# Novel Flanking DNA Sequences Enhance FOXO1a DNA Binding Affinity but Do Not Alter DNA Bending[†]

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*Received December 20, 2007; Revised Manuscript Received April 30, 2008*

ABSTRACT: FOXO1A, a member of the forkhead winged-helix family of proteins is a transcription factor with proapoptotic activities and plays a significant role in insulin and growth factor signaling. As such, FOXO1A is insulin responsive and binds to the insulin response element (IRE). However, multiple forkhead family members with diverse biological functions are also known to bind to the IRE. Therefore, additional DNA sequence elements may be required to provide increased binding affinity and specificity for FOXO1A. We have used the systematic evaluation of ligands by exponential enrichment (SELEX) to systematically identify additional DNA sequences important for FOXO1A binding. We demonstrate for the first time that, in addition to the IRE, two additional sequence elements are important for maximal FOXO1A binding: (1) the reverse complement (5′-GT(A/C)AACA-3′) and (2) the flanking sequence (5′-ACAACA-3′). Although these additional elements do not contribute to the FOXO1A-induced DNA bending angle of 120°, the presence of these additional elements does increase the affinity of FOXO1A DNA binding nearly 9-fold through a 1-to-1 binding stoichiometry. The increased binding affinity subsequently enhances the ability of FOXO1A to activate transcription from a luciferase reporter construct and from promoter regions of endogenous genes known to be direct transcriptional targets of FOXO1A.

FOXO1a is a member of the FOXO family of winged-helix transcription factors, which includes AFX (FOXO4) and FKHR-L1 (FOXO3a). FOXO1A was first identified in humans through its fusion to the myogenic transcription factor Pax3 as a result of the t(2;13) chromosomal translocation, the hallmark of the childhood solid muscle tumor alveolar rhabdomyosarcoma (*1, 2*). FOXO1A is homologous to DAF-16, a transcription factor that functions downstream of insulin-like signals and controls the life-span extension in *Caenorhabditis elegans* (*3*). In *Drosophila*, forkhead is essential for embryonic gut development where its absence leads to inhibition of head involution and the anterior and posterior regions of gut undergo homeotic transformations to spiked head structures or "forks" (*4*). In mammals, the FOXO family of proteins is known to control various cellular processes such as apoptosis, cell cycle control, and the transcription of intercellular signaling molecules such as hormones (*3, 5, 6*). The winged-helix proteins are thus evolutionarily conserved and play important roles in embryogenesis, development, and aging (*7*).

FOXO1A is expressed ubiquitously, with the highest levels of expression in skeletal muscle, ovaries, pancreas, and liver and with barely detectable levels in the colon, brain, and kidney (*8–10*). Consistent with its broad tissue distribution, FOXO1A is involved in a variety of tissue-specific pathways. However, its best studied function is its role in the insulin response whereby FOXO1A binds to the insulin response element (IRE)[1] in the promoter regions of genes such as the insulin-like growth factor binding protein-1 (IGFBP-1), glucose-6-phosphatase (G6Pase), pyruvate dehydrogenase kinase-4 (PDK4), and alkaline phosphatase (ALP) (*3, 11–17*). FOXO1A inhibits the activity of insulin and insulin-like growth factors (IGFs) and thus is an important regulator of glucose homeostasis (*3, 11–14*). In addition to its role in the insulin response pathway, FOXO1A is expressed in the vasculature of developing mice, and disruption of this gene leads to lethality due to vascular defects (*5*). During myogenic differentiation, FOXO1A has both positive and negative effects by first controlling the rate of myotube fusion (*18*) and then impairing the glycemic control in developed skeletal muscles (*19*). Finally, in addition to a wide variety of cellular functions, FOXO1A has been associated as a tumor suppressor gene in prostate cancer (*20*).

The involvement of FOXO1A in multiple different tissue-specific pathways would suggest that there must be some degree of specificity in the ability of FOXO1A to recognize and bind to the promoter elements that it regulates. In addition, multiple forkhead family members with diverse biological functions, such as the hepatocyte nuclear factor 3

[†] Funding for this work was provided by Grant 1-P20-RR020152-01 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), the Louisiana Cancer Research Consortium (LCRC) Pilot Incentive Program, and the LCRC Immediate Response Fund.

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[1] Abbreviations: GST, glutathione *S*-transferase; PCR, polymerase chain reaction; IPTG, isopropyl *β*-D-thiogalactopyranoside; SELEX, systematic evaluation of ligands by exponential enrichment; EMSA, electromobility shift assay; PBS, phosphate-buffered saline; MWCO, molecular weight cutoff; $R_F$, relative mobility; IRE, insulin response element; IGFBP-1, insulin-like growth factor binding protein-1; G6Pase, glucose-6-phosphatase; ALP, alkaline phosphatase; PDK4, pyruvate dehydrogenase kinase-4; LB, Luria broth; SDS−-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi702495m CCC: $40.75 
Published on Web 06/07/2008

(FOXA2), FOXF1, FOXG1, FOXJ1, and FOXP1, have all been demonstrated to recognize and bind to the IRE (*21–25*). Taken together, this information suggests that, in addition to the IRE, additional DNA sequences may be required to provide increased affinity of FOXO1A for its recognition sequences. Consistent with this hypothesis, limited inconclusive studies have suggested that additional DNA sequence elements may in fact be required for maximal FOXO1A binding (*22, 26–29*). Therefore, in this report, we perform a systematic analysis to identify these additional DNA sequences. We demonstrate for the first time that, in addition to the IRE, two DNA sequence elements are required for maximal FOXO1A DNA binding: (1) the reverse complement sequence [5′-GT(A/C)AACA-3′] and (2) the flanking sequence [5′-ACAACA-3′]. Although these additional DNA sequence elements do not contribute to the FOXO1A-induced DNA bending, these elements do increase the affinity of FOXO1A DNA binding and subsequently the ability of FOXO1A to activate transcription from a luciferase reporter construct and from endogenous promoters known to be directly regulated by FOXO1A.

## EXPERIMENTAL PROCEDURES

*Cloning of Glutathione S-Transferase (GST) Tagged FOXO1A.* The full-length FOXO1A coding region was PCR amplified using the pBabe-MSCV-puro-Avi-FOXO1A as a template using primers designed to incorporate unique *Bam*HI and *Xho*I restriction enzyme sites to facilitate directional cloning into the pGEX-5X-1 expression vector (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), which fuses GST onto the amino terminus of FOXO1A. The resulting amplified FOXO1A DNA was purified by gel extraction and TA-cloned into the pCRII cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. The pCRII-FOXO1A vector was digested with *Bam*HI and *Xho*I; the excised FOXO1A fragment was gel purified and subsequently cloned into pGEX-5X-1 using a rapid ligation kit (Roche, Indianapolis, IN) according to the manufacturer's specifications. DNA sequence analysis confirmed that FOXO1A was in-frame with GST and that no additional mutations were introduced by PCR.

