Opposite Orientations of a Transcription Factor Heterodimer Bind DNA Cooperatively with Interaction Partners but Have Different Effects on Interferon-β Gene Transcription

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Background: Nucleoprotein complexes are frequently assumed to adopt a unique configuration.

Results: ATF2-Jun heterodimers bind the interferon-β enhancer in two opposite orientations in association with IRF3 and HMGI. Opposite heterodimer orientations exhibited the same cooperativity of ATF2-Jun-IRF3-HMGI complex formation, but had distinct transcriptional activities.

Conclusion: ATF2-Jun heterodimer orientation did not affect binding cooperativity, but affected transcriptional activity.

Significance: Changes in transcription complex configuration can regulate transcriptional activity.

ATF2-Jun, IRF3, and HMGI recognize a composite regulatory element within the interferon-β enhancer (IFNb). Cooperative ATF2-Jun-IRF3 complex formation at IFNb has been proposed to require a fixed orientation of ATF2-Jun binding. Our results show that ATF2-Jun heterodimers bound IFNb in both orientations alone and in association with IRF3 and HMGI. Two sets of symmetrically located amino acid residues in ATF2 and Jun facilitated the interactions between heterodimers bound in opposite orientations and IRF3 at IFNb. IRF3 and HMGI bound IFNb in association with both orientations of ATF2-Jun heterodimers with the same cooperativity. ATF2-Jun heterodimers that bound IFNb in opposite orientations in vitro had different effects on interferon-β gene transcription when they were co-expressed with IRF3 in cultured cells. These heterodimers had different transcriptional activities at different endogenous genes. Different regions of ATF2 and Jun mediated their orientation-dependent transcriptional activities at different genes. These studies revealed that cooperative DNA binding does not require a unique nucleoprotein complex configuration, and that transcription factor complexes that bind the same enhancer in different configurations can have different transcriptional activities.

Transcription initiation is controlled by multi-protein transcription factor complexes assembled at regulatory elements and at the site of transcription initiation. Cooperative DNA binding by transcription factors that bind to closely juxtaposed sequences (composite regulatory elements) are generally thought to require a specific arrangement of the transcription factors on DNA. Conversely, many mechanisms of transcription activation, including changes in chromatin structure and covalent protein modifications are not thought to require a specific arrangement of transcription factors on DNA.

Some heterodimeric transcription factors can bind their recognition sequences in two opposite orientations (1, 2). