Control of the Orientation of Fos-Jun Binding and the Transcriptional Cooperativity of Fos-Jun-NFAT1 Complexes*

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Heterodimeric transcription regulatory proteins can bind to palindromic recognition elements in two opposite orientations. We have developed a gel-based fluorescence resonance energy transfer assay for quantifying heterodimer orientation preferences. Fos-Jun heterodimers bind in opposite orientations to AP-1 sites with different flanking sequences. The effects of individual amino acid and base pair substitutions on heterodimer binding orientation were quantified. Base pairs at positions ±6 and ±10 relative to the center of the AP-1 site were the principal determinants of Fos-Jun binding orientation. Amino acid residues of opposite charge adjacent to the basic regions of Fos and Jun had independent effects on heterodimer orientation. Exchange of these amino acid residues between the basic region-leucine zipper domains of Fos and Jun reversed the binding orientation. Heterodimers formed by full-length Fos and Jun exhibited the same changes in binding orientation in response to amino acid and base pair substitutions. The preferred orientation of heterodimer binding affected the stability of Fos-Jun-NFAT1 complexes at composite regulatory elements. Changes in heterodimer orientation preference altered the transcriptional activity and the promoter selectivity of Fos-Jun-NFAT1 complexes. Consequently, the orientation of Fos-Jun binding can influence transcriptional activity by altering cooperative interactions with other transcription regulatory proteins.

Regulation of the transcriptional activities of the myriad of genes in mammalian genomes requires combinatorial interactions among multiple transcription regulatory proteins within promoter and enhancer regions (reviewed in Ref. 1). Whether the regulation occurs at the level of chromatin remodeling, coactivator recruitment, or assembly of the transcription machinery, specification of a unique target in the genome requires cooperation among multiple transcription factors (2–5). The cooperative interactions among many transcription regulatory proteins require juxtaposition of contact surfaces that are either part of or tightly coupled to the DNA binding domain (6–10). Interactions among such proteins generally require a specific spacing and orientation of the DNA recognition sequences to allow cooperative complex formation. Many mammalian transcription regulatory proteins form heterodimers that recognize palindromic DNA sequence elements. Such heterodimers can potentially bind to their recognition sequences in either of two opposite orientations. Heterodimers that bind in opposite orientations can differ in their interactions with transcription factors that bind to adjacent regulatory elements. The binding orientations of such heterodimers may be controlled by recognition of asymmetric base pairs flanking the palindromic core sequence element or through interactions with other transcription factors (8, 11–14). Promoter elements are classically defined based on their position- and orientation-dependent transcriptional activities. The orientation of heterodimer binding to asymmetric regulatory elements can influence their transcriptional activities (13, 15–18). The mechanisms whereby the orientation of heterodimer binding influences transcriptional activity remain to be characterized.

Fos and Jun are members of the bZIP1 family of transcription factors and bind to palindromic AP-1 regulatory elements. The x-ray crystal structure of the bZIP domains of Fos and Jun bound to the AP-1 site revealed that the heterodimer could bind to the recognition element in both orientations (19). In solution, Fos-Jun heterodimers bind to different AP-1 sites in opposite preferred orientations (12, 18, 20, 21). The orientation of heterodimer binding is affected by sequences flanking the palindromic core AP-1 recognition element and amino acid residues adjacent to the basic regions of Fos and Jun (12, 14). No direct contacts between the amino acid residues adjacent to the basic regions of Fos-Jun and the base pairs flanking the AP-1 site are observed in the x-ray crystal structure (19). Thus, the orientation of Fos-Jun binding appears to be controlled by indirect recognition of differences in DNA structure between flanking sequences on opposite sides of the AP-1 site.

Fos and Jun activate different genes in different cell types and in response to different extracellular signals (22). These differences in Fos-Jun regulatory specificity are mediated at least in part by cooperative interactions with structurally unrelated transcription factors. Fos-Jun heterodimers can physically and functionally interact with members of the NFAT, Ets, Smad, and nuclear hormone receptor transcription factor families (10, 23, 24). The interaction between Fos-Jun and NFAT1 (NFATp, NFATc2) has been characterized in greatest detail. Cooperative binding by Fos-Jun and NFAT1 to composite regulatory elements in cytokine gene promoters requires a specific orientation of Fos-Jun binding (8, 11). The x-ray crystal structure of the Fos-Jun-NFAT1 complex at the ARRE2 element shows a specific contact interface between NFAT1 and one face of the leucine zipper (7). The interaction with NFAT1 can

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1 The abbreviations used are: bZIP, basic region-leucine zipper; gel-FRET, gel-based fluorescence resonance energy transfer; NFAT, nuclear factor of activated T cells; FL, fluorescent; TR, Texas Red; EP, end preference; PAGE, polyacrylamide gel electrophoresis.
reverse the orientation of heterodimer binding (8). However, the preferred orientation of Fos-Jun binding can influence the stability and the transcriptional activity of Fos-Jun-NFAT1 complexes (18).

We have investigated the structural basis and the functional significance of the opposite orientations of Fos-Jun heterodimer binding at different AP-1 sites. To compare the effects of individual base pairs and amino acid residues on heterodimer orientation, we developed an approach for determination of the free energy of Fos-Jun heterodimer reorientation at different AP-1 sites. The functional consequences of opposite orientations of Fos-Jun heterodimer binding were examined by comparing the stabilities and transcriptional activities of Fos-Jun-NFAT1 complexes formed by heterodimers with opposite orientation preferences.