*Expression and Purification of GST-FOXO1A.* LB medium containing chloramphenicol (34 mg/mL) and ampicillin (50 mg/mL) was inoculated overnight at 37 °C with Rosetta (DE3) (pLysS) bacteria previously transformed with the pGEX-5X-1-FOXO1A vector. The following day, a protease inhibitor cocktail (Roche, Indianapolis, IN) was added to the bacterial culture to a final 1× concentration after which the expression of protein was induced by the addition of 0.1 mM isopropyl $\beta$-D-thiogalactopyranoside (IPTG) (EMD Chemicals, Gibbstown, NJ) for 1 h with continued shaking at 37 °C. After induction, the cells were harvested by centrifugation, washed with 50 mL of ice-cold 1× PBS, and lysed with CelLytic express (Sigma, St. Louis, MO) and 1× protease inhibitor cocktail according to the manufacturer's specifications. The cell debris was removed by centrifugation, and the GST-FOXO1A was purified using MagneSphere GST glutathione magnetic resin (Promega Corp., Madison, WI) as previously described (*30, 31*). Protein bound to the resin and prepared in this manner was then used for the SELEX procedure. For the electromobility shift analysis (EMSA) reactions, GST-FOXO1A was eluted from the resin in 600 $\mu$L of 200 mM Tris-HCl (pH 8.8) containing 100 mM reduced glutathione (Sigma, St. Louis, MO) and incubated at 4 °C with gentle rotation overnight. The next day, the eluted protein was dialyzed extensively against 1× PBS, and the resulting purified protein was analyzed by separation on an 8% SDS−PAGE gel and was found to be greater than 90% pure by Coomassie staining (data not shown).

*Systematic Evaluation of Ligands by Exponential Enrichment (SELEX).* A 72 base-pair random oligonucleotide library was synthesized with the sequence 5′-CGCGGATCCTG-CAGCTCGAG-$(N)_{30}$-CTCTAGAAGCTTGTCGAC-3′, where the N denotes the random insertion of A, T, G, or C in equal proportion. This pool of "randomers" was made double stranded with the use of 20 $\mu$M randomers and 100 $\mu$M each of the forward primer (5′-CGCGGATCCTGCAGCTCGAG-3′) and the reverse primer (5′-CTCTAGAAGCTTGTCGAC-3′) using ExTaqHS DNA polymerase (TakaRa Biosciences, Shiga, Japan) in a 100 $\mu$L PCR reaction according to the manufacturer's specifications. Amplification was performed for three cycles at 94 °C for 1 min, 58 °C for 30 s, and 72 °C for 30 s followed by a final extension of 7 min at 72 °C. The 72 base-pair double-stranded randomer pool was then separated on a 2% agarose gel and purified by gel extraction. Approximately 300 ng (10 $\mu$L) of the eluted double-stranded oligonucleotides was used for the first round of binding and selection. Each round of binding was carried out in a 100 $\mu$L volume containing 10 $\mu$L of GST-FOXO1A protein (approximately 0.5 $\mu$g) prepared as described above. GST alone was immobilized in a similar manner and used in parallel binding reactions as a negative control. Samples were gently agitated at room temperature for 30 min in FOXO1A-SELEX binding buffer (25 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 10 mM $MgCl_2$, and 5% glycerol) containing 5 $\mu$g of poly(dI·dC) and 5 $\mu$g of bovine serum albumin. Nonbound oligonucleotides were removed by washing the resin four times with FOXO1A-SELEX binding buffer, after which the resulting washed resin was resuspended in 50 $\mu$L of water. The bound oligonucleotides were eluted from the protein by boiling for 5 min, and 10 $\mu$L of the supernatant was used as a template for PCR amplification using 100 $\mu$M each of the forward and reverse primers in a 50 $\mu$L reaction for 15 cycles using the cycle conditions as described above. The number of cycles for amplification was determined by visualization of 5 $\mu$L of the PCR reaction on a 2% agarose gel. If a 72 base-pair band was not visible, five additional PCR cycles were performed. If a 72 base-pair band was visible, the entire reaction was then separated by 2% agarose gel electrophoresis and gel extracted as described above. Ten microliters of the eluted fraction was used for subsequent selection. Nine rounds of binding and amplification were performed in the manner just described. The PCR-amplified oligonucleotides from rounds 6, 7, 8, and 9 were separated by 2% agarose gel, gel extracted as described above, cloned into the pCRII TA cloning vector (Invitrogen, Carlsbad, CA), and sequenced.

*Electrophoretic Mobility Shift Assay (EMSA).* The SELEX clones from rounds 6, 7, 8, and 9 were radioactively labeled by PCR incorporation of [$\alpha$-$^{32}$P]dGTP. All binding reactions were carried out at room temperature for 30 min with or without bacterially expressed GST-FOXO1A protein in

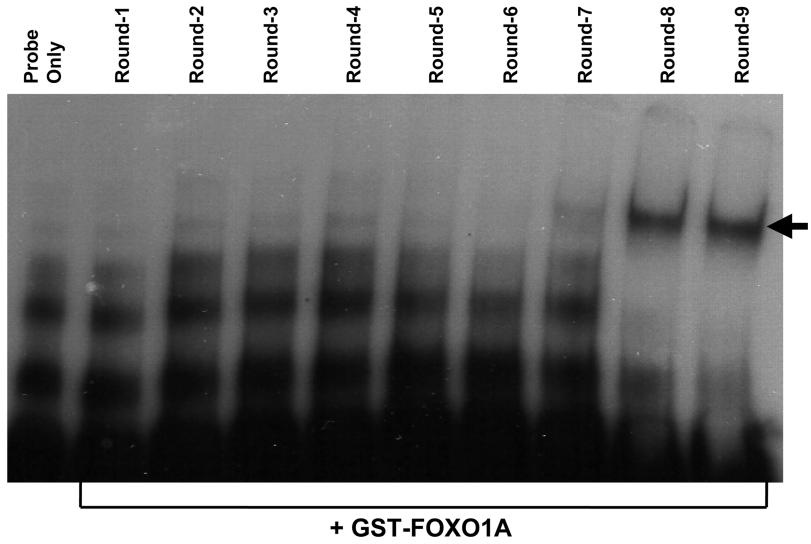


FIGURE 1: Binding analysis of SELEX rounds 1−9. Electromobility shift assay (EMSA) demonstrating the binding of the enriched and PCR amplified oligonucleotides from each round of the SELEX to bacterially expressed and purified GST-FOXO1A. The arrow indicates the specific shift observed in rounds 7, 8, and 9. The enriched oligonucleotides from each round were labeled by PCR incorporation using [α-$^{32}$P]dGTP and bound to 0.85 μg of GST-FOXO1A. The binding reactions were carried out at 25 °C and the protein:DNA complexes were separated by 6% Tris−glycine−glycerol nondenaturing gel at 4 °C, as described in the Experimental Procedures.

