Basic leucine zipper transcription factor Hac1 binds DNA in two distinct modes as revealed by microfluidic analyses

Polly M. Fordyce, David Pincus, Philipp Kimmig, Christopher S. Nelson, Hana El-Samad, Peter Walter, and Joseph L. DeRisi

A quantitative understanding of how transcription factors interact with genomic target sites is crucial for reconstructing transcriptional networks in vivo. Here, we use Hac1, a well-characterized basic leucine zipper (bZIP) transcription factor involved in the unfolded protein response (UPR) as a model to investigate interactions between bZIP transcription factors and their target sites. During the UPR, the accumulation of unfolded proteins leads to unconventional splicing and subsequent translation of HAC1 mRNA, followed by transcription of UPR target genes. Initial candidate-based approaches identified a canonical cis-acting unfolded protein response element (UPRE-1) within target gene promoters; however, subsequent studies identified a large set of Hac1 target genes lacking this UPRE-1 and containing a different motif (UPRE-2). Using a combination of unbiased and directed microfluidic DNA binding assays, we established that Hac1 binds in two distinct modes: (i) to short (6–7 bp) UPRE-2-like motifs and (ii) to significantly longer (11–13 bp) extended UPRE-1-like motifs. Using a genetic screen, we demonstrate that a region of extended homology N-terminal to the basic DNA binding domain is required for this dual site recognition. These results establish Hac1 as the first bZIP transcription factor known to adopt more than one binding mode and unify previously conflicting and discrepant observations of Hac1 function into a cohesive model of UPR target gene activation. Our results also suggest that even structurally simple transcription factors can recognize multiple divergent target sites of very different lengths, potentially enriching their downstream target repertoire.

DNA specificity | microfluidics

The basic leucine zipper (bZIP) proteins form one of the largest families of eukaryotic transcription factors and play roles in a wide variety of biological phenomena, from responding to endoplasmic reticulum (ER) dysfunction to regulating immune responses and oncogenesis (1). Members of this superfamily contain a positively charged DNA binding region composed of basic residues linked to a leucine zipper sequence and homo- or hetero-dimerize via this leucine zipper. Invariant arginine and asparagine residues within the basic DNA binding region (NXAAXXXCR) make direct contact with DNA bases within the major groove and drive binding specificity to palindromic or semipalindromic target sites (2, 3). Although considered to be the simplest known protein-DNA recognition motif, crystal structures of bZIP domains bound to DNA have revealed functional variability in how these conserved residues contact DNA (2, 4), and no universal code linking basic region sequence with target DNA preferences has been developed.

Here, we investigate the mechanisms that drive bZIP target site recognition using Hac1, a Saccharomyces cerevisiae transcription factor involved in the highly conserved unfolded protein response (UPR). During the UPR, cells sense an accumulation of unfolded proteins within the endoplasmic reticulum (ER) and trigger a transcriptional upregulation of genes encoding ER-resident chaperones and protein modifying enzymes, components of ER-associated protein degradation (ERAD), and enzymes for phospholipid biosynthesis (5). In S. cerevisiae, two main proteins are responsible for activating the UPR: Ire1, a transmembrane kinase/endonuclease, and Hac1. Unfolded proteins bind to the Ire1 domain facing the ER lumen, triggering its oligomerization and activation of its cytoplasmic endonuclease domain. Once activated, Ire1 cleaves HAC1 mRNA at two sites and tRNA ligase rejoins the severed exons via an unconventional spliceosome-independent mechanism (5). This splicing removes an intron to produce a new transcript (denoted HAC1i mRNA; “i” for “induced”), thereby relieving translational inhibition exerted by the intron. Following translation of the spliced mRNA, Hac1i is translocated to the nucleus, where it regulates a large set of UPR-responsive genes (6). Despite the central role played by Hac1 in activating the UPR, the rules by which Hac1 recognizes UPR target genes remain unclear.

Initial studies took a candidate-based approach to identify potential Hac1i binding sites within the promoters of known UPR target genes. Analysis of the promoter of KAR2, encoding the major Hsp70-type ER-resident chaperone Kar2 (or BiP), revealed a 22-bp cis-acting unfolded protein response element required for induction of UPR-dependent KAR2 transcription (here referred to as UPRE-1) (7, 8). Subsequent transcriptional activity assays identified a core 7-bp consensus (5′-CAGNGTG-3′; here referred to as cUPRE-1), in which point mutations of palindromic half sites (6 conserved bp) or changes in the half-site spacing severely reduced activity (9). Gel shift assays demonstrated direct binding of Hac1i to the 22-bp UPRE-1, and reporter gene assays confirmed that this element was sufficient to confer UPR-responsive transcriptional activity in an otherwise silent promoter (9, 10). UPRE-1-like motifs were also found in the promoters of four additional known UPR target genes (PDI1, EUG1, FKB2, and LHS1), lending support to its proposed role (11–13).

