

Discovery, optimization and validation of an optimal DNA-binding sequence for the Six1 homeodomain transcription factor

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ABSTRACT

The Six1 transcription factor is a homeodomain protein involved in controlling gene expression during embryonic development. Six1 establishes gene expression profiles that enable skeletal myogenesis and nephrogenesis, among others. While several homeodomain factors have been extensively characterized with regards to their DNA-binding properties, relatively little is known of the properties of Six1. We have used the genomic binding profile of Six1 during the myogenic differentiation of myoblasts to obtain a better understanding of its preferences for recognizing certain DNA sequences. DNA sequence analyses on our genomic binding dataset, combined with biochemical characterization using binding assays, reveal that Six1 has a much broader DNA-binding sequence spectrum than had been previously determined. Moreover, using a position weight matrix optimization algorithm, we generated a highly sensitive and specific matrix that can be used to predict novel Six1-binding sites with highest accuracy. Furthermore, our results support the idea of a mode of DNA recognition by this factor where Six1 itself is sufficient for sequence discrimination, and where Six1 domains outside of its homeodomain contribute to binding site selection. Together, our results provide new light on the properties of this important transcription factor, and will enable more accurate modeling of Six1 function in bioinformatic studies.

INTRODUCTION

A defining characteristic of transcription factors (TFs) is their ability to recognize and bind to specific DNA sequences in the genome, within the regulatory region of

the target genes they control. Multiple structural classes of TFs exist, among which are homeodomain factors. The primary function of the homeodomain is DNA binding. This group of regulatory factors were first discovered due to their involvement in homeotic conversions in *Drosophila*, but were later found to exist in essentially all eukaryote species, from yeast to humans. Bioinformatics analyses indicate that 235 homeodomain TFs are encoded in the human genome (not counting splice variants and pseudogenes) (1). This diversity is manifested by various expression profiles, protein domain composition and interaction partners. However, a large number of homeodomains seemingly recognize the same DNA sequence TAAT, or close variants, leading to the question of how redundancy in binding site selection is avoided by these proteins. The prototypical 60 amino acids long homeodomain assumes a three-dimensional structure composed of an unstructured N-terminal arm followed by three alpha-helices. The N-terminal arm and the third helix contribute most of the DNA sequence-binding specificity, and their amino acid composition are thought to contribute to this property (2,3). Two recent large-scale surveys considered the question of binding specificity of homeodomain TFs from mouse (4) or *Drosophila* (5). It was found that indeed, when homeodomains are considered globally, their amino acid sequence correlates with their DNA sequence-binding preferences within and adjacent to the TAAT core. However, domains residing outside of the homeodomain can also influence DNA binding, either through direct DNA contacts or interaction with dimerization partners (6–9). Therefore, it remains to be established whether homeodomain TF-binding site predictions based solely on *in vitro* DNA-binding preferences are sufficiently accurate to allow to predict which target genes they regulate.

The Six family of homeodomain TFs is conserved from flies to humans, and in mammals counts six members, from Six1 to Six6. Like most homeodomain TFs, they are

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involved in controlling the development of various tissue types; for instance, Six1 and Six4 are involved in the development of the eyes, ears, kidneys and skeletal muscle (reviewed in (10,11)). We became interested in this group of TFs when we found that the DNA motif they recognize, the MEF3 sequence element, is enriched within the promoter region of Myogenic Regulatory Factors (MRFs) target genes, in muscle precursor cells (myoblasts) (12). The MEF3 element is a phylogenetically conserved motif (DNA consensus TCAGGTTTC) that was originally identified within the regulatory region of only a few muscle-specific genes (13,14). The Six1 and Six4 members of the Six family, two factors essential to myogenesis, were subsequently found to bind specifically to the MEF3 sequence within the myogenin (Myog) gene promoter and activate this gene's expression during embryogenesis (15–19). MRFs control myogenesis by activating the expression of a large cohort of genes necessary for differentiation and function of muscle cells, and in some instances they accomplish this task by cooperating with transcription factors of the Mef2 and Pbx families (20,21). The connection between the MRF target genes and MEF3 sites led us to postulate that Six family members can also cooperate with the MRFs to regulate myogenesis. This was confirmed by the genome-wide identification of Six1 target genes in mouse myoblasts and myotubes, and by functional assays which showed that Six factors can activate transcription with the MRFs in a synergistic fashion (22).

The wealth of DNA-binding information contained within our genomic Six1-binding profile (22) gave us the opportunity to examine the DNA sequence-binding preferences of this TF in myoblasts and myotubes and to analyze it in the context of the previously identified MEF3 sequence motif. Here, using *de novo* motif finding and position weight matrix (PWM) optimization, we report that Six1 has a broader than anticipated DNA-binding profile, which extends well beyond the canonical MEF3 consensus sequence. *In vitro* binding data corroborate these *in vivo* DNA-binding sequence preferences, suggesting that sequence-specific DNA binding by Six1 does not require interaction with dimerization partners. However, we find a discrepancy between our results and those of an *in vitro* DNA-binding screen for the Six1 homeodomain, performed by others (4), suggesting that Six1 homeodomain sequence is not the sole determinant of its binding to DNA.

MATERIALS AND METHODS

De novo motif finding

The Amadeus program was used for *de novo* DNA sequence motif discovery (23). The 'bound' sets corresponded to the sequences bound by Six1 in mouse myoblasts (MB, total of 1022 sites) or myotubes (MT, total of 1853 sites) with a false discovery rate (FDR) less than 10% (22). These two sets of bound sequences were broken down into three (for MB) or five (for MT) randomly assigned subgroups, respectively (i.e. MB-A, MB-B, MB-C and so on). The purpose of this

sub-grouping was to obtain one subgroup for motif discovery, and to reserve the other sequences for subsequent validation of the discovered motifs. The Amadeus program was run on each of these eight subgroups, performing 'large' searches of 12 base pairs motifs and examining both DNA strands. For these eight searches, the background sequence set corresponded to a randomly selected subset of the genomic regions surveyed in our ChIP-on-chip analyses (20% of surveyed loci, approximately 108 Mb of sequence). Both sets of sequences were repeat-masked using RepeatMasker (24). The top ranking sequence motif (lowest corrected *P*-value after 25 cycles of bootstrapping where 'bound' and 'background' sequences are randomly interchanged) was retained. Searches for motifs less than 12 base pairs in length were also run and yielded very similar motifs (not shown). Finally, an averaged PWM (Six1_MB+MT) was calculated by aligning the eight PWMs and averaging the frequencies at each position. PWMs were represented graphically using the Weblogo program (25).

Motif abundance within sets of sequences was estimated using the CisGenome program (26). The number of PWM 'hits' within 'bound' and 'background' sequences was determined with a likelihood ratio setting of 500. The bound set of sequences for this purpose was composed of those excluded from the initial motif discovery (e.g. for the matrix identified using subgroup MB-A, the sequences in MB-B and MB-C were combined and used to test its performance). Here the background sequences were a distinct set of 20% of all surveyed loci. The relative enrichment score (the ratio of frequency of PWM hits among bound regions over that in background regions) was calculated. We also calculated the cumulative hypergeometric probability, the chance of finding at least a certain number of hits to a PWM among the bound regions (sample) given that a certain number of hits exist among the background (population) sequences (sample and population sizes expressed as total base pair length). Finally, searches were also repeated on the top 5% most phylogenetically conserved portion of the sequences.

PWM optimization

The methods described by Staden (27) and Bucher (28) were utilized to calculate the PWM. We adopted the base frequencies reported by Amadeus in the *de novo* motif searches (above). These base frequencies were converted into odds scores by dividing the frequencies by expected frequency which is calculated from the Database of Transcription Start Sites (DBTSS) for mouse using the formula described in (27):

$$e_{bi} = \frac{\sum_{i=1}^L n_{bi}}{L}$$

where *b* is one of 4 nucleotides (A,C,G or T) at position *i*, *n_{bi}* is the number of times base *b* occurs at the *i*-th position of the motif and *L* is the length of the sequence.