EMSA binding buffer [20 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 10% glycerol, 200 mM NaCl, 1 μg/μL poly(dI·dC)]. The conditions for individual experiments were modified as follows:

(1) To determine FOXO1A-specific binding in each round of SELEX (Figure 1), the binding reactions were carried out in a 30 μL volume using a constant concentration of GST-FOXO1A (98 nM) and a constant volume of labeled probes (5 μL).

(2) To determine the saturating levels of bound GST-FOXO1A (Figure 3), the reactions were carried out in a 30 μL total volume using a constant concentration of probe and the empirically determined saturating concentration of GST-FOXO1A (data not shown) for clone 9 (20 nM probe, 200 nM protein), clone 14 (20 nM probe, 300 nM protein), and clone 5 (100 nM probe, 1000 nM protein).

(3) The qualitative EMSA reactions (Figure 5) were performed in a 40 μL binding reaction using constant concentrations of labeled probe (35 nM) and protein (98 nM).

(4) The EMSA reactions for the Scatchard analysis (Figure 6) were carried out in a 30 μL binding reaction with a constant concentration of protein (50 nM) and increasing concentrations of clones 9 and 14 (0.5−100 nM) and clone 5 (40−450 nM).

For all reactions, the bound DNA complexes were analyzed as previously described (*30*). The band correlating to the specific shift was excised from the gel and quantified by scintillation counting.

*Calculation of FOXO1A-Induced DNA Bending.* The SELEX DNAs from clone 9 (three binding elements), clone 14 (two binding elements), and clone 5 (one binding element) were cloned into the *Sal*I binding site of the pBend3 cloning vector (*32*). The resulting clones were digested with *Mlu*I, *Bgl*II, *Spe*I, *Dra*I, *Pvu*II, *Stu*I, *Nru*I, or *Bam*HI. These restriction digests will generate a series of fragments of equal length that have the FOXO1A binding element placement varied from one end of the fragment to the other (Figure 4A). The resulting fragments were 5′-end-labeled using [γ-$^{32}$P]ATP and T4 polynucleotide kinase. Binding reactions were carried out as described above in a total of 20 μL using 100000 cpm of labeled DNA fragments and 1 μg of GST-FOXO1A. The free and bound DNA was resolved and visualized as described above. To calculate the bending angle, the relative mobility ($R_F$) of the FOXO1A:DNA complexes was determined relative to the free probe, and the average relative mobility from four independent experiments was graphed as a function of the distance in base pairs of the center of the identified FOXO1A binding site from the 5′-end of the nonconding strand of the DNA fragment. The resulting data were then fit to the second order parabolic equation $y = ax^2 + bx + c$, and the absolute bending center was determined by setting $dy/dx = 0$ and solving for $x$. The magnitude of the protein-induced DNA bending was then calculated using a previously described semiempirical method (*33*).

*Transcriptional Assays.* To determine if FOXO1A is able to activate transcription from the three different FOXO1A binding elements, clones 9, 14, and 5 were independently cloned into the pGL3-Basic firefly luciferase reporter construct (Promega, Madison, WI). HepG2 human hepatoma cells ($2 \times 10^6$) were plated in 60 mm dishes and transfected the following day by the Fugene6 method (Roche, Indianapolis, IN), according to the manufacturer's specifications. The Fugene6/ DNA precipitate consisted of a combination of 200 ng of pcDNA3.1-FOXO1A, 1 μg of either pGL3-Basic or pGL3-Basic containing clone 9, clone 14, or clone 5, and 500 ng of the runilla luciferase (RL) control reporter gene under control of the thymidine kinase promoter (pRL-TK, Promega). Analysis of the luciferase activity was performed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's specifications and light output recorded on a Veritas microplate luminometer. All samples were corrected for the transfection efficiency using the RL activity.

*Real-Time Reverse Transcriptase (RT) PCR.* HepG2 cells were transiently transfected with either the pcDNA3.1-FOXO1 expression vector or the pcDNA3.1 empty vector negative control, as described above. Eighteen hours post-

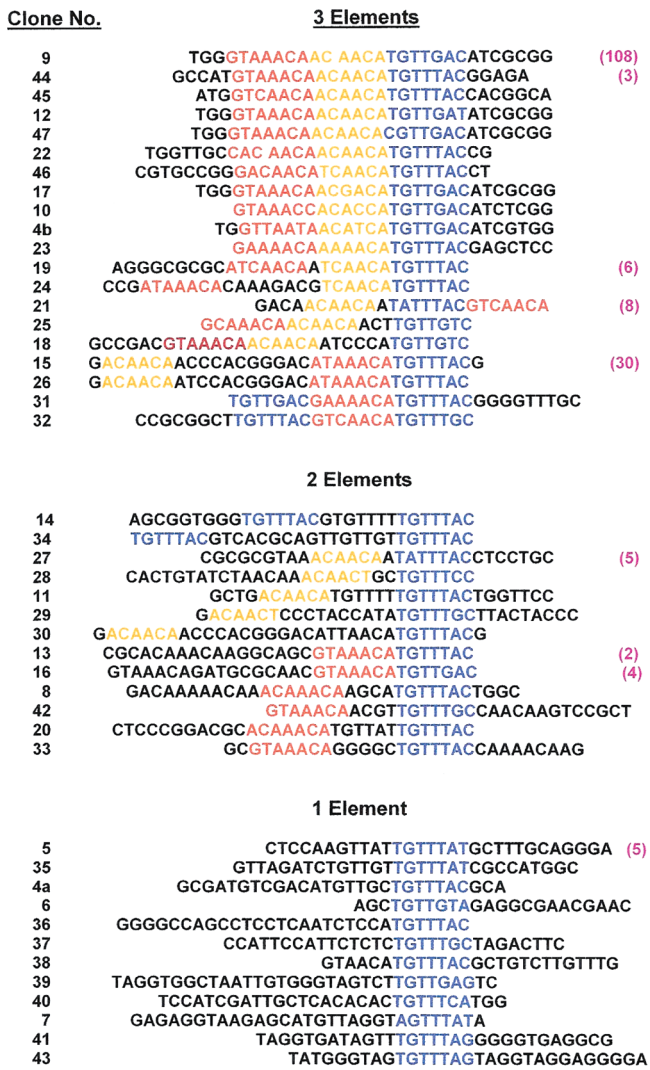


FIGURE 2: Identification of novel flanking FOXO1A DNA binding sequences. Forty-five individual sequences were aligned with respect to the IRE (*3*), (5′-TGTT(T/G)AC-3′ in blue). Through this alignment we identified two additional highly conserved sequences: (1) the reverse complement sequence (5′-GT[A/C]AACA-3′ in red) and (2) the flanking sequence (5′-ACAACA-3′ in yellow). The numbers in parentheses represent the number of times the individual clone was isolated in the SELEX procedure.