**EXPERIMENTAL PROCEDURES**

**Preparation of Fluorescent Oligonucleotides and Proteins—** Oligonucleotides containing the sequences listed in Fig. 6 with symmetrical CTG extensions on both ends were synthesized with fluorescein on the 5' ends. Duplexes formed by one labeled and one unlabeled strand were purified by gel electrophoresis. Proteins encompassing amino acid residues 139–200 of Fos and 257–318 of Jun were expressed and purified to homogeneity as described (8, 12, 14). The amino acid substitutions indicated in the figures replaced residues at positions 139–141 and 257–259, respectively. The proteins were labeled by incubation with Texas Red maleimide (Molecular Probes) and purified as described (8, 12, 14). The FosRI and JunRI calibration standards contained R155I and R273I substitutions, respectively. Full-length Fos and Jun (with or without R155I or R273I substitutions) as well as the DNA binding domain of NFAT1 (residues 396–692) were expressed and purified as described (8, 25, 26).

The gelFRET Assay for the Orientation of Fos-Jun Heterodimer Binding—The orientation of heterodimer binding was determined based on the quantitation analysis of donor fluorophore emissions from donor fluorophores (fluorescein) linked to either end of an oligonucleotide to an acceptor fluorophore (Texas Red) linked to either subunit of the heterodimer. Complexes were formed by incubation of 2–6 μM Fos-Jun heterodimers in which one subunit was labeled with Texas Red with 500 nM oligonucleotides labeled with fluorescein on either the left or the right end. NFAT1 was added to the reactions indicated at 1 μM. The complexes were separated by native PAGE from flanking sequences in either orientation of Fos-Jun heterodimer reorientation indicate that this reaction reaches equilibrium rapidly during incubation of the samples prior to gel electrophoresis (18). The intermediates in the reorientation reaction do not influence our analysis providing that they do not represent a major fraction of the complexes observed under the experimental conditions. The free energy of reorientation (ΔG of i) is calculated based on the fraction of heterodimers in each orientation at equilibrium (ΔG of i = RT ln(f of i/ f of i)). At each AP-1 recognition site, the free energy of reorientation reflects the difference in binding energies (ΔG of i) between the Jun-Fos and Fos-Jun orientation isomers (ΔG of i = ΔG of i - ΔG of i). Fos-Jun heterodimers that bind to the AP-1 site in opposite orientations contact the central base pair using arginines from different subunits (Fig. 1, inset). Replacement of the arginine residue in different orientation isomers results in the binding of opposite DNA directions (12, 20). To use these heterodimers as calibration standards, we assume that contacts to the central base pair influence the free energy of reorientation independent of interactions with flanking sequences (ΔG of i = ΔG of i - ΔG of i). Thus, inversion of the central base pair reverses its effect on the free energy of reorientation (ΔG of i = ΔG of i - ΔG of i). We also assume that substitution of the arginine residues in the basic regions of Fos and Jun does not alter the effect of flanking sequences on the orientation preference (ΔG of i - ΔG of i = ΔG of i - ΔG of i). The end preference values of fully oriented complexes using an iterative fitting procedure requiring that the free energies of reorientation (calculated as described in the previous paragraph) satisfy the constraints (ΔG of i = ΔG of i = ΔG of i) and the careful estimation of the calculated free energy of reorientation of the major complexes was not significantly affected by small changes in the estimated end preference values of fully oriented complexes. Heterodimers labeled on Fos and on Jun were analyzed separately and provide an independent control for the validity of this approach.

**Measurement of Fos-Jun-NFAT1 Complex Dissociation—** Fos-Jun-NFAT1 complexes bound to composite recognition elements were prepared by incubation of 50 nM Fos-Jun (with or without arginine substitutions as indicated) and 100 nM NFAT1 with 20 nM oligonucleotides containing symmetrical sequences (ORI 1) (Fig. 6). The same end preference values for fully oriented complexes were obtained using several different initial values in the fitting algorithm.

The calculated free energy of reorientation of the majority of complexes was not significantly affected by small changes in the estimated end preference values of fully oriented complexes. Heterodimers labeled on Fos and on Jun were analyzed separately and provide an independent control for the validity of this approach.

**RESULTS**

The x-ray crystal structures of Fos-Jun heterodimers bound to an AP-1 site revealed that Fos-Jun can bind to the AP-1 site in two orientations that are related by an ~180° rotation about the dimer axis (Fig. 1) (19). Studies of the orientation of Fos-Jun binding in solution demonstrated that the heterodimer binds to different AP-1 sites in opposite orientations (12). To investigate the nucleic acid and protein determinants of the orientation of Fos-Jun binding, we used gel-based fluorescence resonance energy transfer (gelFRET) to determine the orientation of Fos-Jun heterodimers at different AP-1 sites (Fig. 2).
Influence of Sequences Flanking the AP-1 Site—To determine the relative effects of the core AP-1 recognition element and flanking sequences on the orientation of heterodimer binding, we exchanged the flanking sequences between the left and the right sides of the binding site. The exchange of flanking sequences reversed the binding orientations of all heterodimers (Fig. 2B, \(X_{\text{rev}}\)). The slight asymmetry between the end preferences of complexes at the X and \(X_{\text{rev}}\) sites reflects the influence of the core AP-1 recognition element on heterodimer orientations. Thus, sequences flanking the X site had a much larger effect on heterodimer binding orientations than the asymmetric base pair at the center of the AP-1 site.