This central role for UPRE-1 in upregulating target gene transcription was subsequently called into question by a study employing genome-wide microarray expression profiling to identify all candidate UPR target genes (14). This work identified 381 candidate target genes, representing nearly 5% of all open reading frames in the S. cerevisiae genome and encoding numerous...
proteins required in the ER, the Golgi apparatus, and throughout the secretory pathway. Bioinformatic analysis of the promoter regions of these genes revealed that although most lacked the canonical UPRE-1, many contained one or more of two alternate motifs (UPRE-2, 5′-TAGTGT-3′; UPRE-3, 5′-AGGACAAC-3′) capable of driving Hac1-mediated transcription in reporter assays. Surprisingly, this analysis failed to recover the known UPRE-1 site (15). To account for the target site variety, it was proposed that Hac1 bound to these alternate sites via heterodimerization with Gcn4. Further complicating the picture, a study using protein binding microarrays (PBMs) to probe Hac1 binding preferences among all possible 8-bp nucleotide sequences revealed binding only to UPRE-2 (16).

In vivo studies of Hac1 are complicated by both the very short half-life of the Hac1 isoform derived from the spliced mRNA and the tendency of bZIP transcription factors to homo- and heterodimerize. Therefore by necessity, in vitro approaches provide a particularly valuable tool for accurately defining binding preferences. Here, we probe how Hac1 regulates expression of target genes using microfluidic affinity analysis [MITOMI (17) and MITOMI 2.0 (18)] to identify and characterize Hac1 target genes using microfluidic affinity analysis [MITOMI (17) and MITOMI 2.0 (18)] to identify and characterize Hac1 target genes using microfluidic affinity analysis [MITOMI (17) and MITOMI 2.0 (18)].

To obtain an unbiased assessment of Hac1 binding preferences, we used a microfluidic platform, MITOMI 2.0 (18), to measure relative binding affinities (ΔΔG) between Hac1 and 70-bp double-stranded oligonucleotides containing overlapping instances of all possible 8 bp combinations (Fig. 1A). In previous work, we validated this platform using a panel of 28 S. cerevisiae transcription factors and demonstrated the ability to quantitatively measure relative binding affinities to each oligonucleotide and recover known binding preferences (18).

In brief, each MITOMI 2.0 device contained 4,160 chambers composed of two compartments (“DNA” and “protein”) controlled by three valves (“neck,” “sandwich,” and “button”) (Fig. 1B). Experiments took place in six main steps (Fig. 1C): (i) DNA compartments were programmed with specific Cy5-labeled double-stranded DNA sequences by aligning devices to a spotted DNA microarray; (ii) BODIPY-FL-labeled His-tagged Hac1 was flowed across the protein compartments and recruited to

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**Fig. 1.** MITOMI 2.0 experimental geometry. (A) Three example oligonucleotide library sequences illustrating sequence structure. All sequences contain a “GGC” clamp (gray text), a central variable region composed of overlapping 8-nt candidate binding sites (black text), a “CA” spacer (gray text), and an identical 14-bp sequence (red text) for hybridization and extension of a universal Cy5-labeled oligonucleotide to create dsDNA. Transparent blue boxes show four potential 8-mer binding sites. (B) Photograph of 4,000 unit cell device with a penny for scale (left); close-up view of five individual unit cells (right) showing protein and DNA chambers (yellow), “neck” valve (green), “sandwich” valve (orange), and “button” valve (blue). (C) Schematic showing top and side views of experimental chambers at different points during the experiment. (D) Fluorescence scans showing final Cy5 (DNA, red) and BODIPY-FL (protein, green) intensities in DNA and protein chambers; white arrow highlights DNA recruited by surface-immobilized Hac1 beneath the button valve. (E) Histogram of measured fluorescence intensity ratios (Cy5/BODIPY-FL) on a log-linear scale to highlight outliers; the thick black vertical bar near the y-axis denotes four standard deviations above the background mean. Inset: Zoomed view of background events on a linear scale with a Gaussian fit (black) to the background distribution (x̄2 = 1.19, p = 1.0).
surfaces beneath button valves that were coated with anti-His antibodies; (iii) protein solution was pushed into DNA compart-
ments, solubilizing spotted DNA and allowing Hac1 binding and DNA sequences to interact; (iv) binding interactions were mechanically
trapped at equilibrium by pressurizing button valves to squeeze
out unbound material; (v) neck valves were closed to isolate the
compartments and allow washing away of unbound material in
the protein compartment while preserving equilibrium concen-
trations of both binding partners in the DNA compartment; and
(vi) devices were read using a fluorescence scanner. Final
Cy5 intensities in each DNA chamber were previously shown to
be proportional to the soluble DNA concentration available for
binding (17, 18), and the ratio of Cy5 (DNA) to BODIPY-FL
(Hac1) intensities beneath the button valve reports the protein
fractional occupancy, allowing calculation of interaction Kd and
Δ∆G (Fig. 1C).