The sequences used from DBTSS were of the length 1201 base pairs (−1000 to +201 from the TSS) and were aligned with respect to the TSS.

The weight for each position of the matrix is derived using the formula described in (29):

$$w_{bi} = \ln\left(\frac{n_{bi}}{e_{bi}} + s_i\right) + c_i$$

where b is one of the 4 nucleotides, n_{bi} is the number of times base b occurs at the i -th position of the motif, c_i is a constant providing column maximum value to be zero, s_i is a smoothing parameter preventing the logarithm of zero (or too small a value). We adopted the criteria as: $s_i = 0$ if the first term under logarithm in Formula is larger than $0.01 \times \frac{n}{4 \times e_{bi}}$ and $s_i = 0.01 \times \frac{n}{4 \times e_{bi}}$ otherwise, where $n = \sum_{b=1}^4 n_b$

To calculate the similarity score for a specific sequence within the PWM we used the formula as:

$$S = \sum_{i=1}^L w_{bi}$$

where L is the length of PWM, w_{bi} is the log-odds weight of nucleotide b at position i in the PWM. To optimize the derived PWM, we have used correlation coefficient (CC) also known as Simple Matching Coefficient (SMC) (30) as the objective function. This function takes into account sensitivity and specificity of the predicted TFBS. The process of optimization started with evaluating the performance of the PWM by calculating CC at each cutoff. The CC is calculated as:

$$CC = \frac{(TP \times TN) - (FN \times FP)}{\sqrt{(TP+FN) \times (TN+FP) \times (TP+FP) \times (TN+FN)}}$$

CC is calculated for each cutoff starting from a very stringent threshold and relaxing the threshold until we get the maximal CC. To calculate the CC we have divided the sequences into two different datasets depending on the binding preference of Six1 in myoblasts and myotubes. The sequences where Six1 is found experimentally to bind are regarded as positive and the sequences where Six1 did not bind are regarded as negative dataset. These two datasets were utilized to find out the four parameters to calculate CC: true positives, true negative, false positives and false negatives (TP, TN, FP and FN, respectively). From the above mentioned datasets, we designated TP as the number of sites from the experimental-positive dataset positively identified by the PWM with a given cutoff, and we regarded any sites computationally identified from the experimental-negative dataset where Six1 do not bind as FP. TN is calculated as the difference between the total number of sequences in the negative dataset and FP, while FN is calculated as the difference between the total number of sequences in the positive dataset and TP. The FN was calculated assuming each sequence in the positive datasets should have at least one binding site. The above step is repeated for each cutoff with the increment of 0.1, and maximal CC with respective cutoff was identified. We selected the corresponding cutoff and we further refined the performance of the PWM as follows. The PWM with the previously optimized cutoff shows sensitivity of 54% and specificity

of 76%. This matrix becomes our initial PWM for the next step of optimization. Again we start with the stringent cutoff and refined the motif list used to build the PWM at each 0.1 increment in the cutoff. At each cutoff, the matrix was used to find motifs from the positive dataset. The list of motifs thus obtained from positive dataset was utilized to build the new PWM. With this PWM, we searched motifs in the negative dataset and compared the search results with those obtained from the positive dataset. We subtracted the motifs from the positive search list and rebuilt the PWM with the remaining list. The new PWM was evaluated with the function CC. We repeated the latter step for a large range of cutoffs, from stringent to relaxed, and selected the cutoff where the CC attained the maximum. The resulting matrix provides better discrimination between the positive (bound) and negative (surveyed) datasets. The respective algorithm of PWM optimization is hence named 'Bound/Surveyed sequence Discrimination' (BSD) algorithm. The PWM was optimized using binding data for Six1 in C2C12 myoblasts and in fully differentiated myotubes. For further validation on an independent dataset, we used genomic regions bound by Six1 only at 24 hours of differentiation, but not bound in myoblasts nor in myotubes. This corresponds to a total of 187 DNA sequences with an average length of 1061 bp, which have no overlap with the myoblasts and myotubes datasets.

Recombinant Six1 purification

The full-length coding sequence of mouse Six1 was amplified from C2C12 cells and cloned in frame in the pHIS2 plasmid, which codes for an N-terminal hexa-histidine tag followed by a linker region. The protein was produced in the *E. coli* STAR strain. Cells were grown to an optical density at 600 nm (OD^{600}) of 0.6 and induced to produce the protein with 0.1 mM isopropyl β -D-1-thiogalactopyranoside for 1 h 30 min at 37°C. Cells were pelleted, resuspended in binding buffer (50 mM Tris pH 8.0, 50 mM NaCl, 0.5 mM DTT, 10 mM imidazole and 2 mM phenylmethylsulfonyl fluoride) and sonicated using a microtip sonifier. The lysate was spun at 17000g for 15 min at 4°C, and the supernatant was applied to Nickel-Sepharose beads (GE Healthcare). Beads were washed in binding buffer containing 40 mM imidazole, and elution was carried out using binding buffer containing 500 mM imidazole. The eluate was immediately bound to heparin-Sepharose beads (GE Healthcare). The beads were washed with wash buffer (50 mM Tris pH 8.0, 650 mM NaCl, 0.5 mM DTT and 2 mM phenylmethylsulfonyl fluoride) and eluted in a similar buffer containing 750 mM NaCl. The eluate was dialyzed for 18 hours against a similar buffer reduced to 150 mM NaCl and containing no imidazole, then concentrated using Amicon Ultra (30 kD cut-off, Millipore), aliquoted, frozen in liquid nitrogen and stored at -80°C. Coomassie blue staining of the purified Six1 protein indicated an estimated purity of 90% (Figure 3A). For work with the homeodomain of Six1, amino acids 110 to 201 of mouse Six1 were cloned using the same strategy as for the full-length protein. This

Six1-HD protein therefore contains the homeodomain and 15 amino acids of flanking sequence on each side, which conforms to what Berger *et al.* have used. Purification of the histidine-tagged Six1-HD protein from *E. coli* was performed in the same way as for the full-length protein, except that dialysis and concentration were performed using devices with smaller pore sizes (3 kD cut-off).

Electrophoretic mobility shift assays and calculation of dissociation constant (K_d^{app})

EMSA experiments were performed using His-Six1 and fluorescently-labelled double-stranded DNA probes, which were prepared by end labelling of double-stranded DNA containing a G nucleotide overhang at each end of the molecule, with the Klenow enzyme Exo- (NEB) and Cy5-labelled deoxycytidine triphosphate (dCTP) (GE Healthcare). In all cases, the probe sequence context was that of the mouse myogenin MEF3 site, with the sequence gTTAGAGGGGGCTCAGGTTTCTGTGGCGTTGGC as the top strand. The initial small script 'g' represents the additional nucleotide overhang used for labelling, while the underlined nucleotides constitute the MEF3 site. In order to test the influence of various MEF3 nucleotide substitutions on Six1 binding, and to disregard the putative influence of surrounding nucleotides, we changed the sequence of the MEF3 site while retaining the same surrounding sequence context. EMSA-binding reactions contained varying amounts of His-Six1 protein in a fixed volume (4 μ l in 50 mM Tris, 150 mM NaCl and 1 mM DTT) and 20 fmoles of probe in a final volume of 10 μ l. The binding buffer was composed of Hepes 25 mM pH 7.6, KCl 8 mM, dIdC 1 μ g, MgCl₂ 5 mM, Glycerol 10% v/v. The reactions were set up on ice, then incubated at 37°C for 5 min, and loaded on a 5% w/v acrylamide gel (29:1 ratio acrylamide to bis-acrylamide) containing 2% glycerol, with TGE 0.5 \times (12.5 mM Tris, 95 mM Glycine, 0.5 mM EDTA) as the running buffer. After separation, the gels were rinsed in water, and the fluorescent signal was quantitated using a Typhoon Phosphorimager (GE Healthcare), adjusting the photomultiplier tube voltage so that none of the signal is saturated. The ratio of the volume of shifted probes over that of the total (shifted and free probes) was calculated using ImageQuant software (GE Healthcare) for each concentration of Six1. To determine the K_d^{app} of protein-DNA binding, we determined the concentration of Six1 protein (in nanomolar) required to reach half maximal binding, using the function of one site saturation in SigmaPlot and following recommendations outlined in (31).

