clue to the state of the IGH μ enhancer in PU.1-expressing non-B lymphoid cells comes from our observation that enhancer-driven transcription in vitro requires DNA-bound TFE3, but not a DNase I-hypersensitive site. The presence of PU.1-induced IGH sterile transcripts leads us to speculate that the enhancer may be bound by TFE3 (or a related factor) at the μE3 site. Simultaneous binding of PU.1 plus TFE3 to the enhancer is then the likely explanation for the sterile transcription and the localized restriction enzyme accessibility induced by PU.1 in these cells. It is interesting to note that in vivo footprints of the IGH μ enhancer in B cells, where the enhancer is active and marked by a DNase I-hypersensitive site, reveal occupancy of the TFE3-binding μE3 element, but not of the ETS-binding μA or μB elements (21, 22, 50). These observations are consistent with the idea that emerges from our in vitro analysis that PU.1 binding to the μB site precludes formation of a hypersensitive site.

We have previously shown that the ETS-binding μA and μB motifs are essential for activity of the tripartite μ70 enhancer as well as a larger μ170 fragment of the enhancer. Furthermore, perfect correlation between in vivo function of a series of μB site mutations and PU.1 binding in vitro strongly implies Pu.1 as the functional μB-binding protein that is in-
volved in activation of transcription (23). Finally, ectopic PU.1 expression in non-B lymphoid cells activates sterile IGH transcripts (36). However, PU.1 did not act as a transcriptional activator in the present study. PU.1 is not necessarily always a transcriptional activator, but may act as a transcriptional repressor in other contexts (51, 52). The function of PU.1 is known to be modified by phosphorylation and interaction with other proteins such as Pia/IRF4, Rb, CBP, HDAC1, goosecoid, GATA-1, e-Jun, and eEBPs (51—61). To date, we have not explored these possibilities in our in vitro system.

The μE3 motif is not sufficient for activating transcription in cells. Activity of the Ig μ enhancer is cell-type restricted, even though TFE3-like proteins are expressed ubiquitously. These observations suggest that TFE3-like proteins cannot activate the enhancer in the absence of accessory proteins, which may be ETS proteins. Yet, in vitro TFE3 is sufficient to induce structural changes as well as transcriptional activity. The basis for this difference remains unclear at present. One possibility is that the state of chromatin in vitro is considerably different from the state of the IGH locus in non-B lymphoid cells; TFE3 may target the former but not the latter. These changes could be, for example, histone modifications or other chromatin-bound proteins.

REFERENCES
1. Gillies, S. D., Morrison, S. L., Oi, V. T., and Tonegawa, S. (1983) Cell 33, 717–728.
2. Banerji, J., Olson, L., and Schaffner, W. (1983) Cell 33, 729–740.
3. Jenuwein, T., and Grosschedl, R. (1991) Genes Dev. 5, 912–943.
4. Adams, J. M., Harris, A. W., Finkert, C. A., Corcoran, L. M., Alexander, W. S., Cory, S., Palminter, R. D., and Brinster, R. L. (1985) Nature 318, 533–538.
5. Langdon, W. Y., Harris, A. W., Cory, S., and Adams, J. M. (1986) Cell 47, 11–18.
6. Reik, W., Williams, G., Barton, S., Norris, M., Neuberger, M., and Surani, M. A. (1987) Eur. J. Immunol. 17, 465–469.
7. Schmidt, E. V., Pattengale, P. K., Weir, L., and Leder, P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6047–6051.
8. Ferrier, P., Kripp, B., Blackwell, T. K., Furley, A. J., Suh, H., Winoto, A., Cook, W. D., Hood, L., Costantini, F., and Alt, F. W. (1990) EMBO J. 9, 117–125.
9. Oltz, E. M., Alt, F. W., Lin, W. C., Chen, J., Taccioli, G., Desiderio, S., and Rathbun, G. (1993) Mol. Cell. Biol. 13, 6223–6230.
10. Okada, A., Mendelsohn, M., and Alt, F. (1994) J. Exp. Med. 180, 261–272.
11. Chen, J., Young, F., Bottaro, A., Stewart, V., Smith, R. K., and Alt, F. W. (1993) EMBO J. 12, 4635–4645.
12. Serwe, M., and Sahlymen, F. (1993) EMBO J. 12, 2321–2327.
13. Sakai, E., Bottaro, A., Davidson, L., Sleeman, K., and Alt, F. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1520–1531.
14. Nelson, B., and Sen, R. (1992) Int. Rev. Cytol. 133, 121–149.
15. Ernst, P., and Smale, S. T. (1995) Immunity 2, 427–438.
16. Kluss, M. J., McKercher, S. R., Colada, A., Van Beveren, C., and McKercher, M. A. (1990) Cell 61, 113–124.
17. Henthorn, P., Kilejian, M., and Kadesch, T. (1990) Science 247, 467–470.
18. Murre, C., Varona, A., and Baltimore, D. (1991) Mol. Cell. Biol. 11, 1156–1160.
19. Beckmann, H., St. K., and Kadesch, T. (1990) Genes Dev. 4, 167–179.
20. Roman, C., Matera, A. G., Cooper, C., Artandi, S., Blain, S., Ward, D. C., and Calame, K. (1992) Mol. Cell. Biol. 12, 817–827.
21. Ephrussi, A., Church, G. M., Tonge, D., and Gilbert, W. (1985) Science 227, 134–140.
22. Church, G. M., Ephrussi, A., Gilbert, W., and Tonge, D. (1985) Nature 313, 798–801.
Combinatorial Control of DNase I-hypersensitive Site Formation and Erasure by Immunoglobulin Heavy Chain Enhancer-binding Proteins
Haruhiko Ishii, Ranjan Sen and Michael J. Pazin

J. Biol. Chem. 2004, 279:7331-7338.
doi: 10.1074/jbc.M308973200 originally published online December 1, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308973200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 35 of which can be accessed free at http://www.jbc.org/content/279/8/7331.full.html#ref-list-1