The advantages of ambiguous orientation

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Recent studies have revealed that although it is possible for certain transcription factors to bind in an orientated fashion on DNA, they have not evolved to do so. Rather, they rely on contacts with other proteins to precisely define their mode of binding.

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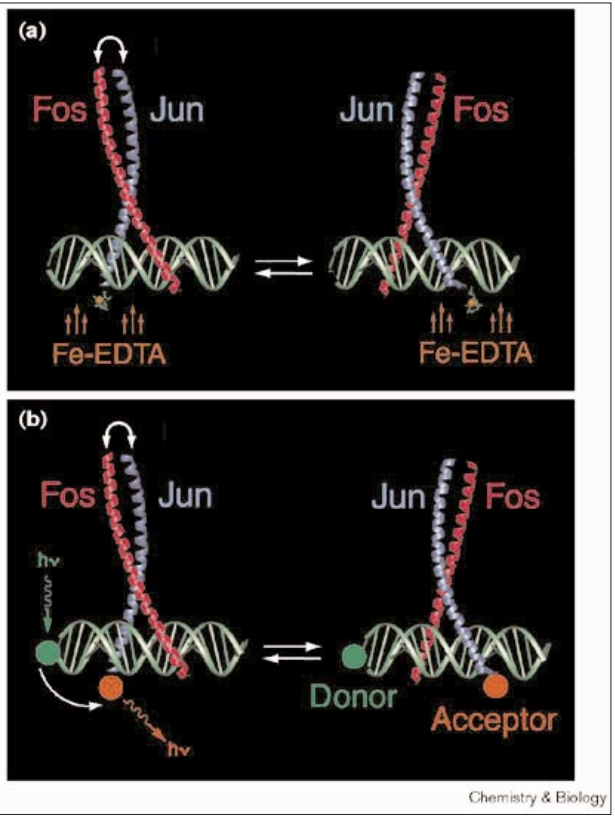
The human genome consists of about 100,000 genes, only a fraction of which are actively transcribed at any given time. Because cells in an organism share the same complement of genes, differences in gene expression are primarily responsible for the profound differences between, for example, spleen cells and neurons. This differential expression is controlled by transcription factors, proteins which bind to specific sites on DNA to direct the expression of genes. Transcription factors also associate with other proteins to form complex multiprotein assemblies that regulate the overall level of gene expression.

Many classes of transcription factors bind to DNA as dimers, including the basic region leucine zipper, or bZip, protein [1,2]. The DNA-binding region of this transcription factor consists of a single α helix. Two of these proteins interact through their α helices to form a Y-shaped dimer in which the 'zipper' regions of the α helices are wound around one another (the stem of the Y), and the positively charged 'basic' regions splay apart and insert into the major groove of DNA (the arms of the Y). Typically seven or eight bases of DNA are contacted by the dimer—three or four bases by each helix.

Heterodimers can conceivably bind to DNA in two different orientations related by a 180° rotation about the center of the site, with a given helix interacting with either the 'upstream' (5′) or 'downstream' (3′) half-site, as shown in Figure 1. The archetypal example of a heterodimeric bZip transcription factor is activator protein-1 (AP-1), which is expressed in a wide range of cell types and is found in organisms as distantly related as yeast [3] and humans. The components of the dimer can consist of more than a dozen individual proteins, two well-characterized members being Fos and Jun. The various AP-1 dimers can also interact with other proteins to regulate transcription. One target for AP-1 is the distal antigen receptor response element (ARRE2) of the interleukin-2 (IL-2) enhancer, where it binds in a highly cooperative manner with a transcription factor known as 'nuclear factor of activated T cells', or NFAT [4,5]. The ARRE2 site is part of the regulatory sequence for the IL-2 gene; binding of the AP-1•NFAT complex is necessary for transcription of IL-2, which is one of the early events in the activation of T cells [6]. AP-1 alone binds only weakly to the ARRE2 site because the consensus AP-1 recognition sequence 5′-TGACTCA-3′ is altered