RESULTS

Identification of a novel MEF3-like motif

We have previously obtained ChIP-on-chip data for Six1 binding in the C2C12 cell line of mouse myoblasts (22). The experiment was performed in proliferating myoblasts and in differentiated myotubes and led to the identification of 1022 and 1853 high-confidence bound genomic loci in these two cell types, respectively. The average length of

Six1-bound loci is 1230 bp; we used Amadeus, a *de novo* motif finding program, to precisely identify the DNA sequence motif most likely recognized by Six1 in these genomic regions. Our assumption is that the most abundant motif in these bound regions should be the one directly bound by Six1. For the purpose of motif discovery and subsequent testing, the search was run multiple times on the Six1 ChIP-on-chip target sequences and partitioned in eight subsets of approximately 330–370 sequences (see materials and methods for details). As shown in Figure 1, we obtained eight similar PWMs with little if any difference between the PWMs of Six1-bound genomic sequences detected in myoblasts and myotubes. Additionally, to summarize these results we also combined these eight PWMs to obtain an average matrix, called Six1_MB+MT (Figure 1 and Supplementary Table S1).

Figure 1 clearly shows that our novel matrices share a strong resemblance with the previously identified MEF3 sequence motif represented in the TRANSFAC database. However, the new PWMs are clearly more degenerate, since at multiple positions more than one nucleotide is allowed; this is most obvious near the center of the motif, at positions 6–8, where the preference for the AGG nucleotides is weaker than in the TRANSFAC motif. In contrast, positions 4 (T) and 12 (C) display the lowest variety. These differences have important implications for the prediction of target gene binding by Six1: using the inflexible TRANSFAC MEF3 element would possibly overlook a large number of true targets.

To determine if these differences are significant and if the increased degeneracy in our novel PWMs has an impact of their predictive value, we evaluated the sensitivity and specificity of the PWMs. We compared the number of matches to each PWM that can be found in bound sequences and in control sequences using the Cisgenome motif mapping program: a larger number of matches indicate higher sensitivity, while the specificity is given by the enrichment ratio (frequency of matches in bound sequences divided by that in control sequences). We first verified that hits to the Six1_MB+MT PWM are substantially enriched among all strata of the Six1-bound loci found by ChIP-on-chip, not solely among the top (highest confidence) loci (Figure 2). Table 1 gives the results of the comparison of our *de novo*-identified motifs and reveals that all nine motifs we generated from our ChIP-on-chip data are present in large numbers among the bound genomic regions (i.e. the PWMs are sensitive) and are characterized by substantial enrichment levels (i.e. they are also specific). Importantly, the results presented in Table 1 clearly show that the novel matrices outperform the TRANSFAC motif in both specificity (higher enrichment levels) and sensitivity (larger number of sites). These analyses were performed giving equal consideration to all genomic sequences. When only the phylogenetically conserved regions of the bound loci were studied, the enrichment level of the PWMs increased, as can be expected for the binding sites of a developmentally important TF (32,33). Here again, our *de novo* PWMs outperformed the TRANSFAC matrix with their

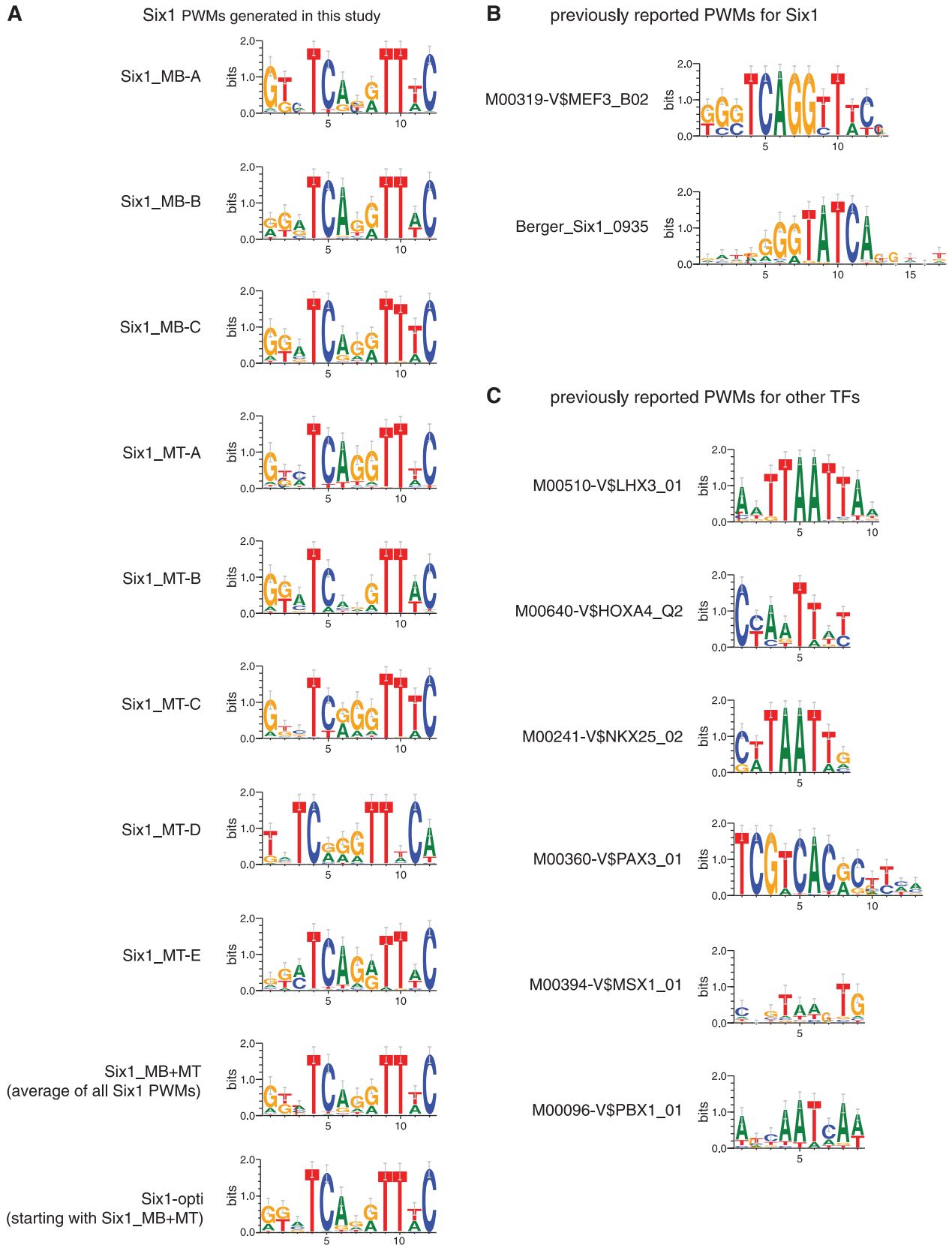


Figure 1. Position weight matrices for Six1 and other homeodomain TFs. (A) PWMs generated in this study. (B) Previously reported PWMs for Six1. (C) The PWMs of other well-characterized homeodomain TFs. The PWMs were represented graphically using sequence logos.