To identify the sequences that determine the orientation of Fos-Jun binding at the X site, we first compared the effects of the amino acid substitutions on the orientation of heterodimer binding at a site with different flanking sequences (Fig. 2B, M). Heterodimers formed by Fos and Jun bZIP domains containing amino acid residues of opposite charge (FosKRR-JunEEE and FosEEE-JunKRR) exhibited opposite end preferences at this site compared with the X site (compare M and X in Fig. 2B). The amino acid substitutions had smaller effects on heterodimer end preferences at the M site, which contained nearly symmetrical flanking sequences. Since the core AP-1 recognition sequences were identical between these two sites, sequences flanking the core AP-1 recognition elements must determine the opposite binding orientations.

The binding orientation reflects the balance between the relative binding affinities of the two subunits in the heterodimer for opposite sides of the binding site. To locate the sequences that determine the opposite binding orientations at the X and M sites, we examined the effects of exchange of flanking sequences on the binding orientation (Fig. 2B). Complexes formed on oligonucleotides that contained flanking sequences from the left side of the X site and the right side of the M site exhibited end preferences more similar to those observed at the X site (Fig. 2B, compare \(X_M\) and X). Conversely, complexes formed on oligonucleotides that contained flanking sequences from the left side of the M site and the right side of the X site exhibited end preferences more similar to those observed at the M site (Fig. 2B, compare \(M_X\) and M). The subunits containing positively charged amino acid residues (FosKRR and JunKRR) exhibited a stronger preference for binding to the left side of the X site than the XM site. Conversely, subunits containing negatively charged residues (FosEEE and JunEEE) exhibited a modest preference for binding to the left side of the M site but had little effect on the orientation preference at the MX site. Thus, both the left and the right sides of the X and M sites affected the orientation of heterodimer binding, but differences between the left sides of the X and M sites contributed more to the opposite binding orientations at these sites.

Influence of Single Base Pair Substitutions—The X and M sites differ at multiple positions flanking the core AP-1 recognition element. Both sites contained symmetrical base pairs to positions ±5 from the center of the AP-1 site. The binding orientations at these sites must therefore be determined by sequences more than 5 base pairs from the center of the AP-1 site. To investigate the contributions of individual flaking
FIG. 2. Sequences flanking the AP-1 site and amino acid residues adjacent to the basic regions of Fos and Jun determine the orientation of heterodimer binding. A, gelFRET analysis of Fos-Jun heterodimer binding orientations at the site used for crystallization of the Fos-Jun-AP-1 complex (X site). Heterodimers formed by Fos and Jun containing the amino acid residues indicated (above the lanes) adjacent to their basic regions were incubated with AP-1 site oligonucleotides labeled with fluorescein on either the left (L) or the right (R) end as indicated below the lanes. Each pair of lanes contained the same heterodimer labeled with Texas Red (TR) on the subunit indicated above the lanes. The complexes (upper bands) were separated by PAGE from free DNA (lower bands) and nonspecific complexes. The gel was scanned using a laser (488 nm) that excites the fluorescein donor, and the donor (green) and acceptor (red) emissions at each position in the gel were measured. The images corresponding to donor and acceptor emissions were superimposed. Thus, orange bands correspond to complexes with high energy transfer, and green bands correspond to complexes with low energy transfer. The diagrams below the gel indicate the preferred binding orientations of the heterodimers in the corresponding lanes. The result is representative of three independent experiments. B, end preferences of heterodimers formed by different Fos and Jun subunits at binding sites containing different combinations of flanking sequences. The end preferences of heterodimers labeled on Fos (red bars) and Jun (blue bars) containing the amino acid residues indicated below the bars adjacent to their basic regions are shown pairwise at the binding site indicated above each graph. A high end preference indicates that the labeled subunit favors binding to the left half-site, whereas a low end preference indicates that the labeled subunit favors binding to the right half-site (arrow on right). The end preferences of control heterodimers containing the amino acid substitutions R155I in Fos and R273I in Jun (indicated by RI) are shown in the subpanels on the right. The X site corresponds to the sequence used for crystallization of the Fos-Jun-AP-1 complex (19). The Xrev site contains the same flanking sequences on the opposite sides of the core AP-1 site. The M site contains nearly symmetrical flanking sequences (italics). The XM and MX sites contain flanking sequences from the left side of the X site and the right side of the M site and vice versa. Standard deviations are shown for end preferences at the X and M sites from three or more independent experiments.
The end preferences of Fos TR-Jun and Fos-Jun TR heterodimers are shown in red and blue, respectively. The base pair substitutions are indicated in larger type above each graph. The end preferences of FosKRR and JunKRR heterodimers are shown in red and blue, respectively.

The differences between the end preferences of various Fos-Jun complexes allow determination of their relative orientation preferences. However, it is not possible to determine the absolute orientation preference of a specific complex without knowing the relationship between the end preference values and the fraction of complexes bound in each orientation. To determine the absolute orientation preferences of Fos-Jun heterodimers, we used calibration standards to estimate the end preferences of fully oriented complexes. The ratio between the end preference values and the fraction of complexes bound in each orientation was then calculated based on the proportionality between differences in end preference and orientation preference (see under "Experimental Procedures"). We describe two independent strategies for calibration of the gel-FRET assay. We used both approaches to quantify the contribution of individual base pairs to the opposite orientations of heterodimer binding at the X and M sites.