We measured Hac1 binding in two independent experiments.
In both cases, Hac1 showed strong preferences for particular
sequences (Fig. 1D arrow and E and SI Appendix, Fig. S1A), with
Z scores of approximately 85 for the highest affinity sequences
(Fig. 1E and SI Appendix, Fig. S1A). Measurements were fairly
reproducible both between replicates within a given experiment
(Pearson r² = 0.73 and r² = 0.77; SI Appendix, Fig. S1B) and
between experiments performed on different days (Pearson
r² = 0.51, SI Appendix, Fig. S1C); therefore, we pooled results
from both experiments for further analysis.

MITOMI 2.0 Analysis Predicts Hac1 Binding Primarily to UPRE-2. Each
70-bp oligonucleotide contained multiple potential overlapping
Hac1 binding sites (Fig. 1D); consequently, additional analysis
was required to deconvolve results and identify the target sites
responsible for Hac1 binding. First, we used fREDUCE (19)
to search for 6-, 7-, and 8-bp motifs whose appearance within
oligonucleotides correlated most strongly with their measured
intensity ratios. Surprisingly, all searches exclusively returned
variants of UPRE-2, with strong correlations between the appear-
ance of this motif and observed intensity values (Fig. 2A and
SI Appendix, Fig. S2 and Table S1). We then assessed the effects
of single nucleotide substitutions in this consensus site on
Δ∆G by using MatrixREDUCE (20, 21) to generate a position-specific
affinity matrix (PSAM). Importantly, PSAMs can be used to
predict binding to any sequence quantitatively, and comparisons
between predicted binding profiles and measured binding profiles
yield additional information: In particular, oligonucleotides
bound more strongly than predicted would indicate binding to
additional motifs, while oligonucleotides bound more weakly
would indicate motifs that repel binding. In our data, compari-
sions between predicted and measured binding showed strong
agreement, suggesting that Hac1 bound nonpromiscuously to
UPRE-2 in vitro and displaying no evidence for binding to addi-
tional sequences present in the oligonucleotide library (Fig. 2A
and SI Appendix, Fig. S2).

Hac1 Binds the UPRE-2 but Not the cUPRE-1. It poses a paradox that
our microfluidic affinity assay data and previous PBM experi-
ments have failed to uncover evidence of UPRE-1 binding, which
was well validated in previous studies (7, 9, 10). This failure could
be explained because either Hac1 does not bind to the cUPRE-1
but requires a longer sequence that is not represented in our
library or by an insufficient sensitivity of the MITOMI 2.0 assay
to pick up low-affinity cUPRE-1 binding.

To distinguish between these possibilities, we directly mea-
sured concentration-dependent binding of Hac1 to a series of
oligonucleotides containing either the cUPRE-1 or the UPRE-2
embedded within random sequence (Fig. 2B). In three separate
experiments, we observed high-affinity binding to the oligonu-

Fig. 2. Hac1 target binding sites revealed by MITOMI 2.0 microfluidic affinity analysis using an 8-mer oligonucleotide library. (A) Top row: Previously
published motifs determined via a candidate-based approach (UPRE-1) (9); bioinformatic analysis of promoters associated with genes upregulated
during the UPR (UPRE-2 and UPRE-3) (15); and in vitro protein binding microarray experiments (UPRE-2) (16). Bottom row, left: 7-bp sequence whose appearance
within oligonucleotide sequences correlates most strongly with measured intensity ratios, as determined using REDUCE (19). Bottom row, middle: PSAM for
this sequence determined using MatrixREDUCE (21). Bottom row, right: Comparison between measured and predicted binding. (B) Measured fluorescence
intensity ratios (gray circles) as a function of soluble DNA concentration for UPRE-2 (blue box) and cUPRE-1 (orange box) embedded within standard MITOMI
library sequence (gray text) in three separate experiments.
cleotide containing the UPRE-2, with no measurable binding above background levels to the oligonucleotide containing the cUPRE-1 (Fig. 2B). Fits to the UPRE-2 binding data yielded a $K_d$ of 427 ± 37 nM, similar to values we previously obtained for other bZIP transcription factors (18).

Reporter assays have suggested that the central C within the cUPRE-1 can be replaced by alternate nucleotides with only a slight reduction in activity (9). We therefore assessed binding to these variants to see if any of the variations restore binding. For all variants, binding remained at the level of random sequence over multiple experimental replicates (SI Appendix, Fig. S3).

**Hac1 Binding to UPRE-1 Requires an Extended Target Site.** Previous work showed that the cUPRE-1 is necessary for transcriptional activity (9). However, it was never shown to be sufficient, and phylogenetic comparisons suggest that cUPRE-1 flanking sequences are important for Hac1 binding. UPRE-1 sites from the promoters of multiple known Hac1 targets (KAR2, EUG1, PDI1, FKB2, and LHS1) show conservation of several nucleotides both upstream and downstream from the 7-bp core, even as the core is imperfectly conserved (11–13) (Fig. 3A). The same pattern is also seen for UPRE-1 sites within the promoters of KAR orthologs from distant species (Fig. 3B).

To test whether flanking sequences are critical for Hac1 binding, we measured concentration-dependent binding for the cUPRE-1 embedded within either a fragment of the KAR2 promoter or within the ERO1 promoter, which typically contains a UPRE-2-like motif (Fig. 3C). Consistent with the notion that

![Fig. 3. Efficient binding of Hac1 to cUPRE-1 requires an additional 2–3 nucleotides both upstream and downstream from the 7-bp core.](image-url)
cUPRE-1 flanking sequences are required, addition of the KAR2 flanking sequences to cUPRE-1 restored high-affinity binding (Fig. 3C). Insertion of the cUPRE-1 into a heterologous flanking context (the ERO1 promoter) did not restore Hac1\(^{i}\) binding (Fig. 3C), establishing that UPRE-1-specific flanking sequences are required.

To identify the precise boundaries of flanking sequences required for Hac1\(^{i}\) binding to the cUPRE-1, we started with the cUPRE-1 embedded within heterologous ERO1 flanking sequences and systematically substituted these sequences with increasing portions of KAR2 sequences in the upstream and/or downstream direction (Fig. 3D). Restoration of upstream or downstream flanking sequences alone did not significantly increase Hac1\(^{i}\) binding affinity to cUPRE-1 (Fig. 3D, top and middle rows, respectively). By contrast, simultaneous addition of both upstream and downstream KAR2 flanking sequences had a strong effect on binding (Fig. 3D, bottom row).

Addition of one nucleotide on either side of the cUPRE-1 increased affinity fivefold, and addition of two nucleotides on either side of the core restored affinity to that measured for the cUPRE-1 in its native KAR2 context (Fig. 3E). Inclusion of additional KAR2 flanking sequence did not significantly alter binding affinities, suggesting that the 12-bp sequence 5′-GGA-CAG-GTG-GTC-3′ (hereafter termed the extended core UPRE-1, or xcUPRE-1) is sufficient for Hac1\(^{i}\) binding. Further corroboration of the importance of these nucleotides comes from the observation that single point mutations 2-bp upstream and downstream from the cUPRE-1 in the KAR2 promoter previously caused a reduction in transcriptional activity (7, 9).

**Systematic Mutation of xcUPRE-1 and UPRE-2 Target Motifs Reveals Two Distinct Target Motifs.** Understanding precisely how xcUPRE-1 and UPRE-2 differ requires a comprehensive map of individual nucleotide preferences for each binding mode. To create such a map, we measured concentration-dependent binding curves for systematic substitutions of all possible nucleotides at each position within both targets (Fig. 4A and SI Appendix, Table S2). In each case, we performed 3–4 experimental replicates (SI Appendix, Figs. S4–S7) and computed the average relative binding affinity for each substitution (Fig. 4A). From these relative affinities, we then computed an average PSAM (Fig. 4B and SI Appendix, Tables S3 and S4 and Fig. S8) for each motif.

The relative nucleotide preferences for UPRE-2 derived from these measurements agree well with those from our MITOMI 2.0 analysis (compare Fig. 4B and Fig. 2A). Taken together, these results establish that the complete UPRE-2 is short, subtending 6–7 nucleotides, with little degeneracy tolerated at most positions. The UPRE-2 appears to be an imperfect palindrome: attempts to create a more fully symmetric site by either adding a 5′ C (Fig. 4A) or by altering multiple nucleotides (SI Appendix, Fig. S9) do not lead to statistically significant increases in binding affinity. By contrast, mutations at nearly all positions within A

![Fig. 4. Maps of nucleotide binding preferences at each position within xcUPRE-1 and UPRE-2. (A) Measured relative binding affinities for all possible single nucleotide substitutions at each position within both UPRE-2 (top, blue bars) and xcUPRE-1 (bottom, orange bars) sites. Values represent the average affinity for each substitution relative to the previously reported canonical sequence (shown at top) measured over multiple replicates; errors reported are the standard error on the mean. Canonical UPRE-2 and xcUPRE-1 sequences are from the ERO1 and KAR2 promoters, respectively. (B) Affinity Logos\(^{21}\) for UPRE-2 (left) and xcUPRE-1 (right) PSAMs derived from relative affinities.](www.pnas.org/cgi/doi/10.1073/pnas.1212457109)
xcUPRE-1 have significant effects on affinity, further confirming that xcUPRE-1 subtends on the order of 11–12 bp (Fig. 4A and B). In addition, the overall composition of the motif is different: xcUPRE-1 appears to be composed of two palindromic dyad repeats (5′-G[A/C][A/C]-3′) separated by a central degenerate nucleotide.

The absolute affinity for UPRE-2 in these experiments was slightly higher than the absolute affinity for xcUPRE-1 (UPRE-2 $K_d = 497 \pm 60$ μM; xcUPRE-1 $K_d = 720 \pm 80$ μM) (SI Appendix, Table S5). However, the range of affinities measured for all UPRE-2 and xcUPRE-1 variants largely agree, with the strongest binding measured to be $220 \pm 30$ μM and $360 \pm 40$ μM, respectively (SI Appendix, Table S5).