Figure 1



Fos and Jun are capable of binding to DNA in either of two orientations related by a 180° rotation. Two methods to determine the orientations are shown. The structures are adapted from the crystal structure of AP-1 bound to DNA [9]. **(a)** In the affinity-cleavage technique, a reactive metal species (brown sphere surrounded by green ligand) attached to Jun causes DNA strand cleavage (brown arrows). The DNA can be analyzed by gel electrophoresis to determine the sites of cleavage, and thus the location of the metal probe. **(b)** In the fluorescence resonance energy transfer (FRET) technique, a donor (green) is irradiated with light of one wavelength and transfers this energy in a distance-dependent manner to an acceptor (red), which fluoresces. The efficiency of the energy transfer provides a measure of the distance between donor and acceptor. In the orientation on the left, the donor and acceptor are in close proximity, so will show a larger FRET signal than in the orientation on the right. Adapted from Erlanson *et al.* [21].

to 5′-TG*TT*TCA-3′ (nonconsensus differences in italics). When NFAT binds to an adjacent upstream site, however, AP-1 shows enhanced affinity for this sub-optimal site [7,8].

Members of the AP-1 family share significant sequence homology in their bZip domains, and, because the canonical recognition site for AP-1 is pseudopalindromic, there was some question as to whether it would be possible for AP-1 to orient itself uniquely on DNA. This is important for the assembly of the rest of the components of the transcription-initiation complex. In fact, the first crystal structure of AP-1 bound to DNA contained complexes oriented in both directions on the DNA (i.e., with either Jun or Fos interacting with 5′-TGAC-3′) [9]. A slight bias was observed — one of the orientations was favored over the other by a factor of 2:1. However, as crystal packing forces could influence the orientational preference of AP-1, some means of identifying the orientation of the complexes in solution was required.

Methods for determining orientation of transcription factors on DNA in solution

Affinity cleavage is an established method for examining the position of a protein relative to DNA [10–13]. In this technique, a protein is derivatized site-specifically with a chelating agent capable of binding a reactive metal ion such as iron. Under redox conditions, reactive oxygen species are generated that cleave nearby DNA. The sites of DNA cleavage can readily be determined by using high-resolution gel electrophoresis, and, therefore, the site of the metal ion and associated protein can be inferred.

The application of affinity cleavage to the AP-1 system is illustrated in Figure 1a. When Jun is derivatized with the affinity-cleaving reagent Fe(II)–EDTA on the amino terminus of the protein (the 'basic' DNA-binding portion of the protein) and allowed to bind to DNA under redox conditions, cleavage occurs at sites in close proximity to the metal chelate. If AP-1 binds in a preferred orientation, cleavage will be observed in two regions on the DNA, whereas if AP-1 shows no orientational preference cleavage will be observed in three regions.

Another method for determining orientation relies on fluorescence resonance energy transfer (FRET), as shown in Figure 1b [14]. In this technique, an oligonucleotide is derivatized site-specifically with a fluorescent 'donor', whereas the protein of interest is site-specifically derivatized with a fluorescent 'acceptor'. Irradiation of the donor with light of a specific wavelength induces fluorescence in the acceptor, but this energy transfer is distance dependent. The position (or orientation) can therefore be inferred from the strength of the FRET signal. In Figure 1b, the complex on the left would show a stronger FRET signal than the complex on the right. If AP-1 shows no orientational preference, the FRET signal would be intermediate between the two complexes shown.

Orientation of AP-1 alone on DNA

The affinity-cleavage technique was used in initial investigations into the orientation of AP-1 on DNA [15]. The AP-1 binding site chosen was a modified version of ARRE2. The naturally occurring site contains two deviations from the AP-1 consensus site, so these were mutated to the consensus sequence to enhance AP-1 affinity. The cleavage pattern produced by derivatized Fos mixed with unmodified Jun was very similar to that observed with unmodified Fos mixed with derivatized Jun and to that of derivatized Fos mixed with derivatized Jun (Figure 1a). These results suggested that neither of the two orientations is preferred.

Similar results were observed using the FRET assay on this DNA site: no orientational preference was observed [16]. A modest degree of orientational preference was detectable, however, when DNA bases flanking the AP-1 core binding site were mutated [17]. Depending upon the exact sequence, one orientation was preferred over the other by a ratio of at most 4:1. This is consistent with the crystal structure [9], which also showed a modest preference for one orientation over the other.