The cooperative interaction between Fos-Jun and NFAT1 requires a specific orientation of Fos-Jun heterodimer binding (8, 9, 11). Thus, quaternary Fos-Jun-NFAT1 complexes provide calibration standards that are predicted to exhibit a strong preference for binding in one orientation. One limitation of this calibration strategy is that the heterodimers can be oriented in only one of the two opposite orientations. Furthermore, heterodimers with strong orientation preferences in the absence of

**Fig. 3. Effects of single base pair substitutions on heterodimer binding orientations.** The end preferences of heterodimers formed by Fos and Jun subunits containing different amino acid residues adjacent to their basic regions (indicated below each graph) were determined at binding sites in which base pairs were exchanged between the X and M sites. The base pair substitutions are indicated in larger type above each graph. The end preferences of FosKRR and JunKRR heterodimers are shown in red and blue, respectively.
NFAT1 can influence the end preferences of Fos-Jun-NFAT1 complexes (8). NFAT1 binding may also influence energy transfer through mechanisms unrelated to the orientation of heterodimer binding. For purposes of these experiments, we assumed that 1) the end preferences of Fos-Jun-NFAT1 complexes represented a good estimate of the end preferences...
of fully oriented Fos-Jun heterodimers and that 2) the end preferences of complexes bound in opposite orientations were equivalent for complexes labeled on different subunits.

To test the validity of the calibration of the gelFRET assay using Fos-Jun-NFAT1 complexes, we compared the effects of symmetry-related single base pair substitutions on opposite sides of the AP-1 site on the orientation of heterodimer binding (Fig. 4). These base substitutions were predicted to have effects of equal magnitudes but opposite directions on the orientation preference. The base pairs in the NFAT recognition sequence had a moderate effect on heterodimer end preferences in the absence of the base substitutions (Fig. 4A, N). Single base pair substitutions at the −6 and +6 positions shifted the end preferences in opposite directions (Fig. 4A, N−6T and N+6A). These base substitutions had converse effects on the end preferences of all heterodimers. The subunits containing negatively charged amino acid residues adjacent to the basic region (FosEEE and JunEEE) favored binding to the half-sites proximal to the base substitutions, whereas the subunits containing positively charged residues (FosKRR and JunKRR) favored binding to the distal half-sites. The end preferences of quaternary Fos-Jun-NFAT1 complexes were virtually unaffected by the base substitutions flanking the AP-1 site (Fig. 4B, N−6T, N and N+6A). The fraction of Fos-Jun heterodimers bound in each orientation was calculated based on the end preferences of the Fos-Jun heterodimer in the absence of NFAT1 and the same Fos-Jun heterodimer in the Fos-Jun-NFAT1 complex (see under “Experimental Procedures”).

The Free Energy of Heterodimer Reorientation—To facilitate comparison of the orientation preferences of different complexes, we define the free energy of reorientation (ΔGORI) as the difference in Gibbs free energies between complexes formed by opposite orientations of heterodimer binding (Jun-Fos versus Fos-Jun). This difference is equivalent to the difference in binding energies between heterodimers bound in opposite orientations to the same recognition site (ΔGORI = ΔGbind,Fos-Jun − ΔGbind,Fos-Jun)The free energy of reorientation was calculated based on the assumption that the fraction of heterodimers bound in each orientation represents the equilibrium distribution under the experimental conditions (see under “Experimental Procedures”). This assumption is consistent with the relatively rapid Fos-Jun reorientation (18) compared with the time required for gel electrophoresis and fluorescence imaging.

To compare the effects of individual amino acid or base pair substitutions on the orientation preference, we calculate the difference in the free energy of reorientation between complexes with and without the substitution (ΔΔGORI). This difference represents the change in the free energy of reorientation caused by the substitution in the specific context examined. The changes in the free energy of reorientation caused by the symmetry-related −6T and +6A substitutions at the N site were nearly identical in magnitude but of opposite signs, indicating that these base substitutions had equal effects in opposite directions on the orientation preferences of all complexes (Fig. 4C).

The use of Fos-Jun-NFAT1 complexes for calibration of the gelFRET assay is directly applicable only to binding sites that can be placed adjacent to an NFAT recognition sequence. To develop a more general calibration strategy, we exploited the effects of amino acid (RI) substitutions in the basic regions of Fos and Jun that shift the binding orientation in opposite directions (Figs. 2, 3, and 4D). The end preferences of these complexes were used to calibrate the gelFRET assay (see under “Experimental Procedures”). To test the validity of calibration of the gelFRET assay using heterodimers in which the arginine residue in either subunit was replaced, we compared the changes in the free energy of reorientation (ΔΔGORI) calculated based on FosRI-Jun and Fos-JunRI calibration standards (Fig. 4E) with that obtained by calibration using Fos-Jun-NFAT1 complexes (Fig. 4C). The effects of the flanking base pair substitutions on the free energy of reorientation were virtually identical based on both approaches. The symmetry-related base substitutions on opposite sides of the AP-1 site had equivalent effects in opposite directions on the orientation preferences of all heterodimers. The effects of the −6T and +6A substitutions were therefore independent of other asymmetric base pairs in the N site. Fos labeled with Texas Red exhibited a slightly greater preference for occupying the half-site distal from the base substitution. This may reflect a small effect of the fluorophore on the orientation preferences of the heterodimers. However, this trend does not interfere with analysis of either the effects of amino acid substitutions or the influence of flanking base pairs on the orientation of heterodimer binding since the effect was small and affected all heterodimers to a similar extent. The comparable results from calibration of the gelFRET assay using Fos-Jun-NFAT1 complexes as well as FosRI-Jun and Fos-JunRI heterodimers validate the quantitative analysis of the orientation of Fos-Jun heterodimer binding.