**Prediction of Potential Genomic Targets Using xcUPRE-1 and UPRE-2 PSAMs.** An advantage of PSAMs over position weight matrices (PWMs) is that they allow de novo prediction of protein binding affinities to arbitrary sequences. To test the performance of our xcUPRE-1 and UPRE-2 PSAMs, we compared measured and predicted Hac1$^+$ binding affinities for a variety of genomic UPREs, including those present within the ERO1, KAR2, EUG1, LHS1, FK22, SEC66, and PDF1 promoters (SI Appendix, Fig. S10). Measured and predicted affinities showed relatively strong agreement ($r^2 = 0.64$, $p = 0.03$), confirming our ability to accurately predict binding to novel sequences. Next, we calculated predicted binding affinities to all annotated promoters within the yeast genome as well as to known UPR target genes (14) (SI Appendix). Promoters predicted to be bound with high affinity via UPRE-2-like binding were more likely to be present within the original UPR-induced data set (14) (SI Appendix, Table S6), with the top 20 hits including multiple known UPR targets (ULI1, TRA1, SFB3, MCD4, SNF11, HNT1, and KIC1). Known UPR target promoters included 10, 19, and 20 genes with predicted affinities within the top 10% of the distribution for the xcUPRE-1 PSAM alone, the UPRE-2 PSAM alone, or both PSAMs, respectively.

To test the ability of binding affinities measured in vitro to predict in vivo transcriptional response, we compared levels of expression of green fluorescent protein (GFP) in two S. cerevisiae strains following addition of dithiothreitol, which impairs the formation of disulfide bonds and leads to induction of the UPR. In one strain, GFP expression was driven by a synthetic promoter containing four repeats of the full KAR2 UPRE-1 (14); in the second strain, cUPRE-1 sequences were replaced by UPRE-2 sequences, resulting in higher measured in vitro affinities (SI Appendix, Fig. S11). In both strains, basal GFP expression was low and identical. Following induction of the UPR, GFP levels in the strain containing the UPRE-2 substitutions were approximately twofold higher (SI Appendix, Fig. S11), establishing that changes in affinity measured here predict target promoter activity in vivo.

**A Region Of Extended Homology N-Terminal to Basic Region Is Required for Dual Site Recognition.** Given that xcUPRE-1 and UPRE-2 differ significantly both in their overall length and relative nucleotide compositions (Fig. 4A), recognition of each motif must accommodate distinct arrangements of contacts between Hac1$^+$ and target site nucleotides. If this is the case, it should be possible to create Hac1$^+$ mutants that disrupt binding to one site while largely preserving binding to the other. In the Maf subfamily of bZIP transcription factors, a region of extended homology positioned N-terminal to the basic DNA binding domain is critical for recognition of extended (13–14 nucleotide) target sites (4). Phylogenetic alignment of Hac1 orthologs across ascomycetes reveals a similar region of extended homology (SI Appendix, Fig. S12), suggesting that these residues may be important for DNA specificity.

To identify mutants with altered binding preferences, we used a genetic screen to assess levels of binding via each mode. To do so, we used error-prone PCR to generate a library of Hac1$^+$ constructs containing random mutations to the protein between the N terminus and the first heptad repeat of the leucine zipper (Fig. 5A and SI Appendix, Fig. S13). We transformed this library into a yeast strain containing two synthetic promoters controlling the expression of two fluorescent proteins. The first promoter consists of four repeats of the KAR2 UPRE-1 motif driving mApple expression, and the second consists of four repeats of the UPRE-2 driving GFP expression (Fig. 5B). To generate the 4x-UPRE-2 promoter, we mutated two nucleotides within the KAR2 UPRE-1 to create a UPRE-2 target site (Fig. 5B). Importantly, the PSAMs derived here (Fig. 4B and SI Appendix, Tables S3 and S4) predict that these mutations are sufficient to switch Hac1$^+$ binding toward the UPRE-2 recognition mode (SI Appendix, Fig. S14). To ensure that differences in fluorescence intensity were due to changes in Hac1$^+$ binding and not indirect effects from other UPR components, we ectopically expressed both wild-type Hac1$^+$ and this mutant library using an estradiol-inducible system (22) (SI Appendix, Fig. S15).

Using this approach, we identified multiple Hac1$^+$ mutants with altered levels of binding to either one or both target promoters relative to wild-type constructs (Fig. 5C and SI Appendix, Figs. S16 and S17). Remarkably, constructs sharing a given mutation displayed the same fluorescence phenotype (SI Appendix, Fig. S17 and Tables S7 and S8). Most constructs with altered binding retained the ability to recognize xcUPRE-1 even as UPRE-2 recognition was impaired; this tendency could reflect the fact the xcUPRE-1 recognition appears to take place via both binding modes (SI Appendix, Fig. S14) or could simply be due to the increased length and tolerance of degeneracy within xcUPRE-1 (Fig. 4A and B). Mutations in positively charged arginines or lysines within the extended homology region or near the N terminus of the basic DNA binding region preferentially reduced UPRE-2 binding while maintaining xcUPRE-1 binding (Fig. 5D). Interestingly, a single arginine within the basic region plays a crucial role in xcUPRE-1 recognition (Fig. 5D). The diversity of these binding phenotypes and their emergence from individual mutations strongly argues that Hac1$^+$ binds DNA via distinct binding modes, with individual protein residues playing different roles within each interaction.

**An N-Terminal Truncation Mutant Lacking Extended Homology Regions Binds UPRE-2-Like Sequences with Reduced Affinity.** To further probe this idea and test the notion that residues within the Hac1$^+$ extended homology region are required for UPRE-2 recognition, we created Hac1$^+$ constructs with truncations at different locations within the extended homology region and mapped their xcUPRE-1 and UPRE-2 binding preferences using microfluidic affinity analysis. One truncation mutant (N25) retained three residues identified as being important for UPRE-2 binding, while the other truncation mutant (N35) lost these residues (Fig. 6A). Although comparisons between relative binding affinities for nearly full-length (N10) Hac1$^+$ and the N25 truncation mutant showed strong agreement ($r^2 = 0.90$; Fig. S18A), similar comparisons between nearly full-length (N10) Hac1$^+$ and the N35 truncation mutant showed much weaker agreement ($r^2 = 0.40$; SI Appendix, Fig. S18B), suggesting a change in binding preferences. Calculation of the difference in binding preference relative to average xcUPRE-1 and UPRE-2 behaviors for each construct reveals that although all constructs show similar binding preferences for oligonucleotides containing xcUPRE-1 and single-site substitutions (Fig. 6B), the N35 construct shows dramatically reduced binding for UPRE-2 and single-site substitutions (Fig. 6C). In particular, the N35 UPRE-2 PSAM shows a decreased tolerance for nucleotide substitutions at the 5′ end of
Yeast strains used in flow cytometry assays contained (mutant Hac binding. In support of this hypothesis, mutational analysis reveals a region of Hac that Hac binds two divergent DNA binding sites, a compact 6- or 7-bp UPRE-2 site and a significantly longer 11-bp palindromic 5′-G[A/C]CAC-3′ dyad repeats separated by a central bp that is relatively degenerate (Fig. 4A), with mutations at this position having little effect on transcriptional activity (11). These differences in both site length and nucleotide composition suggests that Hac binds two divgent DNA binding sites, a compact 6- or 7-bp UPRE-2 site and a significantly longer 11-bp palindromic 5′-G[A/C]CAC-3′ dyad repeats separated by a central bp that is relatively degenerate (Fig. 4A), with mutations at this position having little effect on transcriptional activity (11). These differences in both site length and nucleotide composition suggest that Hac must contact each site via distinct modes of binding. In support of this hypothesis, mutational analysis reveals that a region of extended homology N-terminal to the basic DNA binding domain is required for Hac1′ dual site recognition, and microfluidic affinity analysis confirms the importance of these residues for UPRE-2 recognition. Based on these conclusions, Hac1′ emerges as the first natural bZIP transcription factor shown to operate in at least two different modes.

The idea that the xcUPRE-1 subtends 11–12 bp is supported by multiple previous observations. Although necessary for UPRE-responsive transcriptional activation, the 7-bp cUPRE-1 was not sufficient for activation, and mutations in flanking nucleotides outside of this core motif caused severe reductions in reporter assay activity (7, 9). Such a long recognition sequence may also explain the prior failure of short word-based bioinformatic analysis of promoters to recover this motif from known UPR target genes (15). Our results are therefore consistent with previous observations and clarify our understanding of Hac1′ function. Several arguments suggest that the binding observed here reflects the behavior of Hac1′ alone and not of Hac1′ heterodimers, as previously proposed (15). In our experiments, 6x-His tagged Hac1′ was produced in an in vitro translation system that was then flowed over a surface coated with anti-His antibodies, effectively concentrating and purifying Hac1′ on-chip prior to affinity measurements. We consider it likely that Hac1′ produced in this manner exists as an equilibrium of monomeric and homodimeric species. In addition, the shapes of the concentration-dependent binding curves suggest that both xcUPRE-1 and the motif, suggesting a shift towards a more extended binding site (Fig. 6C and SI Appendix, Fig. S19).

The N25 and N35 truncation mutants showed twofold and 10-fold decreases in overall binding affinities, respectively (SI Appendix, Fig. S20). As a result, mapping N35 binding preferences required that experiments be performed at fourfold higher DNA concentrations to accurately measure affinities. Comparisons between relative affinities measured for the N10 construct at both concentrations showed good agreement (r² = 0.76; Fig. S18C), signifying that changes in binding preferences do not result merely from changes in experimental conditions. These results lend additional support to the idea that residues within the extended homology region are required for dual-mode binding of Hac1′ to target sites.