Inspection of the crystal structure of AP-1 bound to DNA reveals that the DNA contacting residues in Fos are also present on Jun. Interestingly, a highly conserved arginine residue present at equivalent positions in both Fos (Arg155) and Jun (Arg279) contacts the central base of the recognition site. One arginine, however, makes a nonspecific electrostatic interaction with the phosphate backbone of the DNA, whereas the corresponding arginine on the other protein makes very specific and well-ordered bidentate hydrogen-bonding interactions with the central guanine residue. Mutating this arginine in one of the two proteins would abolish the ability of the mutant protein to interact with the central guanine, possibly causing one orientation to predominate. Indeed, when a complex consisting of wild-type Jun and Fos containing the mutation Arg155→Ala (Fos_{R155A}•Jun) bound to DNA, the complex oriented itself such that the arginine from Jun could interact with the central guanine, as judged by affinity-cleavage experiments [18]. Similarly, when a complex consisting of Jun containing the mutation Arg279→Ala and wild-type Fos (Fos•Jun_{R279A}) bound to DNA, the complex assumed the opposite orientation so that the arginine from Fos interacted with the central guanine. Finally, when the AP-1 binding site was reversed (5′-TGACTCA-3′ to 5′-TGAGTCA-3′), the orientation of mutant AP-1 binding to the site was also reversed.

Significantly, Fos•Jun_{R279A} had a similar DNA-binding affinity to Fos•Jun, whereas Fos_{R155A}•Jun had a slightly lower affinity, but Fos_{R155A}•Jun_{R279A} did not show any specific DNA binding. These results suggest that, although the arginine residue is dispensable in either of the two proteins individually, it must be present in at least one of the proteins. The fact that this arginine residue is highly conserved

among all AP-1 proteins [1,2] also argues that AP-1 has evolved to use this arginine to increase binding affinity, not as a means of achieving a unique orientation on DNA.

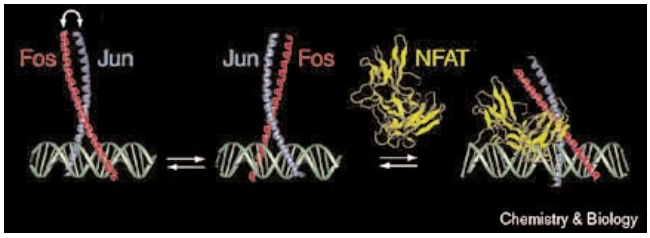
Similar results were observed using FRET analyses and slightly different mutants, in which the arginines were mutated to isoleucine instead of to alanine [16]. Furthermore, in contrast to the native proteins, these mutant proteins were not sensitive to the sequence of DNA bases outside of the core AP-1 binding site; they showed strong orientational preferences dictated by the central base pair, regardless of the flanking sequences. These results argue that these mutants are highly predisposed to binding in a single orientation, and emphasize that the effect of flanking sequence on orientation is relatively minor.

Orientation of AP-1 on DNA in the presence of NFAT

Given that Fos and Jun can readily be engineered to bind in an oriented fashion, why did they evolve to bind in either of two orientations, rather than developing high specificity for one particular orientation? Part of the answer lies in the fact that transcription factors interact not only with DNA, but with other proteins as well. As discussed above, NFAT is critical for AP-1 binding at the ARRE2 site. The effect of NFAT on the orientation of AP-1 is profound. Both affinity cleavage [15] and FRET [16] experiments reveal that, when AP-1 binds to ARRE2 in the presence of NFAT, it binds in a single orientation (Figure 2). This is true for both the nonconsensus AP-1 site present in wild-type ARRE2 as well as the mutated consensus site discussed above. In fact, the orientational effect of NFAT on AP-1 is even sufficient to override the innate orientation of mutants of AP-1 in which arginine was changed to isoleucine [16].

Mutational studies revealed specific residues of Fos, Jun and NFAT that are important for binding [19,20] and orientation [16,21]. A crystal structure [22] as well as a nuclear magnetic resonance (NMR) structure [23] of NFAT bound to DNA in the presence of AP-1 largely confirmed the mutational studies and provided high-resolution views of the complex. The structures reveal a well-ordered and highly evolved protein–protein contact area between NFAT and the zipper regions of Fos and Jun. This explains why NFAT strongly directs the orientation of AP-1 on DNA. It also suggests a reason why AP-1 has not evolved to bind selectively in a single orientation: doing so would not confer any advantage. By relying on contacts with other proteins, AP-1 can forego innate specificity. Indeed, given the fact that there are many members of the AP-1 protein family, a very efficient evolutionary strategy is to keep the DNA-binding domain constant while allowing the zipper domain to evolve to maximize interactions with other proteins. AP-1 shows both positive and negative cooperativity with a number of other proteins besides NFAT, including Ets, Smad, NF-κB, ABP, the glucocorticoid receptor, Oct, IP-1 and the estrogen receptor [1,2,6]. In virtually all of these cases the orientation of AP-1 has yet to be determined.

Figure 2

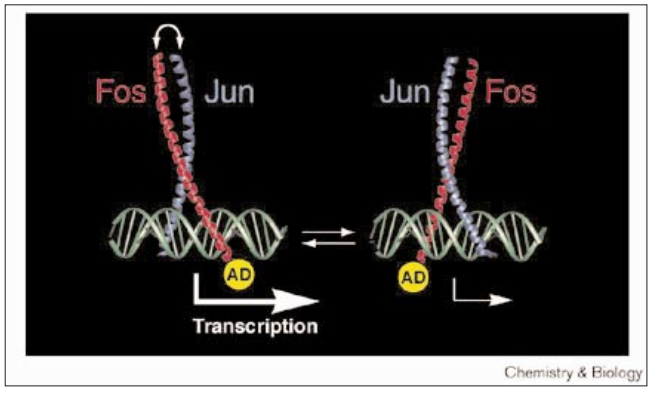


Fos and Jun have no orientational preference for the ARRE2 site in the absence of NFAT, but are strongly oriented by NFAT. The structure on the right is taken from the crystal structure of the quaternary complex [22]. Adapted from Chytil *et al.* [18].

Orientation and transcription

The observation that AP-1 can bind to DNA in either orientation is interesting from a biophysical standpoint, but is it biologically relevant? In fact, the orientation of AP-1 on DNA does have an effect on gene activation (Figure 3). This was demonstrated by fusing a strong transcriptional activation domain to the bZip domain of wild-type Fos and expressing this protein, along with Jun_{R279A} or wild-type Jun, in yeast [18]. The yeast was equipped with a plasmid containing a reporter gene downstream of the consensus AP-1 binding site in either the forward (5′-TGACTCA-3′) or reverse (5′-TGAGTCA-3′) orientation. Because the transcriptional activation domain fused to Fos strongly activates transcription, any enhanced activation of the reporter gene could be attributed to the introduced Fos and Jun proteins rather than endogenous yeast AP-1 proteins. In the case of wild-type Jun, both AP-1 site orientations produced equivalent signals, confirming that wild-type AP-1 is not able to orient itself on this DNA sequence. In the case of mutant Jun_{R279A} the forward-oriented AP-1 site produced a signal

Figure 3



Transcriptional activation of AP-1 is orientation dependent. AD, activation domain. Adapted from Chytil *et al.* [18].

of similar intensity to that seen in wild-type Jun. The signal was, however, an order of magnitude weaker for the reporter plasmid containing the reverse-oriented AP-1 site [18]. One orientation of AP-1 therefore causes much greater transcriptional activation than the other.

Other groups have also reported that AP-1 orientation affects transcriptional activation. The Anders group [24] investigated the effect of changing the orientation of the AP-1 consensus site in a reporter plasmid transfected into mammalian cells stimulated to express native, full-length AP-1. In this case, one site orientation showed about a fivefold enhancement in transcriptional activation over the other orientation. Because this was done using wild-type AP-1, the implication is that in this system the AP-1 protein is able to orient itself on DNA, perhaps by cooperating with other proteins. Similar results have been reported in other systems [25].

Outlook and implications

A recurring theme in evolution is that simple solutions to difficult problems are repeated in a wide variety of settings. The bZip family of transcription factors is a very successful example of a small domain that binds to DNA and also presents a binding surface to other proteins. By allowing some orientational degeneracy, AP-1 maintains a high degree of binding flexibility while site-specificity can be maintained through interactions with other proteins. The complexity of cellular transcriptional machinery suggests that regulation could be most effectively controlled through the use of multiple subunits, each of which has only modest affinity and specificity for a particular site, but as an ensemble are capable of exquisite discrimination [2,26–29]. Indeed, recent affinity-cleavage studies with the TATA-box-binding protein suggest that even this central player in transcription may not be capable of uniquely orienting itself on DNA [30].

It seems likely that many other DNA-binding proteins have evolved with low or nonexistent innate orientational bias, but that cooperation with other proteins locks their orientation. The biochemical techniques described here, affinity cleavage and FRET experiments, provide direct methods for studying the orientation of proteins on DNA. In combination with transcriptional activation assays, these tools will allow us to examine the precise stereochemical arrangement of complex protein assemblies in their operationally relevant forms.

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