Independent Effects of Amino Acid Substitutions in Fos and in Jun on Orientation Preference—Amino acid residues adjacent to the basic regions of both Fos and Jun influence the orientation of heterodimer binding. To determine if these amino acid residues affected the binding orientation independently or interacted with each other, we compared the effects of all amino acid substitutions in complexes formed with different dimerization partners (Fig. 5). The effect of each amino acid substitution was calculated based on the difference in the free energy of reorientation (ΔΔGORI) between heterodimers that differ only in the amino acid residues adjacent to the basic region of one subunit. Each amino acid substitution caused a characteristic change in the free energy of reorientation that was virtually unaffected by the dimerization partner, indicat-
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Quantitative Effects of Base Substitutions on Orientation Preference—Calibration of the gelFRET assay makes it possible to compare the quantitative effects of base substitutions at different binding sites on the orientation preferences of Fos-Jun heterodimers. We determined the change in the free energy of reorientation caused by the exchange of charged amino acid residues between Fos and Jun at each binding site (Fig. 6). The changes in the free energy of reorientation were determined separately for heterodimers labeled on Fos and on Jun to eliminate the possibility that the differences in orientation preferences were caused by the fluorophore label and to obtain independent verification of the results.

As shown by the qualitative analysis of end preferences, substitution of the the T:A base pair at the −6 position of the X site by a G:C base pair from the M site had a larger effect on heterodimer orientation than the substitutions at the −7 or −8 positions (Fig. 6, X-6G, X-7T, and X-8A). The complementary substitution of a G:C base pair at the −6 position in the M site by a T:A base pair had a reciprocal effect on heterodimer orientation (Fig. 6, M-6T). Likewise, complementary base substitutions at the −7 and −8 positions of the M site had small effects on heterodimer orientation (Fig. 6, M-7C and M-8C). Exchange of the asymmetric base pairs at the ±10 positions of the M site reversed the change in the free energy of reorientation caused by the charged amino acid residues (Fig. 6, M-10C and M+10T). Likewise, exchange of the sequences flanking the X site reversed the effect of electrostatic interactions on the free energy of reorientation (Fig. 6, Xrev). Substitution of the G:C base pair at the −5 position of a symmetrical binding site by an A:T base pair from the X site had little effect on heterodimer binding orientations (Fig. 6, compare S and S-5A). A binding site containing asymmetric base pairs at all positions with the exception for the ±6 and ±10 positions exhibited little effect of the exchange of amino acid residues on heterodimer orientation (Fig. 6, MX). Thus, the asymmetric base pairs at the ±6 and ±10 positions were the principal determinants of the difference in heterodimer binding orientations at the X and M sites.

To investigate the influence of sequence context on the effects of asymmetric base pairs on heterodimer orientation, we compared the effects of base substitutions at the ±6 positions in different binding sites. The base substitutions at the −6 and −6 positions in the N site had effects of similar magnitudes on heterodimer binding orientations (Fig. 6, compare N-6T and N+6A versus N). Thus, the other asymmetric base pairs in the N site did not alter the effects of these base substitutions. The same base substitutions in the M-6T and X-6G sites had slightly smaller effects on heterodimer orientations (compare with the M and X sites). To investigate the effects of these base pairs in a different sequence context and at a different position flanking the AP-1 site, we inserted these base pairs at the ±6 and ±7 positions of otherwise symmetrical binding sites. Insertion of the asymmetric (T:A and C:G) base pairs at the ±6 positions favored the same orientation of heterodimer binding as they did at the N-6T, M-6T, X, and XM sites. However, the effect of the asymmetric base pairs at the ±6 positions on the change in the free energy of reorientation at this binding site was smaller than their effect at other binding sites (Fig. 6, I-6T I+6C). Insertion of the same asymmetric base pairs at the ±7 positions also favored heterodimer binding in the same orientation. Moreover, the effect of the asymmetric base pairs at ±7 positions on the change in the free energy of reorientation at this binding site was similar to their effect at the ±6 positions (Fig. 6, I-7T I+7C). Thus, the same asymmetric base pairs at the ±6 and ±7 positions can have similar effects on heterodimer orientation preferences, and neighboring base pairs can influence the magnitudes of these effects.

### Table 1: Quantitative Effects of Base Substitutions on Orientation Preference

| Site   | Sequence | ΔΔGobs (kJ mol⁻¹) |
|--------|----------|------------------|
| X      | ACTTTTGGATTGGACTGCCT       | -10.1 -9.6       |
| X-6G   | ACTTTTGGATTGGACTGCCT       | -5.1 -4.8        |
| X-7T   | ACTTTTGGATTGGACTGCCT       | -13.6 -9.3       |
| X-8A   | ACTTTTGGATTGGACTGCCT       | -9.4 -9.9        |
| M      | ACTTTTGGATTGGACTGCCT       | 1.0 3.4          |
| M-6T   | ACTTTTGGATTGGACTGCCT       | -3.2 -3.6        |
| M-7C   | ACTTTTGGATTGGACTGCCT       | 1.3 2.2          |
| M-8C   | ACTTTTGGATTGGACTGCCT       | 0.4 1.7          |
| M-10C+10T | ACTTTTGGATTGGACTGCCT       | -1.4 -3.9       |
| Xrev   | ACTTTTGGATTGGACTGCCT       | 11.3 13.4        |
| XM     | ACTTTTGGATTGGACTGCCT       | -5.2 -5.1        |
| MX     | ACTTTTGGATTGGACTGCCT       | -1.1 0.5         |
| S-5A   | ACTTTTGGATTGGACTGCCT       | -0.1 0.3         |
| S      | ACTTTTGGATTGGACTGCCT       | 0.4 0.1          |
| N      | ACTTTTGGATTGGACTGCCT       | 1.9 2.0          |
| N-6T   | ACTTTTGGATTGGACTGCCT       | -6.0 -7.0        |
| N+6A   | ACTTTTGGATTGGACTGCCT       | 8.8 9.1          |
| I-6T+6C| ACTTTTGGATTGGACTGCCT       | -3.4 4.2         |
| I-7T+7C| ACTTTTGGATTGGACTGCCT       | -4.5 3.8         |

**Fig. 6.** Quantitative effects of sequences flanking the AP-1 site on the orientation of Fos-Jun binding. The change in the free energy of reorientation caused by the exchange of charged amino acid residues between Fos and Jun (ΔΔGobs; FosEEE-JunEEE vs. FosKRR-JunKAE, see Fig. 5) was determined at each binding site. The free energy change was calculated independently for heterodimers labeled on Fos (Fos+Jun) and on Jun (Jun+Jun). Single base pair substitutions are indicated in boldface. Asymmetric base pairs at the ±6 and ±10 positions are emphasized by enclosing the base pairs favored by the negative charged subunits (FosEEE and JunEEE) in boxes.
Orientation Preferences of Full-length Fos and Jun—Previous studies of the orientation of Fos-Jun binding have been limited to analysis of truncated peptides encompassing the bZIP domains (8, 11, 12, 20). These truncated peptides differ from native Fos and Jun both in length and in amino acid sequence. Regions outside the bZIP domains of Fos and Jun are essential for transcription activation (28) and influence DNA structure in the Fos-Jun-AP-1 complex (21, 26). To investigate the orientation preferences of full-length Fos and Jun, we examined the end preferences of heterodimers formed by full-length Fos and Jun with labeled Fos and Jun bZIP domains (Fig. 7). The end preferences of heterodimers formed by full-length Fos and Jun with the corresponding bZIP domains were similar to those of the same heterodimers formed by FosKRR and JunEEE (Fig. 7, compare upper and lower panels). Amino acid substitutions adjacent to the basic regions of the bZIP domains had comparable effects on the end preferences of heterodimers formed by both full-length and truncated Fos and Jun. Symmetry-related base substitutions on opposite sides of the AP-1 site had converse effects on the end preferences of heterodimers formed by wild type Fos-Jun as well as heterodimers in which the arginine that can contact the central guanine was replaced by an isoleucine in either subunit. The dissociation rates were compared at binding sites containing central C:G or G:C base pairs that favor opposite orientations of binding by heterodimers in which one arginine was replaced (12, 20). In the absence of NFAT1, there was no significant difference (less than 2-fold) between the dissociation rates from these two binding sites for any of the heterodimers (data not shown). In the presence of NFAT1, the dissociation rate of wild type Fos-Jun heterodimers was reduced by ~100-fold at both binding sites (Fig. 8A, upper panels). The similar dissociation rates at both sites are consistent with the weak orientation preference of Fos-Jun heterodimer binding at these sites in the absence of NFAT1 (8) and the small effect of the central base pair on the orientations of heterodimers formed by wild type Fos and Jun.

In contrast, Fos-Jun-NFAT1 complexes formed by heterodimers in which the arginine in Fos was replaced by an isoleucine (FosRI-Jun) exhibited a more than 10-fold slower dissociation rate at the binding site containing a central G:C base pair than at the site containing a C:G base pair (Fig. 8A, middle panels). The effect of the central base pair was even

Functional Effects of the Orientation Preference of Fos-Jun Binding—The preferred orientation of Fos-Jun heterodimer binding may influence interactions with transcription factors that bind to adjacent recognition sequences. We examined the influence of the preferred orientation of heterodimer binding on cooperative DNA binding and transcription activation by Fos-Jun and NFAT1. The dissociation of Fos-Jun heterodimers from composite regulatory elements was measured in the presence and absence of NFAT1 by monitoring the time-dependent change in fluorescence resonance energy transfer following addition of unlabeled competitor DNA (Fig. 8A). We examined the dissociation rates of heterodimers formed by wild type Fos-Jun as well as heterodimers in which the arginine that can contact the central guanine was replaced by an isoleucine in either subunit. The dissociation rates were compared at binding sites containing central C:G or G:C base pairs that favor opposite orientations of binding by heterodimers in which one arginine was replaced (12, 20). In the absence of NFAT1, there was no significant difference (less than 2-fold) between the dissociation rates from these two binding sites for any of the heterodimers (data not shown). In the presence of NFAT1, the dissociation rate of wild type Fos-Jun heterodimers was reduced by ~100-fold at both binding sites (Fig. 8A, upper panels). The similar dissociation rates at both sites are consistent with the weak orientation preference of Fos-Jun heterodimer binding at these sites in the absence of NFAT1 (8) and the small effect of the central base pair on the orientations of heterodimers formed by wild type Fos and Jun.