transfection, the medium was changed and incubated for 6 h to recover the cells, after which FOXO1A was maximally activated by the addition of IL-6 (25 ng/mL). After 24 h, the cells were harvested, mRNA was isolated, and real-time RT-PCR was performed on 30 ng of mRNA using primers specific for ceruloplasmin, IGFBP-1, or G6Pase. Real-time RT-PCR and the subsequent fold FOXO1A-dependent gene expression were determined as previously described (*34*).

## RESULTS

*Identification of Novel FOXO1A DNA Binding Elements.* In order to identify novel flanking FOXO1A DNA binding elements, we created a random oligonucleotide library comprised of a 72 base-pair oligonucleotide with the central 30 base pairs being completely random with respect to the insertion of A, T, G, or C, which is standard for the SELEX procedure and has been successfully used by others (*35*–*39*). Bacterially expressed and purified GST-FOXO1A fusion protein was then used to select specific sequences from the

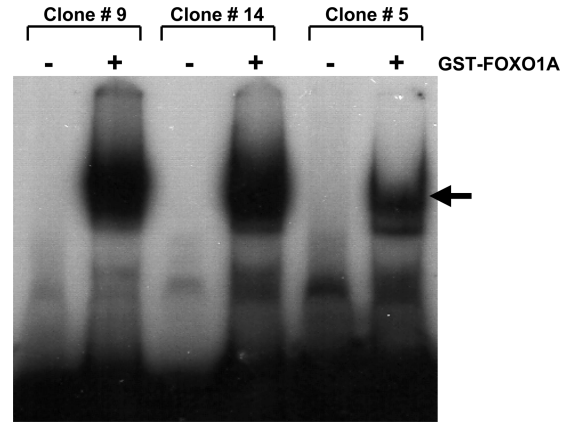


FIGURE 3: Oligonucleotides with three, two, or one DNA sequence element bind equal number of molecules of GST-FOXO1A. Representative electromobility shift assay demonstrating the binding of bacterially expressed and purified GST-FOXO1A to clones 9 (three DNA sequence elements), 14 (two DNA sequence elements), and 5 (one DNA sequence element), with sequences as illustrated in Figure 2. The probes were labeled by PCR incorporation with [$\alpha$-$^{32}$P]dGTP. A constant concentration of radiolabeled probe (clones 9 and 14, 20 nM; clone 5, 100 nM) was bound to saturating concentrations of bacterially expressed and purified GST-FOXO1A (clone 9, 200 nM; clone 14, 300 nM; clone 5, 1000 nM). The saturating protein concentrations were empirically determined for each individual clone (data not shown). The binding reactions were carried out at 25 °C, and the protein:DNA complexes were separated by 6% Tris−glycine−glycerol nondenaturing gel at 4 °C, as described in the Experimental Procedures. The arrow indicates the shift observed in the presence of the protein.

random oligonucleotide library using the SELEX procedure, as described in the Experimental Procedures. We carried out nine rounds of binding selection and amplification and confirmed the absence of nonspecific amplification by using GST only as a negative control in parallel reactions (data not shown). The selected and amplified oligonucleotides from rounds 6, 7, 8, and 9 were subsequently cloned into the pCRII TA cloning vector. After nine rounds of selection and amplification, a standard EMSA was performed to confirm and validate the selection of the SELEX procedure. We observed a faint shift of the labeled probes in the presence of protein in round 7 with an increase in the intensity of the bound DNA in rounds 8 and 9 (Figure 1). This result directly confirms that our SELEX procedure provided a gradual and progressive selection and amplification of oligonucleotides capable of specifically binding GST-FOXO1A.

The amplified and selected oligonucleotides from rounds 6−9 were then cloned into the pCRII TA cloning vector and sequenced. Out of the 299 sequenced oligonucleotides, 204 (68%) contained the insulin response element (IRE) (*3*), which served as an internal positive control to validate our SELEX procedure. In addition, we found that of the 204 IRE-containing sequences, several of these sequences were isolated multiple times. In particular, clone 9 was isolated 108 individual times throughout rounds 7−9, indicating that this clone may contain the optimal sequence for FOXO1A binding (Figure 2). Because we were interested in identifying novel DNA sequences that flanked the IRE, we aligned the 45 unique sequences relative to this sequence (Figure 2). Using this alignment, we identified two additional sequence elements that were highly conserved throughout a majority of the clones. In addition to the IRE, we also identified a reverse complement sequence, which is an exact reverse

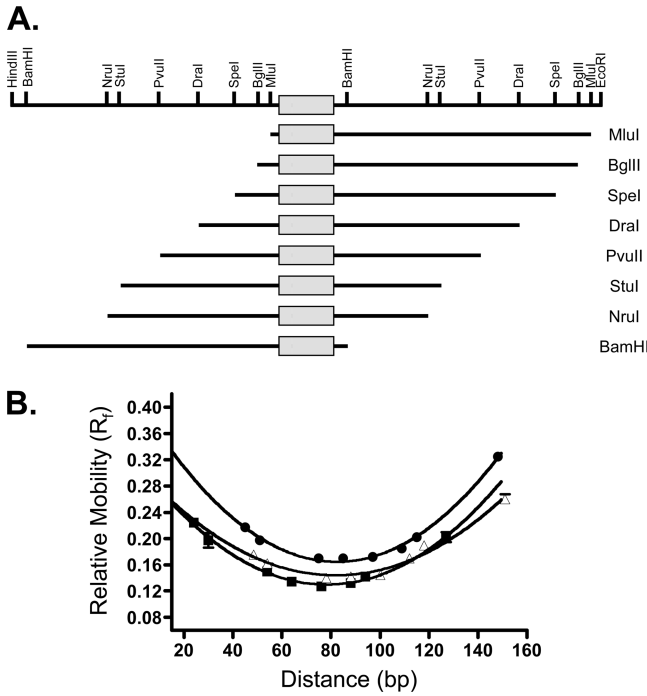


FIGURE 4: DNA bending by FOXO1A. (A) Illustration of the series of circularly permuted DNA fragments used to determine the DNA bending ability of FOXO1A. Fragments were produced by digestion of pBend3-clone 9, pBend3-clone 14, or pBend3-clone 5 with *Mlu*I, *Bgl*II, *Spe*I, *Dra*I, *Pvu*II, *Stu*I, *Nru*I, or *Bam*HI. The stippled box represents the location of the FOXO1A DNA binding elements relative to the ends of the molecule. (B) Analysis of DNA bending. The data were fit to the following second-order equations: clone 9 (closed square), $y = 0.3194 - 0.00484X + (3.094 \times 10^{-5})X^2$; clone 14 (closed triangle), $y = 0.3127 - 0.004117X + (2.515 \times 10^{-5})X^2$; clone 5 (closed circle), $0.4158 - 0.006089X + (3.691 \times 10^{-5})X^2$. The bending angles (α) were calculated as described in the Experimental Procedures.

complement of the IRE, [5′-GT(A/C)AACA-3′], and a "flanking" sequence (5′-ACAACA-3′), which in a majority of the clones is present immediately 5′ of the IRE (Figure 2). We found that clone 9, which was isolated 108 individual times, contained all three of these elements, further suggesting that optimal FOXO1A binding occurs when all three elements are present. Our alignment analysis also revealed that the isolated clones contained either all three of these sequence elements, two of the three elements in various different combinations, or the core IRE sequence alone (Figure 2).

*Probes with Three, Two, or One DNA Sequence Element Bind Equal Numbers of Molecules of GST-FOXO1A.* We then wanted to investigate the effects of these additional sequences on FOXO1A binding. The presence of two IRE sequences in some clones, either as the IRE or as the reverse complementary sequence (Figure 2), suggested that more than one molecule of FOXO1A may bind to the DNA when multiple copies of the IRE are present. We used a representative clone that contained all three of the sequence elements (clone 9), two of the elements (clone 14), and a clone that contained the IRE sequence alone (clone 5) in a qualitative EMSA. The EMSA was performed using constant concentrations of probe and the empirically determined saturating concentrations of protein (data not shown). Under these experimental conditions the binding of two molecules of GST-FOXO1A (99 kDa) would be expected to result in a

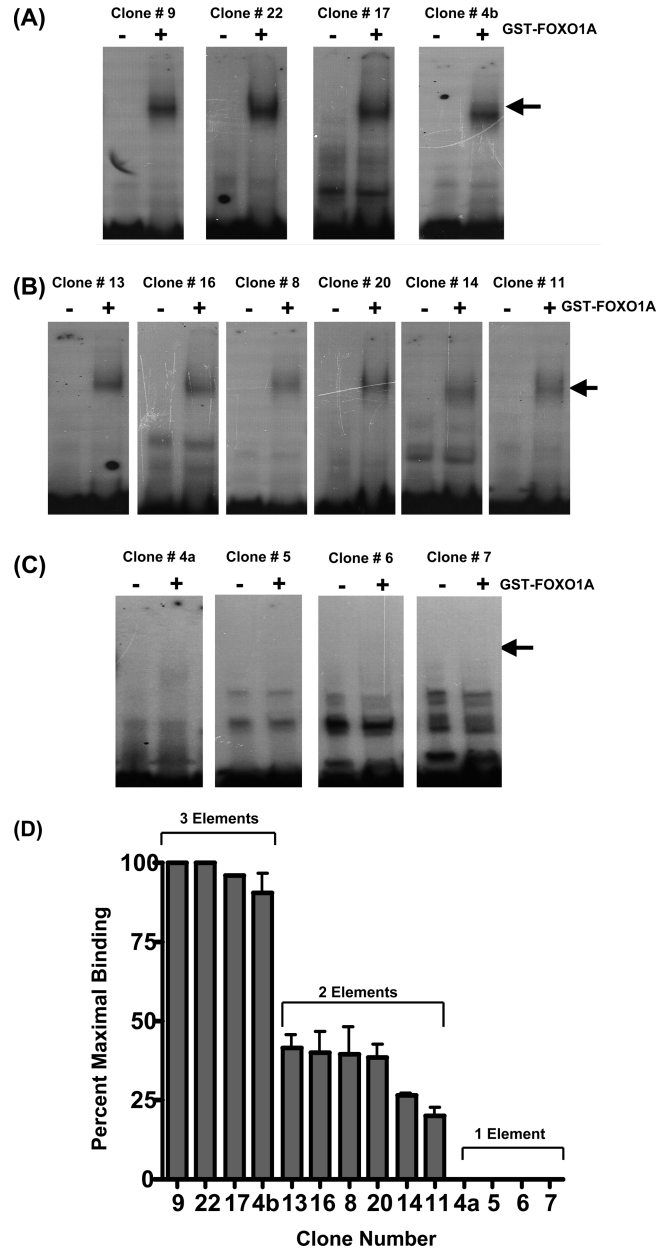


FIGURE 5: Qualitative differences in the binding of the three classes of DNA flanking elements. A representative qualitative electromobility shift assay demonstrating the binding of GST-FOXO1A to clones containing three elements (A), two elements (B), or one element (C). The probes were labeled by PCR incorporation using [α-$^{32}$P]dGTP. Binding reactions were carried out using a constant concentration of probe (35 nM) and a constant concentration of protein (98 nM) for all reactions as described in the Experimental Procedures. The arrow indicates the mobility of the specific shift (A and B) or the expected mobility if a shift were to be observed (C). (D) The band correlating to the specific shift observed for each clone was excised from the gel, the radioactivity was quantified as described in the Experimental Procedures, and the concentration of the bound DNA was determined. The binding of clone 9, which contains three DNA binding elements and was isolated 108 individual times from the final four rounds of SELEX, was identified as 100% binding. The remaining clones are illustrated as the percent maximal binding relative to clone 9. The error bars represent the standard deviation from the mean of at least three independent determinations.

greater shift of the radiolabeled probe than for the binding of one molecule of GST-FOXO1A. Instead, we observed that GST-FOXO1A shifted all three probes to the same extent irrespective of the number of DNA sequence elements

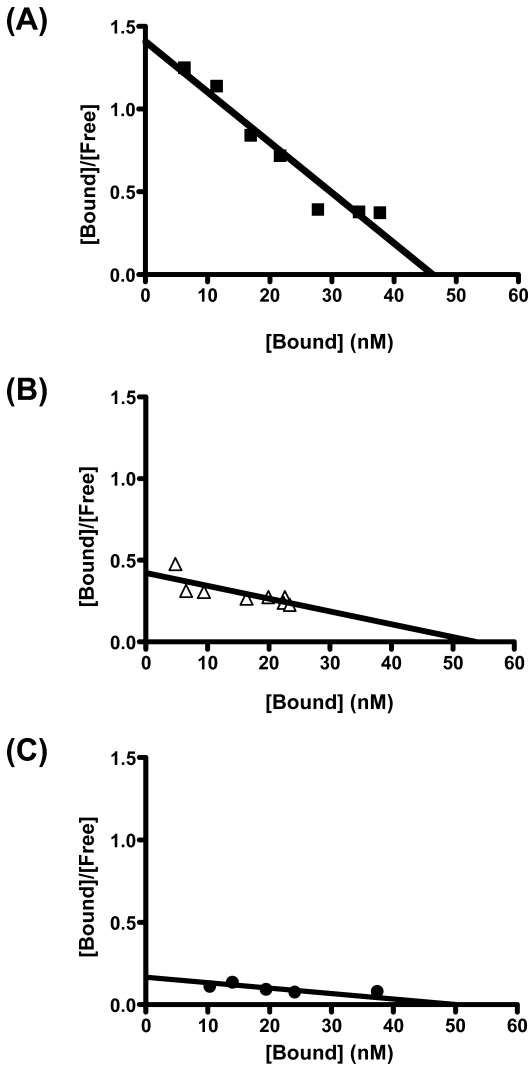


FIGURE 6: Binding affinity of the three classes of FOXO1A flanking DNA binding elements. Increasing concentrations of clones 9 (A), 14 (B), and 5 (C) were labeled by PCR incorporation using [α-$^{32}$P]dGTP and bound to a constant concentration of GST-FOXO1A (50 nM), as described in the Experimental Procedures. The specific shift was excised from the gel, the radioactivity of bound DNA was determined, the concentration of bound DNA was calculated and normalized for the specific activity of FOXO1A, and the resulting data were analyzed according to a standard Scatchard analysis (*41*) with each point resulting from an average of at least three independent determinations. The dissociation constants for clone 9 ($K_D$ = 33 ± 4 nM), clone 14 ($K_D$ = 126 ± 15 nM), and clone 5 ($K_D$ = 298 ± 32 nM) were calculated from a linear regression of the data according to the standard Scatchard equation (*41*).

present in the probe (Figure 3). The absence of a supershift when saturating levels of GST-FOXO1A are bound to probes containing multiple sequence elements directly demonstrates that an equal number of molecules of GST-FOXO1A bind to probes containing multiple elements as bind to probes containing a single element.

*Binding of GST-FOXO1A Induces Similar DNA Bending.* To date, the angle of DNA bending induced upon the binding of FOXO1A to DNA has yet to be determined. Therefore, we used the circular permutation polyacrylamide gel retardation assay (*40*) to calculate the angle of bending when FOXO1A binds to the core IRE sequence and to determine if the presence of the novel flanking DNA elements alters this angle. This assay is based on the theory that DNA bending results in a reduction of gel mobility of the DNA: protein complex and that this reduction in mobility is dependent on the angle of bending, which can be described by the semiempirical equation, as described in the Experimental Procedures. The representative clones described above (clones 9, 14, and 5) were centrally cloned into the pBend3 bending vector (*32*) and subsequently digested with a series of restriction endonucleases that generate DNA fragments of identical size but have the FOXO1A DNA binding site circularly permuted along the DNA fragment (Figure 4A).

Figure 4B shows the results of the gel retardation assays of the fragments using clones 9, 14, and 5. All three clones demonstrated different mobilities depending on the location of the FOXO1A binding site on the DNA fragment, indicating that FOXO1A induces a significant bend in the DNA regardless of the number of DNA sequence elements that are present. More importantly, the differences in the mobilities were similar for all three clones, indicating minimal differences in the angle of bending. Each gel retardation assay was repeated in quadruplicate, and the angle of bending was calculated for each individual assay. Consistent with the similar mobilities between the three different clones, the angles of DNA bending were calculated as 118 ± 4° for clone 9 (three DNA sequence elements), 112 ± 6° for clone 14 (two DNA sequence elements), and 120 ± 4° for clone 5 (the core IRE sequence alone). Therefore, FOXO1A induces essentially the same angle of bending in the DNA regardless of the number of DNA sequence elements that are present.

*The Novel Flanking DNA Elements Enhance FOXO1A DNA Binding Affinity.* We next wanted to determine if the presence of the novel flanking DNA sequences altered the FOXO1A DNA binding affinity. First, to ascertain if a qualitative difference in binding exists, we performed an EMSA analysis using several representative clones from each group (Figure 5). In order to make direct comparisons in the qualitative binding of FOXO1A to each of the clones, the binding reactions were carried out using a constant concentration of probe (35 nM) and GST-FOXO1A protein (98 nM). Using these experimental conditions, we observed striking differences in the extent of binding between each of the three groups of clones (Figure 5A−C). In order to quantify the observed differences, the bands corresponding to the specific shift were excised, and the concentration of the bound DNA was determined by scintillation counting (Figure 5D). Clone 9, which contains three binding elements and was isolated 108 individual times from the SELEX (Figure 2), consistently had the highest level of binding. Therefore, we assigned clone 9 as 100% maximal binding and calculated the remaining clones with respect to this clone. We observed between 90−100% maximal binding for clones containing all three elements, 20−40% maximal binding for clones containing two elements, and no apparent binding for clones containing the IRE alone. Although the clones containing just the IRE did not show a shift under the conditions used for the experiment, we did observe a shift when utilizing higher concentrations of protein (Figure 3) or higher concentrations of probe (Figure 6). Therefore, the novel flanking DNA sequences significantly affect the qualitative binding of FOXO1A to DNA.