Discussion

Here, we show that Hac1′ binds two divergent DNA binding sites, a compact 6- or 7-bp UPRE-2 site and a significantly longer 11- or 12-bp xcUPRE-1 site. While the compact UPRE-2 appears to be a slightly asymmetric half-site, the xcUPRE-consists of two palindromic 5′-G[A/C]CAC-3′ dyad repeats separated by a central bp that is relatively degenerate (Fig. 4A), with mutations at this position having little effect on transcriptional activity (11). These differences in both site length and nucleotide composition suggest that Hac1′ must contact each site via distinct modes of binding. In support of this hypothesis, mutational analysis reveals that a region of extended homology N-terminal to the basic DNA binding domain is required for Hac1′ dual site recognition, and microfluidic affinity analysis confirms the importance of these residues for UPRE-2 recognition. Based on these conclusions, Hac1′ emerges as the first natural bZIP transcription factor shown to operate in at least two different modes.

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Here, we show that Hac1′ binds two divergent DNA binding sites, a compact 6- or 7-bp UPRE-2 site and a significantly longer 11- or 12-bp xcUPRE-1 site. While the compact UPRE-2 appears to be a slightly asymmetric half-site, the xcUPRE-consists of two palindromic 5′-G[A/C]CAC-3′ dyad repeats separated by a central bp that is relatively degenerate (Fig. 4A), with mutations at this position having little effect on transcriptional activity (11). These differences in both site length and nucleotide composition suggest that Hac1′ must contact each site via distinct modes of binding. In support of this hypothesis, mutational analysis reveals that a region of extended homology N-terminal to the basic DNA binding domain is required for Hac1′ dual site recognition, and microfluidic affinity analysis confirms the importance of these residues for UPRE-2 recognition. Based on these conclusions, Hac1′ emerges as the first natural bZIP transcription factor shown to operate in at least two different modes.

The idea that the xcUPRE-1 subtends 11–12 bp is supported by multiple previous observations. Although necessary for UPRE-responsive transcriptional activation, the 7-bp cUPRE-1 was not sufficient for activation, and mutations in flanking nucleotides outside of this core motif caused severe reductions in reporter assay activity (7, 9). Such a long recognition sequence may also explain the prior failure of short word-based bioinformatic analysis of promoters to recover this motif from known UPR target genes (15). Our results are therefore consistent with previous observations and clarify our understanding of Hac1′ function. Several arguments suggest that the binding observed here reflects the behavior of Hac1′ alone and not of Hac1′ heterodimers, as previously proposed (15). In our experiments, 6x-His tagged Hac1′ was produced in an in vitro translation system that was then flowed over a surface coated with anti-His antibodies, effectively concentrating and purifying Hac1′ on-chip prior to affinity measurements. We consider it likely that Hac1′ produced in this manner exists as an equilibrium of monomeric and homodimeric species. In addition, the shapes of the concentration-dependent binding curves suggest that both xcUPRE-1 and the motif, suggesting a shift towards a more extended binding site (Fig. 6C and SI Appendix, Fig. S19).

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UPRE-2 motifs are bound by Hac1 complexes with identical stoichiometries: All curves in a given experiment asymptote to an identical fluorescence intensity ratio, establishing that the number of DNA molecules bound per labeled protein molecule remains constant. The notion that Hac1 binds as a homodimer is further supported by detection of Hac1 constructs in PBM experiments employing Hac1 proteins expressed in an *Escherichia coli* system that does not contain potential orthologous binding partners (16, 23). Moreover, gel shift assays performed in yeast extracts showed indistinguishable shifts for Hac1 bound to oligonucleotides containing either xcUPRE-1 or UPRE-2 motifs (15). Finally, the palindromic structure of the xcUPRE-1 target site is consistent with expectations of homodimeric binding.

How does Hac1 bind both long xcUPRE-1 and compact UPRE-2 DNA target sites? Although most bZIP transcription factors are thought to bind relatively compact binding sites (SI Appendix, Fig. S21), Maf subfamily transcription factors recognize unusually long motifs (13–14 bp) via an unconventional conformation of the invariant arginine and asparagine residues within the basic region of all bZIP proteins (4). Similarly, a crystal structure of Pap1, a *S. pombe* bZIP transcription factor, complexed with DNA demonstrated that Pap1 target site specificity was also due to alternate positioning of these two residues (2). In our genetic screen, none of the constructs with altered binding affinities were found to have mutations in these invariant residues (Fig. 5D and SI Appendix, Fig. S17 and Table S8), although such mutations were found in colonies lacking fluorescence in either channel (SI Appendix, Table S7). We therefore suggest that these invariant residues could be required for recognition of both target sites, with changes in their conformation leading to recognition of one site or the other. Notably, the ability to recognize two closely related sites via conformational shifts has previously been proposed for a minimal bZIP construct (24). In a manner analogous to the Maf proteins, we propose that the extended homology region could stabilize invariant bZIP residues in the conformation required for UPRE-2 recognition. With the exception of MafG, most bZIP crystal structures have been based on constructs truncated to include only 1–9 nucleotides N-terminal to the basic DNA binding region (4, 25–28) (SI Appendix, Fig. S22). It remains to be seen whether N-terminal regions of extended homology facilitate binding of alternate sites by other bZIP proteins.