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larger for heterodimers in which the arginine in Jun was replaced by an isoleucine (Fos-JunRI), but the relative stabilities of Fos-Jun-NFAT1 complexes at the two binding sites were reversed (Fig. 8A, lower panels). In both cases, the slower dissociation rate was observed for complexes in which the preferred orientation of heterodimer binding in the absence of

FIG. 8. Functional effects of the preferred orientation of Fos-Jun heterodimer binding. A, effects of the preferred orientation of heterodimer binding on the dissociation rates of Fos-Jun-NFAT1 complexes. The changes in fluorescence emissions from fluorescein (green) and Texas Red (red) were monitored following the addition of competitor DNA to Fos-Jun-NFAT1 complexes at the binding sites shown above the graphs. The Fos-Jun-NFAT1 complexes were prepared using heterodimers with opposite orientation preferences as indicated by the diagrams in the graphs (a circular arrow above the heterodimer indicates no orientation preference). Some heterodimers contained substitutions at the arginine residues that can contact the central base pair in either Fos (FosRI) or Jun (JunRI). The changes in fluorescence emissions were normalized to the same range to facilitate comparison of the rates. The average half-life of each complex is indicated. The non-reciprocal changes in fluorescein and Texas Red fluorescence after prolonged incubation were caused by bleaching of fluorescein. B, effects of the preferred orientation of heterodimer binding on the transcriptional activities of Fos-Jun-NFAT1 complexes. The in vitro transcriptional activities of heterodimers formed by full-length Fos and Jun with or without NFAT1 were measured at promoters containing the composite regulatory elements indicated on the left. Each reaction contained two templates that differed only by the single base pair indicated in large type at the center of the AP-1 site and by the length of the transcription unit. An AdML template was used as an internal reference. The nuclear extract transcription reactions were supplemented by the proteins indicated above the lanes, and the transcripts were analyzed by denaturing PAGE followed by autoradiography. A shorter exposure of the last lane is shown to the right of the upper panel. The templates used in the experiment shown in the lower panel contained the NFAT recognition sequence on the opposite side of the AP-1 site as shown on the left. In addition, the complexes were incubated for 1 min in the presence of competitor oligonucleotides (2 μM) containing either the NFAT site alone (lanes 2–4) or the composite NFAT-AP-1 site (lanes 1 and 5–7) prior to addition of nuclear extract and nucleotides.
NFAT1 was the same as the orientation in the Fos-Jun-NFAT1 complex. Thus, different combinations of the same amino acid and base pair substitutions have distinct effects on the stability of the Fos-Jun-NFAT1 complex depending on their effects on the orientation of heterodimer binding. The larger difference in the stabilities of complexes at the two binding sites for Fos-JunRI heterodimers compared with FosRI-Jun heterodimers is consistent with the preferential interaction between the central guanine and the arginine in Fos (12, 20). Consequently, the preferred orientation of Fos-Jun heterodimer binding can influence cooperative interactions with other transcription factors at composite regulatory elements.

The differences in Fos-Jun-NFAT1 complex stabilities caused by opposite orientations of Fos-Jun binding may influence the transcriptional activities of Fos-Jun-NFAT1 complexes. We examined transcription activation by full-length Fos-Jun and NFAT1 using in vitro transcription reactions containing two templates with composite NFAT-AP-1 regulatory elements that favored opposite orientations of heterodimer binding (Fig. 8B). The promoters on the two templates differed by a single base pair at the center of the AP-1 site that controls the prefered binding orientations of heterodimers in which one of the arginines that can contact this base pair has been replaced (12, 20). The promoters were linked to transcription units of different lengths to allow comparison of the transcriptional activities of the two promoters in the same reaction. In the absence of added NFAT1, heterodimers formed by wild type Fos and Jun as well as subunits containing arginine substitutions exhibited modest activation of both promoters. In the presence of NFAT1, wild type Fos-Jun heterodimers exhibited robust activation of both promoters. In contrast, heterodimers in which the arginine in Fos was replaced (FosRI-Jun) preferentially activated transcription from the promoter containing a central G:C base pair. Heterodimers in which the arginine in Jun was replaced (Fos-JunRI) exhibited an even greater bias but selectively activated transcription from the promoter containing a central C:G base pair. Thus, heterodimers with opposite orientation preferences exhibited reciprocal patterns of transcription activation at promoters that differed by single base pairs. The ratio between the relative efficiencies of transcription activation by heterodimers with opposite orientation preferences at the two promoters was 6. The difference in the lengths of the transcription units did not influence the efficiencies of transcription activation since the relative amounts of the two transcripts were reversed by exchange of the promoters between the two transcription units (data not shown). Thus, the orientation preference of Fos-Jun heterodimer binding can influence both transcriptional activity and promoter selectivity.