In order to quantify the binding affinities, we performed a Scatchard analysis to determine the dissociation constant ($K_D$) for the representative clones described above (clones

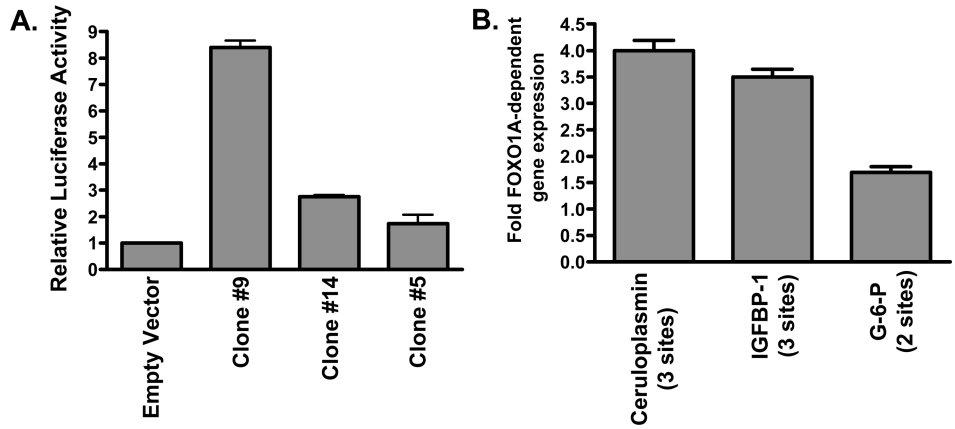


FIGURE 7: Transcriptional activity of DNA binding elements containing three, two, or one DNA binding elements. (A) HepG2 cells were transiently transfected with pcDNA3.1-FOXO1A and the pGL3-basic luciferase reporter construct alone or with the pGL3-basic reporter construct containing clone 9, clone 14, or clone 5. After 48 h luciferase activity was determined as described in the Experimental Procedures. All values were normalized for cotransfected pRL-TK vector and reported relative to the luciferase activity of the empty pGL3-basic luciferase reporter construct. Error bars represent the standard deviation from six independent determinations. (B) HepG2 cells were transiently transfected with pcDNA3.1-FOXO1a or the empty pcDNA3 control vector and subsequently treated with IL-6 to maximally activate FOXO1 as described in the Experimental Procedures. Real-time RT-PCR was performed on mRNA isolated from the cells using primers specific for ceruloplasmin (three FOXO1 binding elements [5′-GTAAAAAGCTACATGTTTTG-3′]), IGFBP-1 (three FOXO1 binding elements [5′-GCAAAACAAACTTATTTTG-3′]), or G6Pase (two FOXO1 binding elements [5′-TGTTTTTGTGTGCCTGTTTTG-3′]). The fold FOXO1-dependent gene expression was determined relative to the negative control, and error bars represent the standard deviation from three independent determinations.

9, 14, and 5). We titrated a constant concentration of protein with increasing concentrations of probe, as described in the Experimental Procedures, determined the concentration of bound DNA as described above, and after normalizing the results for the specific activity of the protein, performed a Scatchard analysis on the resulting binding data (Figure 6). A linear regression of the data fit to the standard Scatchard equation (*41*) provided apparent dissociation constants of $K_D = 33 \pm 4$ nM for clone 9 (three binding elements), $K_D = 126 \pm 15$ nM for clone 14 (two binding elements), and $K_D = 298 \pm 32$ nM for clone 5 (the IRE alone). In addition, all three of the clones have a saturating concentration of binding of approximately 50 nM (Figure 6). When normalized for the concentration of GST-FOXO1A used in the reaction (50 nM), this result directly demonstrates a 1:1 binding stoichiometry between GST-FOXO1A and the probe regardless of the number of DNA binding elements that are present.

*In Vitro Binding Affinities Correlate to in Vivo Transcriptional Activity.* In order to determine if the increase in FOXO1A DNA binding affinity correlates with the ability of FOXO1A to activate transcription from these same DNA binding elements, we cloned the representative clones (clones 9, 14, and 5) into the pGL3-Basic luciferase reporter construct. HepG2 cells were transfected with the pGL3-Basic luciferase reporter constructs, FOXO1A, and the pRL-TK runilla luciferase vector to control for transfection efficiency. A Western blot analysis confirmed that equal amounts of FOXO1A were being expressed in these cells (data not shown). The ability of FOXO1A to activate transcription from these three different reporter constructs was determined using the luciferase assay, as described in the Experimental Procedures. In this cellular context, the FOXO1A-dependent transcriptional activity from clone 9, which contains all three DNA binding elements, was approximately 9-fold above background. Consistent with our *in vitro* binding affinity results, FOXO1A-dependent transcriptional activity from clone 14 (two binding elements) and clone 5 (the core IRE alone) was approximately 3-fold and 6-fold lower than that observed for clone 9, the optimal binding sequence (Figure 7A).

Finally, to correlate our *in vitro* binding and transcriptional assay results with the *in vivo* expression of known FOXO1A target genes, we performed a real-time RT-PCR analysis to determine the ability of FOXO1A to activate the expression of endogenous genes directly regulated by FOXO1A. We found an approximately 3.5−4.0-fold increase in the expression of genes whose promoters contained all three of the FOXO1A DNA binding elements (ceruloplasmin and IGFBP-1) (*42*) (Figure 7B). Consistent with our *in vitro* binding data and our transcriptional assays, G6Pase, which contains two FOXO1A DNA binding elements (*17*), has FOXO1A-dependent expression approximately 2.5−3.0-fold lower than IGFBP-1 or ceruloplasmin (Figure 7B). We did not observe any increase in gene expression for PDK4, which both contain only the IRE sequence (data not shown). However, consistent with our binding and transcriptional assay results, we would not expect to see any changes under the conditions used for this analysis.

## DISCUSSION

FOXO1A, a winged-helix transcription factor, is known to have several tissue-specific functions whose best studied function is its role in the insulin response pathway. In this pathway FOXO1A is known to bind and regulate the transcription of genes through its binding to the DNA sequence of the IRE (*3*). However, multiple other unrelated forkhead family members with diverse biological functions also bind to the IRE, suggesting that additional sequence elements are required to enhance FOXO1A DNA binding and provide it with needed specificity. In the present report we have used the SELEX procedure to systematically identify these additional sequence elements. Using this analysis we have identified two highly conserved sequence elements that act together with the IRE to contribute to FOXO1A DNA

binding affinity. We demonstrate that there is a 1 to 1 binding stoichiometry between FOXO1A and DNA and that there is a similar extent of FOXO1A-induced DNA bending regardless of whether all three binding elements are present or if only the IRE is present. More importantly, we demonstrate that FOXO1A binds to DNA containing all three sequence elements with a 3- or 9-fold greater affinity than to DNA with only two elements or the IRE alone, respectively. These differences in affinity correlate with the greater transcriptional activity of FOXO1A from an artificial reporter or and from biologically relevant promoters.