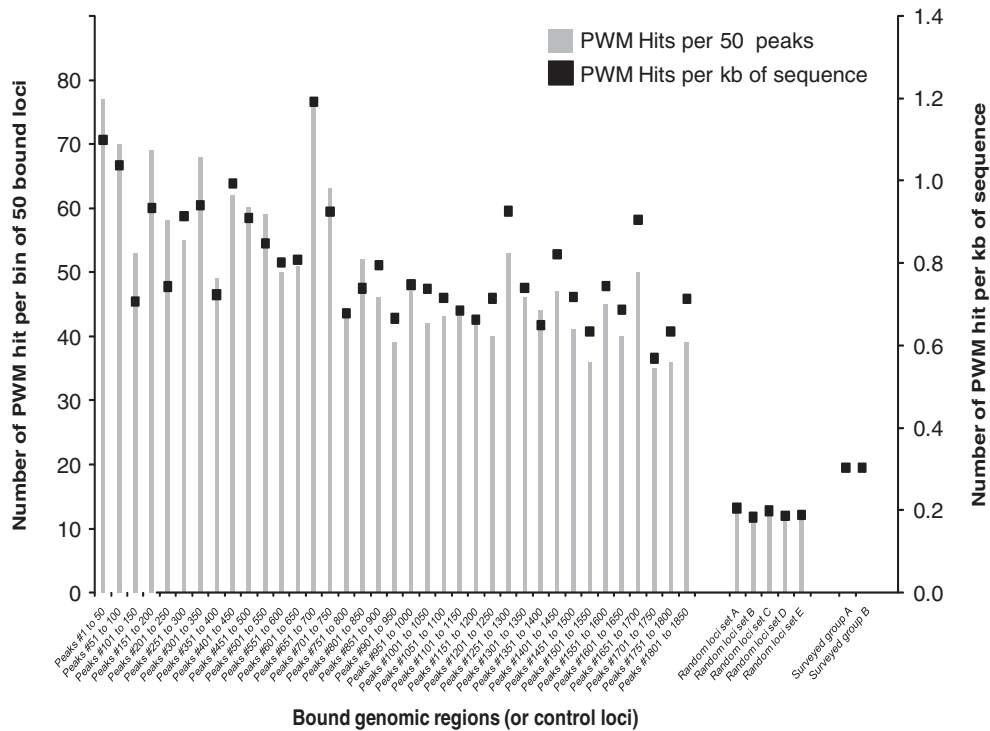


Figure 2. Enrichment of the Six1_MB+MT matrix hits within the loci bound by Six1 in myotubes, as a function of their score in the ChIP-on-chip experiment. The 1853 genomic loci bound by Six1 in myotubes were ranked in decreasing order of ChIP enrichment and subdivided in bins of 50 regions. Matches to the Six1_MB+MT matrix were identified, and the numbers of PWM hit per bin (left-hand y axis) or per base pair (right-hand y axis) were calculated. As controls, five sets of randomly selected genomic regions were also scanned for PWM hits. Additionally, two sets of sequences totaling 108 Mb and originally surveyed in the ChIP-on-chip experiments were also scanned here.

higher enrichment levels (Table 1, enrichment—conserved sites only).

Secondly, we also compared our novel matrices to that identified by Berger *et al.* (4) by probing protein-binding microarrays with the bacterially expressed Six1 homeodomain in isolation, excluding the N-terminal Six domain as well as the C-terminal region (11). Again, our *de novo* matrices outperform this motif, both when all sequences or only the conserved subset were considered (Table 1). We note that the similarity between our and Berger's matrices is limited to positions 4–6 of our motif (10–12 of their motif, consensus TCA).

Finally, we also verified whether the binding sites of other homeodomain transcription factors, including some that are involved in controlling myogenesis (Nkx2.5, Msx1, Pbx1, Pax3), are enriched among the genomic sites bound by Six1. None of these were enriched to a significant level within the Six1-bound genomic regions (Table 1). The canonical 'ATTA' (reverse-complement of TAAT) DNA sequence motif recognized by homeodomain transcription factors (e.g. Nkx2.5, in Figure 1) is observed in the Six1_MB+MT matrix at positions 8 to 11 (consensus (A/G)TT(T/A)). However, among the matches to the PWM that we have identified, only 222 out of 1873 conform to the canonical TAAT sequence at these positions; this sub-motif ranks fourth in frequency, behind GTTT, GTTA and ATTT (536, 374 and 268 hits respectively, Supplementary Table S2). Together, the results of this analysis suggest that

Six1-bound DNA elements are not limited to the canonical 'ATTA' DNA sequence motif shared by several homeodomain TFs and provide a PWM that characterizes Six1 DNA-binding preferences with improved accuracy over all other existing matrices.

Broad sequence specificity of DNA binding by Six1

The Six1 PWM that we established is substantially different from other homeodomain TFs as well as from the PWMs previously reported for Six1 (TRANSFAC and Berger *et al.*). We were especially intrigued by the rather degenerate nature of the central portion of the matrix. Consequently, we used electrophoretic mobility shift assays (EMSA) to probe Six1's ability to bind a range of DNA sequences that is wider than previously expected, avoiding the contribution of other confounding factors.

First, we set out to determine the apparent equilibrium dissociation constant (K_d^{app}) of purified Six1 for the MEF3 site present in the Myog proximal promoter (15,22,34,35), a site that is identical to the consensus DNA motif established by the TRANSFAC PWM, and which is to date the best characterized Six1-binding site. EMSA reactions were performed in the presence of fixed amounts of Cy5-labelled probe and increasing amounts of the Six1 protein (Figure 3A). We found that Six1 binds to the mouse Myog MEF3 site with a K_d^{app} of 35 nM (Figure 3B). Next, we aimed to verify whether the sequence preferences given by our PWM reflect the

Table 1. Results of the sensitivity and specificity searches for new and existing Six1 PWMs

Name	Target list ^a	No. of sites (all) ^b	Enrichment (all sites) ^c	<i>P</i> -value (all sites) ^d	No. of sites (conserved only) ^e	Enrichment (conserved sites only)	<i>P</i> -value (conserved sites)
Six1_MB-A_m01.mat	MB_BC	485	4.73	<1E-16	139	6.49	<1E-16
Six1_MB-B_m01.mat	MB_AC	449	4.45	<1E-16	141	7.21	<1E-16
Six1_MB-C_m01.mat	MB_AB	471	5.23	<1E-16	144	7.31	<1E-16
Six1_MT-A_m01.mat	MT_BCDE	977	2.58	<1E-16	246	4.60	<1E-16
Six1_MT-B_m01.mat	MT_ACDE	1043	2.96	<1E-16	260	4.52	<1E-16
Six1_MT-C_m01.mat	MT_ABDE	888	2.73	2.2E-16	240	4.70	<1E-16
Six1_MT-D_m01.mat	MT_ABCE	891	3.08	<1E-16	248	4.92	1.3E-15
Six1_MT-E_m01.mat	MT_ABCD	895	2.36	<1E-16	242	3.93	<1E-16
Six1_MB+MT.mat	MB_ABC	1144	3.50	<1E-16	321	4.99	<1E-16
	MT_ABCDE	1873	2.93	3.3E-16	489	4.67	7.8E-16
Berger_Six1_0935.mat	MB_ABC	308	1.38	2.8E-08	51	1.52	2.6E-03
	MT_ABCDE	544	1.25	2.5E-07	84	1.54	9.5E-05
M00319-V\$MEF3_B02.mat	MB_ABC	26	2.29	1.2E-04	9	5.02	8.6E-05
	MT_ABCDE	51	2.29	8.5E-08	11	3.77	1.7E-04
M00510-V\$LHX3_01-Lhx3a.mat	MB_ABC	350	0.67	1.0E+00	116	0.67	1.0E+00
	MT_ABCDE	782	0.77	1.0E+00	203	0.72	1.0E+00
M00640-V\$HOXA4_Q2-HOXA4.mat	MB_ABC	697	0.89	1.0E+00	185	0.99	5.9E-01
	MT_ABCDE	1429	0.93	1.0E+00	317	1.04	2.6E-01
M00241-V\$NKX25_02-Nkx2-5.mat	MB_ABC	472	0.77	1.0E+00	116	0.69	1.0E+00
	MT_ABCDE	866	0.72	1.0E+00	188	0.68	1.0E+00
M00360-V\$PAX3_01-Pax-3.mat	MB_ABC	27	1.13	2.9E-01	2	0.39	9.7E-01
	MT_ABCDE	63	1.35	1.3E-02	14	1.67	4.5E-02
M00394-V\$MSX1_01-Msx-1.mat	MB_ABC	241	1.00	5.2E-01	52	0.80	9.5E-01
	MT_ABCDE	528	1.12	6.4E-03	105	1.00	5.2E-01
M00096-V\$PBX1_01-Pbx1a.mat	MB_ABC	541	0.80	1.0E+00	147	0.94	7.7E-01
	MT_ABCDE	997	0.76	1.0E+00	211	0.83	1.0E+00