Several recent studies have noted plasticity in bZIP binding preferences, although to date, this plasticity has been confined to tolerance for binding multiple related sites of the same or very similar lengths. A synthetic bZIP protein composed of the Gcn4 basic region fused to the C/EBP leucine zipper was shown to bind with high affinity to both cognate and alternate sites, indicating that protein architecture beyond the basic region can affect binding preferences (24). In addition, a recent study employing PBMs to characterize the DNA binding specificities of multiple bZIP TFs noted that several proteins (Yap1, Yap3, and Sko1) possessed the ability to bind closely related dyad repeat sites with variable length (1–2 bp) spacers or extensions at either end (23). This study also noted that the DNA binding domain for Hac1 shares multiple residues with the basic regions of bHLH proteins,
For the glucocorticoid receptor, DNA sequences can act as allos-
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To what purpose does Hac1 recognize multiple distinct sites? For the glucocorticoid receptor, DNA sequences can act as allo-
targets, underscoring the importance of integrating both biophysical and biological data to understand transcriptional regulation.

Materials and Methods
DNA Library and Hac1 Production. DNA libraries for MITOMI 2.0 experiments were synthesized as described previously (18). Briefly, all possible 65,536 8-bp DNA sequences were assembled into a compact DNA library spread over 1,457 oligonucleotides, each of which contained an identical 3 nt 5′ ‘CCG’ clamp and an identical 14 nt 3′ universal sequence allowing hybridization of a single Cy5-labeled primer (Fig. 1A). Following hybridization, all sequences were extended using Klenow exo- (New England Biolabs). Prior to printing, 8-mer libraries were dried down and resuspended to a final concentration of 1.25 μM in 3X SSC containing polyethylene glycol (PEG) (Fluka) and D(-)-trehalose dihydrate (Fluka) to improve spot visibility and solubilization. Libraries for measuring concentration-dependent binding behavior were synthesized largely as described previously (18) with final preprinting concentrations of 10 μM, 6.7 μM, 4.4 μM, 3.0 μM, 2.0 μM, 1.3 μM, 0.9 μM, and 0.4 μM. For truncation mutant experiments, final preprinting concentrations were 16 μM, 10.7 μM, 7.1 μM, 4.7 μM, 3.2 μM, 2.1 μM, 1.4 μM, and 0.94 μM; each truncation was cloned into a single integration oligonucleotide relative to the most strongly bound xcUPRE-1-like or UPRE-2-like oligonucleotide and (ii) computing the average relative affinity over all replicates. Differences in binding preferences for Hac1 truncation mutants were calculated by subtracting measured relative binding affinities (calculated relative to the most strongly bound oligonucleotides) from the average relative binding affinities given in the xcUPRE-1 and UPRE-2 PSAMs (SI Appendix, Tables S3 and S4).

Error-Prone PCR. The Hac1 mutant library was created using error-prone PCR (34), using a total of 48 cycles and 11 serial dilution steps (performed every four cycles). All sequence alignments and phylogenetic alignments were created using Geneious v4.8.2 (35).

Yeast Strains and Plasmids. Standard cloning and yeast techniques were used for construction, transformation, and integration of the plasmid within strain W303. The transcription reporters used here controlled expression of the blue fluorescent proteins mApple and GFP via a crippled cy7 promoter containing four repeats of a 22-bp UPR-responsive cis element (xcUPRE-1 for mApple or UPRE-1 for GFP). TheUPRE-2 reporter was generated by site directed mutagenesis of 4aUPRE-1 (Fig. S8 and SI Appendix, Fig. S14). The xcUPRE-1-mApple plasmid was cloned into a single integration, HIS3-marked vector (pNH603), while the mUPRE-1-GFP was cloned into a single integration, LEU2-marked vector (pNH605).

Flow Cytometry. A dual reporter strain containing xcUPRE-1-mApple (integrated in his3) and mUPRE-1-GFP (integrated in leu2) also expressed a chimeric estradiol-responsive transcriptional activator with an N-terminal activation domain derived from Msn2 and a C-terminal DNA binding domain from Gal4. This parent strain was then transformed with either wild-type HAC1 or the mutant hac1 library cloned into a single integration, TRP1-marked vector under the control of the GAL1 promoter). Cells were cultured in 2x SDC at 30 °C in 96 well plates (2 μL) in an Innova plate shaker at 900 rpm. After induction with estradiol (100 nM), cells were sampled after 4 h using a BD LSR-i equipped with a high throughput sampler, a 488 nm 150 mW laser, 532 nm 150 mW laser, FITC and PE-Texas red emission filters, and FACS DIVA software. Flow cytometry data were analyzed using custom software written in Python. Reported mean fluorescence intensities for all strains were calculated via Gaussian fits to binned intensity distributions for individual cells.

ACKNOWLEDGMENTS. We thank Doron Gerber, Dan Tran, and Stephen Quake for assistance with fabrication of microfluidic devices and early microfluidic assays; Marshall Burke for photographs of microfluidic devices; and Matthew Larson and Florencia Caro for careful reading of the manuscript. P.M.F was supported by a Howard Hughes Medical Institute/Helen Hay Whitney Foundation Postdoctoral Fellowship. J.D.R. and P.W. are investiga-
tors of the Howard Hughes Medical Institute.