It is interesting to note that we observed an increase in the FOXO1A DNA binding affinity in the presence of additional flanking sequences that can be either an exact copy or a palindromic copy of the IRE (Figure 2). It is commonly known that the apparent affinity of a transcription factor for its DNA recognition sequence will increase when multiple copies of this recognition sequence are present. However, the increase in the apparent affinity usually arises from the cooperative homodimerization of the transcription factor to the multiple sites (*43, 44*). Unlike the cooperative binding model, we have conclusively demonstrated both through qualitative gel shifts (Figure 3) and through quantitative Scatchard analysis (Figure 6) that there is a 1 to 1 binding stoichiometry between FOXO1A and DNA regardless of the number of sequence elements that are present. Therefore, homodimerization of FOXO1A cannot occur on DNA, and the increase in binding affinity must be mediated through additional protein:DNA contacts between the FOXO1A DNA binding domain and the additional flanking DNA sequences.

At present, the exact solution structure of FOXO1A is not known. However, the members of the forkhead winged-helix family share a conserved 100 amino acid DNA binding domain containing three major α-helices (H1, H2, and H3), three $\beta$-strands, and two wing-like loops (W1 and W2) with highly similar crystal structures (*7, 28, 45–47*). Molecular modeling studies have been performed on FOXO4 bound to DNA, a FOXO family member that is 89% identical and 95% similar to FOXO1A in the DNA binding domain (*29*). Consistent with the results presented in this study, the molecular modeling analysis of FOXO4 demonstrated that in addition to the primary contact between H3 and the IRE, both wings (W1 and W2) and the N-terminal region of the DNA binding domain contact flanking DNA sequences. These additional contacts encompass approximately 20 base pairs of DNA, a result further supported by two independent FOXO1A DNA footprinting studies demonstrating the protection of nearly 20 to 25 base pairs upon the binding of FOXO1A to DNA (*13, 48*). Further consistent with the results presented for FOXO1A, the individual deletion of the N-terminal portion or W1 and W2 of FOXO4, a situation that would mimic binding of FOXO1A to two sequence elements (*29*), reduced FOXO4 DNA binding affinity approximately 3−4-fold. In addition, the deletion of both the N-terminal portion and W1/W2, which would reduce binding to interactions between H3 and the IRE alone, reduced FOXO4 DNA binding affinity nearly 10-fold (*29*). Both of these results are consistent with the 3−4-fold and nearly 10-fold reduction in DNA binding affinity that we observed for FOXO1A binding to two elements or the IRE alone. Therefore, taken together these studies provide compelling evidence to support our conclusions that additional sequence elements play a role in enhancing the FOXO1A DNA binding affinity, most likely through providing additional protein:DNA contacts with other regions of the FOXO1A DNA binding domain.

Consistent with the correlations described above for FOXO4, it has been demonstrated that in addition to the third α-helix (H3) wing 2 (W2) of the FOXO1A DBD is also critical for DNA binding (*11*). Therefore, the requirement of additional protein elements within the FOXO1A DBD combined with the molecular modeling studies of a closely related forkhead FOXO family member supports our conclusion that, in addition to the IRE sequence, flanking sequences are important for maximal FOXO1A binding. We have demonstrated that a single molecule of FOXO1A interacts with a single molecule of DNA irrespective of the number of DNA sequence elements that are present. Therefore, our results and results from the literature support a model by which minimal binding between FOXO1A and DNA and the FOXO1A-induced DNA bending (see below) is mediated through the third α-helix of the FOXO1A DNA binding domain and the IRE sequence. The reverse complement and flanking sequences would then be capable of interacting with the winged structures of the FOXO1A DNA binding domain, providing stronger interactions between the protein and the DNA resulting in our experimentally determined binding affinities.

In addition to enhancing the DNA binding affinity of FOXO1A, it was previously believed that the flanking DNA sequences may promote different extents of DNA bending (*22, 26–28*). In this report we provide the first demonstration of the determination of the angle of FOXO1A-induced DNA bending (Figure 4). FOXO1A induces a surprisingly large angle of DNA bending of approximately 120°. This angle of bending is similar to that seen for architectural transcription factors such as LEF-1 and integration host factor (IHF) (*49*), factors that act to change the shape of DNA control regions so that other proteins can successfully interact with the promoter. Therefore, it is conceivable that part of the transcriptional activity of FOXO1A is achieved through its ability to drastically alter the shape of the promoter regions to which it binds. Irrespective of the biological function of the DNA bending, we determined that the angle of bending was identical regardless of whether all three DNA sequence elements were present or if only the IRE was present (Figure 4). Because the IRE alone is capable of inducing the observed angle of bending, we conclude that interaction between the third α-helix of the FOXO1A DNA binding domain and the core IRE is sufficient to induce DNA bending and the subsequent interactions between the additional DNA sequence elements and FOXO1A contribute to increase the binding affinity.

Finally, an analysis of the promoter regions of genes known to be direct transcriptional targets of FOXO1A demonstrates that the various combinations of the core IRE, reverse complement, and the flanking sequences are present in biological systems (Figure 7B). In addition, we have noted that the level of FOXO1A-dependent gene expression of endogenous genes correlates directly with our experimentally determined affinities and transcriptional activities of FOXO1A for the various combinations of sequences. For example, IGFBP-1 and ceruloplasmin, whose promoter regions contain all three FOXO1A DNA binding elements in a nearly optimal

configuration (Figure 7B), both have a 3.5−4.0-fold increase in FOXO1A-dependent gene expression. G6Pase, whose promoter contains two core elements, has FOXO1A-dependent gene expression that is approximately 3-fold less than that of IGFBP-1 and PDK4, which contains only the core IRE sequence, was undetectable under the assay conditions used, as was expected based on our binding and transcriptional data (Figure 7B). These numbers directly correlate to our experimentally determined binding affinities and transcriptional activities. Our results demonstrate that FOXO1A binds to and activates transcription from DNA containing all three binding elements approximately 3-fold stronger that it does to DNA containing only two elements and two elements approximately 2-fold stronger than one element (Figures 6 and 7). Although additional factors are most likely involved in regulating the expression of FOXO1A transcriptional targets in biological systems, the direct correlation between our results and *in vivo* expression data provides compelling evidence to support the idea that the presence of different combinations of FOXO1A binding elements provide different levels of binding affinity to ultimately result in different levels of gene expression.

## ACKNOWLEDGMENT

We thank Dr. Gerard Grosveld, St. Jude Children's Research Hospital, for kindly providing us with the pBabe-MSCV-puro-Avi-FOXO1A retroviral construct and Dr. Sankar Adhya, National Cancer Institute, for kindly providing us with the pBend3 circular permutation vector. We also thank San-San Ng and Angela Flynn of the Genomics Core Facility at Louisiana State University Health Sciences Center, New Orleans, and the CRC-DNA sequencing facility at the University of Chicago, Chicago, IL, for assistance with sequencing.

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BI702495M