Enrichment of binding sites predicted by PWMs discovered for Six1, for existing Six1 PWMs and for other homeodomain transcription factors has been illustrated.

^aList of target genomic regions scanned with a given PWM. MB indicates Six1-bound targets in myoblasts, and MT those bound in myotubes. Subgroups of targets are given as letters (e.g. MB_AB refers to the combination of myoblast targets subgroups A and B).

^bNumber of sites corresponding to 'hits' to the PWM, irrespective of their phylogenetic conservation.

^cThe enrichment is given as the ratio of hits found in the indicated target set over those found in a fraction of the ChIP-surveyed sequence space, pro-rated by the length of each group of sequences in base pairs.

^dThe *P*-value represents the cumulative hypergeometric probability subtracted from 1.

^eSame as for ^b, but limited to genomic regions among the top 5% most phylogenetically conserved among 45 vertebrate species.

affinity of the protein to DNA. Accordingly, we designed a library of Cy5-labelled DNA duplexes corresponding to MEF3 site derivatives with a focus on the sequences diverging between Six1_MB+MT and the MEF3 PWM from TRANSFAC (Table 2). We found that, as suggested by the relative degeneracy of our novel MEF3-like matrix, many sequences differing from the TRANSFAC motif can be bound with high avidity by Six1 (Table 2, Myog_mut 07-11). These results further support the ideas that the TRANSFAC PWM is too stringent, and that *de novo* motif more accurately captures the DNA sequence preference of the Six1 transcription factor. Interestingly, we found that the C nucleotide 'suffix' of the motif (TCAG GTTTC) is essential for high affinity binding of Six1 to DNA; mutation to any other nucleotide leads to a sharp decrease in binding (Table 2, Myog_mut 14 to 16, and Figure 3C). This is an important observation considering that a shorter MEF3 element, amputated of this cytosine suffix, has often been described (36–38). Other variants are also indicative of Six1-binding preference. For example, even though the Six1_MB+MT has a high level of degeneracy at positions 2-3 and 6, changing the prefix GGC to GAT (Mut03, at positions 2-3) or position 6 from an A to a T (Mut06) abolishes binding.

Regions outside the homeodomain contribute to DNA-binding sequence specificity

In their large-scale study of mouse homeodomains, Berger *et al.* reported a DNA sequence motif preferred by the Six1 homeodomain (Six1-HD, Figure 1B) that is rather different from the one we report here for the full-length protein (Figure 1A). This has important implications for the possible mode of DNA binding by Six1, and suggests that protein regions outside of its homeodomain may participate in binding site selection. We therefore addressed this question using EMSA, by comparing the affinities of Six1-HD and Six1 for certain DNA sequences.

First, we tested binding of the two proteins on the Myog wild-type and mut02 probes, since the latter conforms to the Berger *et al.* preferred sequence. We observed that while binding of Six1-HD on the mut02 probe occurs with a K_d^{app} of 690 nM, only very weak binding occurred between the homeodomain and the wild-type Myog MEF3 site (K_d^{app} 7700 nM, Figure 4A). This is consistent with the fact that the Berger *et al.* PWM gives a substantial importance to the GTA prefix, which is present in mut02 but absent in the wild-type probe. In contrast, the full-length Six1 protein binds both sequences with comparable affinities (Table 2, 34.7 and 28.7 nM for WT and

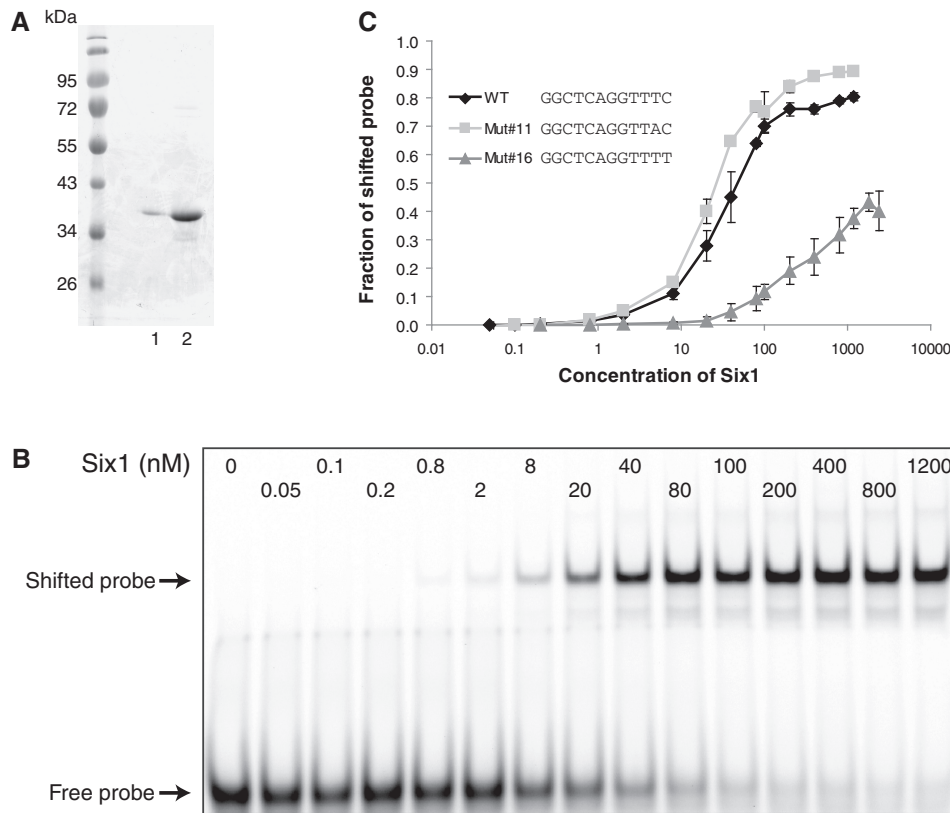


Figure 3. EMSA gels using recombinant Six1 on selected DNA sequences. (A) SDS-PAGE gel stained with coomassie blue, showing 200 ng (lane 1) or 2.0 μ g (lane 2) of Six1 protein. (B) Increasing amounts of Six1 were incubated with fixed amounts of fluorescently labelled double-stranded DNA probes, and electrophoresed on non-denaturing polyacrylamide gels. The gel shown is from an experiment performed with the wild-type Myog MEF3 site. (C) The fluorescent signals corresponding to the free and shifted probes were measured in each lane, and the proportion of shifted probe over shifted free probe were plotted as a function of the concentration of Six1 present in each lane. The concentration of Six1 to reach half maximal binding represents the K_d^{app} value for that probe sequence. The complete set of results is reported in Table 2.

mut02, respectively), in accordance with the fact that our Six1_MB+MT PWM attributes considerably less importance to the prefix sequence (Figure 1).