The influence of the preferred orientation of Fos-Jun binding on transcriptional activity at these promoters was presumably due to their cooperative interaction with NFAT1. However, heterodimers in which the arginine residue in one subunit was replaced exhibited differential activation of the two promoters even in the absence of added NFAT1 (Fig. 8B, upper panel, lanes 3 and 4). To confirm that the influence of heterodimer orientation preference on transcriptional activity reflected the interaction with NFAT1, we examined transcription activation on templates where the NFAT recognition sequence was moved to the opposite side of the AP-1 site by substitution of 2 base pairs on each side of the AP-1 site (Fig. 8B, lower panel). To reduce the effects of endogenous activators in the nuclear extract, transcription was performed in the presence of competitor oligonucleotides. Under these conditions, heterodimers in which the arginine residue was replaced exhibited comparable activation of both promoters in the absence of added NFAT1. In the presence of added NFAT1, the heterodimers in which different arginine residues were replaced exhibited selective activation of different promoters. The ratio between the relative transcriptional activities of heterodimers with opposite orientation preferences at the two promoters was 15. Significantly, the relative activities of promoters containing central C:G and G:C base pairs were reversed by transfer of the NFAT recognition sequence to the opposite side of the AP-1 site. Consequently, the orientation preference of heterodimer binding can influence the synergistic activation of transcription by Fos-Jun and NFAT1 at composite regulatory elements.

**DISCUSSION**

Synergistic control of gene expression frequently depends on a specific arrangement of regulatory elements within promoter and enhancer regions (2, 3, 5). The structural organization of transcription factor complexes is therefore likely to have a crucial role in the control of transcriptional activity. The sequence of the DNA binding site can influence the conformation of transcription regulatory proteins (29). The functions of transcription factor complexes can be affected by conformational changes caused by differences in the sequence of the regulatory element (30–32). Opposite orientations of heterodimer binding represent perhaps the most dramatic conformational change in terms of its effects on interactions with adjacent regulatory proteins. The gelFRET assay provides a quantitative tool for analysis of conformational variation in nucleoprotein complexes.

Quantitative analysis of the orientation preferences of Fos-Jun heterodimers at different AP-1 sites demonstrated that base pairs that are far from the core AP-1 recognition sequence can influence the orientation of Fos-Jun heterodimer binding. The sequences flanking AP-1 sites in different promoters and enhancers are distinct, suggesting that the preferred orientation of Fos-Jun binding varies between different regulatory regions. It is also possible that transcription factor binding to sequences adjacent to the AP-1 site can influence the orientation of Fos-Jun binding even in the absence of direct contacts between the proteins. This mechanism could contribute to the regulatory interactions that have been observed between Fos-Jun and a variety of structurally unrelated transcription factors that bind to adjacent recognition elements (10, 23, 24).

The effects of base substitutions at the ±6 positions as well as amino acid substitutions adjacent to the basic regions on heterodimer orientation are highly correlated with their effects on DNA bending in the Fos-Jun-AP-1 complex (12, 14, 20). The largest effects of asymmetric base pairs on heterodimer orientation were observed at the ±6 and ±10 positions where the major or minor groove faces the same side of the DNA helix as the amino acid residues adjacent to the basic region that control the binding orientation. This is consistent with the preferred mode of DNA bending by roll into the major and minor grooves (33). Base pairs flanking the core AP-1 recognition element affected both the binding orientation and DNA bending by Fos and Jun without the requirement for direct amino acid-nucleotide contacts (12). One mechanism whereby flanking DNA sequences influence the orientation of Fos-Jun binding involves electrostatic interactions between charged amino acid residues in Fos-Jun and the negatively charged phosphodiester backbone of DNA (14).

The free energy of Fos-Jun heterodimer reorientation at all binding sites examined was relatively small compared with the free energy of Fos-Jun binding to the AP-1 site. It was also smaller than the free energy of Fos-Jun interactions with NFAT1 since NFAT1 can reverse the orientation of Fos-Jun binding at all composite regulatory elements examined (8). Thus, the preferred orientation of heterodimer binding does not...
prevent formation of Fos-Jun-NFAT1 complexes at binding sites where Fos-Jun alone bind in the disfavored orientation. However, the orientation preference of Fos-Jun binding affected the relative stabilities of Fos-Jun-NFAT1 complexes at different composite regulatory elements. Moreover, the orientation preference affected the relative transcriptional activities of templates containing composite regulatory elements with opposite preferred orientations of heterodimer binding. Thus, the orientation preference of heterodimer binding can control both the transcriptional activity and promoter selectivity of Fos-Jun-NFAT1 complexes.

The arginine substitutions that shift the preferred orientation of Fos-Jun heterodimer binding also reduce the binding affinity of Fos-Jun heterodimers. However, the reduction in binding affinity did not cause the orientation-dependent transcriptional activity since the same change in binding affinity was observed regardless of the central base pair, and the heterodimer with a higher binding affinity (Fos-JunRI) exhibited the greater orientation dependence. Moreover, transcription activation by Fos-Jun-NFAT1 complexes formed by wild type Fos-Jun heterodimers was also affected by the preferred orientation of heterodimer binding (18). A Fos-Jun-NFAT1 complex at a particular regulatory element is formed in competition with all other accessible sites. Thus, the orientation preference of Fos-Jun heterodimer binding is likely to influence the occupancy and the transcriptional activity of composite regulatory elements in the cell.

The influence of the orientation of heterodimer binding on transcriptional activity and promoter selectivity emphasizes the importance of conformational variation among transcription regulatory proteins for transcriptional activity. Such conformational variability can influence both cooperative interactions among transcription factors bound to composite regulatory elements as well as interactions with coactivators and corepressors (13, 31, 32). The conformational variations of transcription regulatory proteins are likely to represent equilibria among several conformations. The balance of such equilibria can be controlled by relatively subtle structural determinants. This provides the potential for control of transcriptional activity through shifts in the equilibrium between different conformational states.

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