Secondly, we designed new Myog mutant probes (mut32, mut33 and mut34), which conform to the Berger *et al.* PWM at the prefix (GTA instead of GGC), but they deviate from the wild-type sequence near the 3' end (Table 2). Using these new probes, we again compared Six1-HD and Six1 by EMSA. We found that Six1-HD is mostly unaffected by these mutations, including replacement of the suffix C by a G (mut34, Figure 4B, top row). This is consistent with the reported PWM, which does not dictate any sequence preference at these positions. On the other hand, two of these mutations greatly affect full-length Six1 binding (Figure 4B, bottom row). Based on these substantial differences in DNA sequence preferences between Six1-HD and Six1, we conclude that binding site selection by Six1 involves not only its homeodomain but also regions outside of it.

Computational optimization of the Six1 PWM

As outlined above the novel Six1_MB+MT PWM discovered *de novo* outperforms other existing matrices. However, we postulated that it could still be improved

using bioinformatic approaches, considering a potential 'dilution effect' due to the length of peaks from the ChIP-on-Chip technique (a stretch of 12 bp motif in peaks of 1230 bp on average). We employed for this purpose a novel BSD approach (Bound/Surveyed Discrimination) developed in this study (see Methods for details). Using our ChIP-on-chip data, we sought to determine if changes to the nucleotide weight values within the matrix could be introduced and further increases its specificity and sensitivity to discriminate between the 'bound' and 'surveyed' sets of sequences. In doing this, we made the assumption that matches to the PWM in 'bound' sequences represent biologically true binding sites, while matches to the 'surveyed' regions represent mostly 'not-bound' sites. We note, however, that the 'bound' set of sequences is a subset of the 'surveyed' sequences and so surveyed sequences do contain truly bound sites. This led to the generation of the Six1-opti matrix (Figure 1A, bottom). A receiver-operator characteristics curve analysis of the two PWMs reveals that indeed, the optimized PWM performs better than the original Six1_MB+MT matrix (or the TRANSFAC and Berger PWMs, Figure 5A), since at any given level of specificity, the optimized PWM has enhanced sensitivity. The benefits of using our optimization procedure are unlikely to

Table 2. Summary of EMSA experiments

Name ^a	Sequence ^b	Rationale ^c	K _d ^{app} (nM) ^d
WT			
Myog_WT	gTTAGAGGGG GGCTCAGGTTTC TGTGGCGTTGGC	Wild-type sequence	34.7 ± 7.9
Prefix changes			
Myog_mut01	gTTAGAGGGGGGATCAGGTTTCTGTGGCGTTGGC	Most frequent prefix	28.7 ± 4.3
Myog_mut02	gTTAGAGGGGGTATCAGGTTTCTGTGGCGTTGGC	Prefix conforms to Berger <i>et al.</i>	16.8 ± 2.4
Myog_mut03	gTTAGAGGGGGATTCAGGTTTCTGTGGCGTTGGC	Least frequent prefix	>350
Core changes			
Myog_mut04	gTTAGAGGGGGGCTCGGGTTTCTGTGGCGTTGGC	G is second most frequent after A	40.9 ± 4.1
Myog_mut05	gTTAGAGGGGGGCTCCGGTTTCTGTGGCGTTGGC	C is least frequent nucleotide	63.8 ± 7.7
Myog_mut06	gTTAGAGGGGGGCTCTGGTTTCTGTGGCGTTGGC	T is third most frequent after A	>350
Myog_mut07	gTTAGAGGGGGGCTCAGATTCTGTGGCGTTGGC	Very frequent dinucleotide	52.3 ± 5.6
Myog_mut08	gTTAGAGGGGGGCTCAAGTTTCTGTGGCGTTGGC	Very frequent dinucleotide	34.2 ± 1.8
Myog_mut09	gTTAGAGGGGGGCTCATGTTTCTGTGGCGTTGGC	Very frequent dinucleotide	29.4 ± 2
Myog_mut10	gTTAGAGGGGGGCTCAAATTTCTGTGGCGTTGGC	Very frequent dinucleotide	32.4 ± 2.1
Myog_mut11	gTTAGAGGGGGGCTCAGGTTCTGTGGCGTTGGC	A is second most frequent after T	24.2 ± 1.3
Myog_mut12	gTTAGAGGGGGGCTCAGGTTCTGTGGCGTTGGC	Rare nucleotide	81.6 ± 10
Myog_mut13	gTTAGAGGGGGGCTCAGGTTGCTGTGGCGTTGGC	Rare nucleotide	72.2 ± 3.1
Suffix changes			
Myog_mut14	gTTAGAGGGGGGCTCAGGTTTATGTGGCGTTGGC	Very rare nucleotide	>350
Myog_mut15	gTTAGAGGGGGGCTCAGGTTTGTGTGGCGTTGGC	Very rare nucleotide	>350
Myog_mut16	gTTAGAGGGGGGCTCAGGTTTGTGTGGCGTTGGC	Very rare nucleotide	>350
Multiple changes			
Myog_mut32	gTTAGAGGGGGTATCAGGGTTCTGTGGCGTTGGC	mut02 with change near 3' end	>350
Myog_mut33	gTTAGAGGGGGTATCAGGTTCTGTGGCGTTGGC	mut02 with change near 3' end	130 ± 50
Myog_mut34	gTTAGAGGGGGTATCAGGTTTGTGTGGCGTTGGC	mut02 with change at 3' end	>350
Tests of Six1-opti PWM prediction of binding sites (changes to the core, suffix and/or prefix)			
Myog_mut17	gTTAGAGGGGATCTCATATTACTGTGGCGTTGGC	Unique to Six1-opti	25 ± 3.3
Myog_mut18	gTTAGAGGGGAGATCACATTTCTGTGGCGTTGGC	Unique to Six1-opti	39.4 ± 2.1
Myog_mut19	gTTAGAGGGGAGATCACATTACTGTGGCGTTGGC	Unique to Six1-opti	48.2 ± 1.1
Myog_mut20	gTTAGAGGGGTTCTCAAATTTACTGTGGCGTTGGC	Unique to Six1-opti	46.7 ± 1.1
Myog_mut21	gTTAGAGGGGGTATAAAATTTCTGTGGCGTTGGC	Unique to Six1-opti	74.3 ± 9
Myog_mut22	gTTAGAGGGGAGCTCTGGTTACTGTGGCGTTGGC	Unique to Six1-opti	85.4 ± 10
Myog_mut23	gTTAGAGGGGAGATCAGGTTTATGTGGCGTTGGC	Unique to Six1-opti	69.3 ± 8.2
Myog_mut24	gTTAGAGGGGGGGTCAAGTGTACTGTGGCGTTGGC	Unique to Six1-opti	>350
Myog_mut25	gTTAGAGGGGATATCAGATATCTGTGGCGTTGGC	Unique to Six1-opti	29.1 ± 5.3
Myog_mut26	gTTAGAGGGGGTATCAAATAACTGTGGCGTTGGC	Unique to Six1-opti	10.8 ± 3
Myog_mut27	gTTAGAGGGGGCCCTCGGGTTTCTGTGGCGTTGGC	Unique to Six1_MB + MT	>350
Myog_mut28	gTTAGAGGGGGGATCGGGTTCTGTGGCGTTGGC	Unique to Six1_MB + MT	42.4 ± 7.6
Myog_mut29	gTTAGAGGGGGTTTCAGGTTCTGTGGCGTTGGC	Unique to Six1_MB + MT	75.4 ± 8.3
Myog_mut30	gTTAGAGGGGGTCTCGGTTTCTGTGGCGTTGGC	Unique to Six1_MB + MT	>350
Myog_mut31	gTTAGAGGGGGATTCAGGTTTCTGTGGCGTTGGC	Unique to Six1_MB + MT	>350

^aMyog_WT is the Myog probe with wild-type MEF3 consensus in the center. Myog_mut01 to Myog_mut30 are probes with various mutations in the MEF3 consensus. Myog_mut31 is the same probe as Myog_mut03 cited as a different rationale.

^bMutated nucleotides in the MEF3 consensus are highlighted in black. The lower cap 'g' nucleotide was added for fluorescent labelling purposes. The natural sequence would be a 'C' at that position.

^cRationales to choose the corresponding sequences are listed. Mut01 the most frequent MEF3 sequence found in Six1_MB and Six1_MT binding data. Mut02 contains TA at position 2 and 3, which is found in the Berger *et al.* study. Myog03 has the least frequency of dinucleotides (AT) at position 2 and 3. The MEF3 in Mut05 is found in the Myod core enhancer region. Mut07 to 10 are selected with different dinucleotide combination at position 7 and 8. Mut04, 06, and 11 to 16 are chosen based on the frequency of the nucleotide at a certain position. Mut17 to 26 are MEF3 sequences found only using Six1-opti MEF3 motif. Mut27 to 31 are MEF3 sequences found only using Six1_MB+MT MEF3 motif. Of note, Mut03 and Mut31 contain the same MEF3 sequence.

^dDissociation constant (K_d^{app}) and standard error of mean are calculated for each probe based on at least three independent experiments. >350 nM, not accurately determined due to very weak binding.

originate from having started with a poor initial PWM generated by Amadeus, since PWMs obtained by MEME-ChIP (39) and Weeder (40), two popular motif finding programs, did not perform any better than the one obtained with Amadeus (Six1_MB + MT).

The improved Six1-opti PWM allowed us to identify new potential binding sites that may have been missed using the original Six1_MB + MT matrix. At similar sensitivity and specificity (~60% and ~73%, respectively), the Six1-opti matrix identified 322 novel putative binding sequences (occurring a total of 505 times among

our Six1-bound genomic loci) that were missed with the starting matrix (Figure 5B). On the other hand, the starting matrix identified only 11 sequences (17 occurrences) not found by the optimized PWM. It is also noteworthy to consider these results in terms of putative target gene identification, since this is a common use of PWM scanning programs. Using the Six1-opti PWM would allow to identify 1051 target genes (i.e. sequences with at least one hit to the PWM), while the original matrix would only recognize 747 of them. This represents a 40.7% increase in sensitivity.

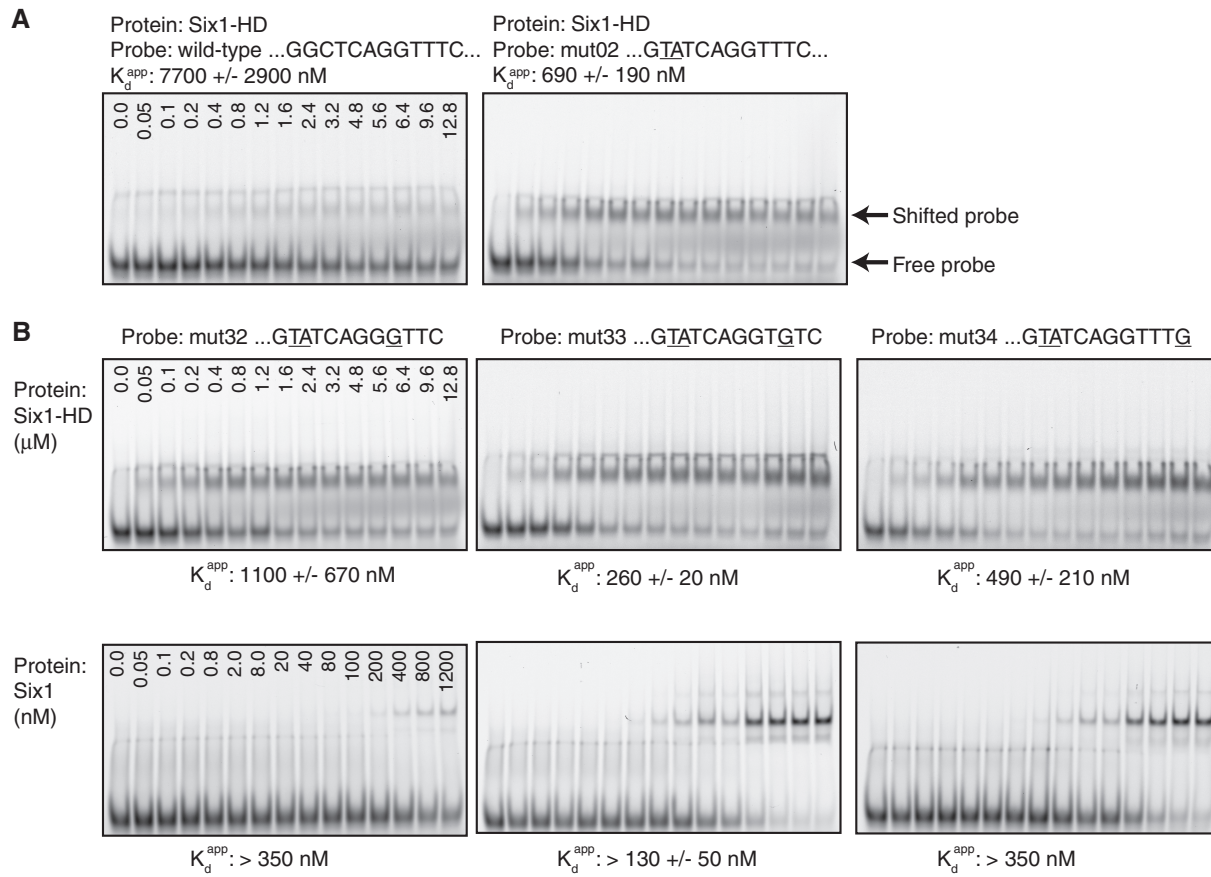


Figure 4. Substantial differences in DNA sequence selectivity between Six1-HD and Six1. (A) EMSA gels were performed with increasing amounts of Six1 homeodomain (Six1-HD) using the Myogenin WT probe (left), or the mut02 probe (right), which conforms to the consensus reported by Berger *et al.* using protein-binding microarrays. (B) EMSA experiments performed with the Six1-HD (top row) or Six1 (bottom row) proteins, on three derivatives of the mut02 probe (mutated positions are underlined, compared with the WT probe). Note that because the Six1-HD has a relatively low affinity for DNA in these assays, the amounts of protein used are higher than those used for the full-length Six1 protein. The K_d^{app} values (all in nanomolars) for each protein on each probe are given underneath the respective gel images.

To determine if Six1 binds the new sequences identified by the optimized matrix, we used EMSA to assess the binding affinity of Six1 to them. We selected 10 novel sites unique to Six1-opti and 5 sites uniquely identified with Six1_MB+MT for validation (Table 2). Interestingly, 9 out of 10 sites unique to Six1-opti have comparable affinity to the Myog probe, whereas only 2 out of 5 sites unique to Six1_MB+MT are bound by Six1 with a measurable K_d^{app} , suggesting that our optimization approach improved the discriminatory power of our Six1 PWM. Finally, as an ultimate test of the relevance of the binding site predictions made by the optimized PWM, we repeated the search on an independent set of Six1 target loci that are bound by Six1 24 hours after the onset of myoblast differentiation, but not in myoblasts or myotubes (see materials and methods). As expected, with 49 common binding sequences out of 187 Six1-bound regions, 23 binding sites were identified solely with the Six1-opti PWM, while only 2 binding sequences were uniquely defined with Six1_MB+MT (Figure 5C). This confirms the superiority of the optimized PWM over the original matrix.

DISCUSSION

Using bioinformatic analysis of the genomic binding profile of Six1 in muscle cells, we have found that this transcription factor has the ability to bind to a much broader range of DNA sequences than anticipated. While the previously reported MEF3 sequence motif is clearly enriched among genomic regions bound by Six1, other sequences that diverge substantially from this DNA element are also found preferentially at Six1-bound loci. We used an *in vitro* binding assay with recombinant Six1 to confirm that the protein indeed binds to these divergent sequences with high affinity, thereby ruling out an artifactual effect of the *de novo* sequence discovery algorithm we used. The novel PWM should prove to be useful to studies that employ TF target site prediction to elucidate the structure of regulatory networks (41). For Six1, a TF involved in the genesis of multiple tissue types, a more precise DNA binding motif may contribute to discovering novel direct targets, elucidating composite regulatory networks and rationalizing its implication in diseases such as breast cancer or Branchio-oto-renal syndrome (7,42,43).

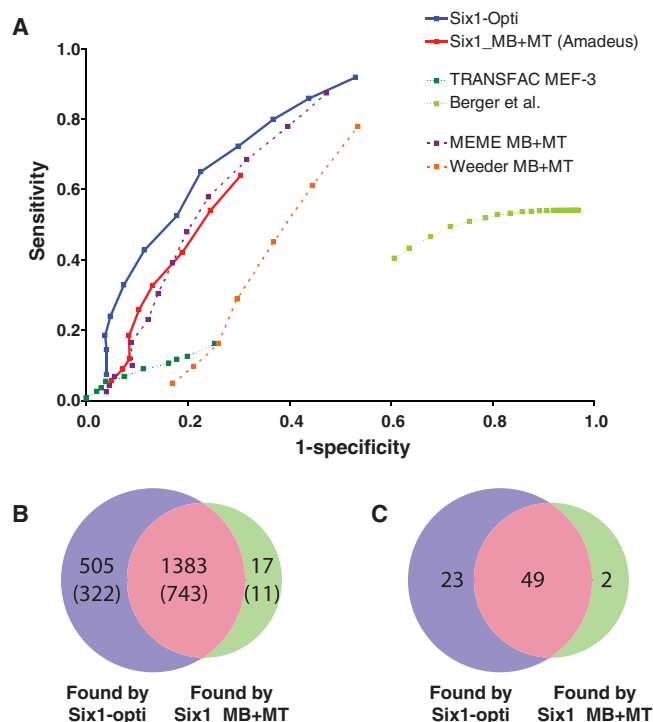


Figure 5. Performance comparison of the Six1_MB+MT and Six1-opti PWMs. (A) ROC curves. The y-axis is the sensitivity and the x-axis is the 1-specificity value. Performances of the original TRANSFAC MEF3 and Berger *et al.* PWMs for Six1 are also given for comparison. (B) Venn diagram indicating the number of hits to each PWM, and their overlap, at their respective optimal thresholds, among loci targeted by Six1 in myotubes. Numbers in parentheses are the number of unique sequences (one sequence can occur more than once). (C) Results for the comparison between predictions made with the Six1-opti and Six1_MB+MT PWMs, on sequences bound by Six1 only at 24 hours post-differentiation.

The results of our analyses provide useful information that may guide structure-function studies of Six1-DNA interactions. The Six family homeodomains, including that of Six1, belong to the K50 class of homeodomains, as they differ from the majority of other homeodomains classes by the change of a key DNA-binding residue, asparagine at position 50 of the HD, to a lysine. The importance of that residue in encoding DNA-binding specificity has been highlighted by a number of biochemical and structural studies (44–48). Other residues implicated in DNA-binding specificity or stability, for example those within the N-terminal arm, differ between Six and other homeodomains or within Six family members [discussed in (49–51)]. Yet, it remains to be determined precisely how DNA-binding specificity is established by Six family TFs, and by Six1 in particular. The various family members have been shown to bind to different sequences: Six3 and Six6 can bind to the canonical TAAT sequence that is bound by most homeodomain TFs, while Six1/2/4/5 have all been shown to be able to bind to sequences resembling the MEF3 element [reviewed in (11)]. Some Six proteins can also bind DNA as heterodimers with Eya family proteins, and these interactions are thought to enhance their affinity for

DNA (7,51). Most TFs, including several homeodomain factors, bind to DNA *in vivo* as homo- or heterodimers, and stabilization of such oligomeric states has been put forth as one possible mechanism to explain the influence of Eya proteins in regulating Six proteins (51). In our experiments, one predominant oligomeric state of Six1 was detected in EMSA; however, the oligomeric state of Six1 in solution remains unclear (i.e. whether Six1 binds DNA as a monomer or as a multimer in our assays), although at very high protein concentrations slower migrating complexes became visible on EMSA gels (data not shown). We cannot tell at this point if these species represent aggregates or functionally and physiologically relevant oligomers. Further analysis of the precise mode of DNA recognition by Six1, the influence of interaction partners and possible involvement of oligomeric states, should help us understand the function of this protein and rationalize its implication in diseased states.

ChIP-on-chip analysis captures a snapshot of protein-DNA interactions as they occur in live cells, and although chromatin is immunoprecipitated with an antibody against Six1, putative DNA-binding partners of Six1 were possibly involved in the interactions we have discovered. It would be interesting to determine the genomic binding profiles of Eya proteins in myoblasts to see if indeed these proteins tend to bind DNA along with Six1, and if co-binding with Eya proteins alters DNA-binding preferences in any way. However, these experiments may prove excessively difficult to perform *in vivo*: while it is possible to ChIP a specific Eya-Six chromatin-bound complex (using sequential ChIP assays), it would be much more challenging to devise a way of pulling-down only Six1 chromatin complexes that do not contain Eya proteins. Interestingly, however, the results of our *in vitro* experiments corroborate those of our ChIP-on-chip experiments: the variety of sequence motifs enriched among Six1-bound loci is reflected in EMSA using DNA probes and Six1 alone. This leads us to postulate that protein domain(s) within Six1 itself are the main determinant of DNA sequence selection by Six1 *in vivo* (at least in muscle cells, where our analysis was done). As shown by others and noted above, Eya binding could influence predominantly the binding affinity rather than sequence selectivity (7,51).

The PWM we have generated is fairly close to the initial MEF3 PWM reported more than 15 years ago. The stringency (and therefore low sensitivity) of the TRANSFAC MEF3 PWM comes from the fact that it was derived from the DNA sequences of only five sites within muscle gene promoters. One can easily imagine that with larger sampling, the PWM would perform better at predicting Six1-binding sites.

The significant discrepancy between our data and those reported by Berger *et al.* was more puzzling. The authors used protein-binding microarrays to determine the sequence specificity of the mouse Six1 homeodomain and reported a Six1 PWM that has only limited resemblance to our PWM or to the TRANSFAC MEF3 element (4). We reason that these differences originate from the fact that only the homeodomain region of Six1 was studied, whereas our ChIP-on-chip and binding studies were

performed with full-length Six1. Indeed, we confirmed with EMSA experiments that Six1-HD exhibits a DNA sequence preference that is in line with what Berger *et al.* reported, but that is substantially different from that exhibited by the full-length protein. We therefore conclude that regions outside of the Six1 homeodomain participate in DNA binding, either through direct DNA contacts or indirectly, perhaps by enabling structural stabilization.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–2.

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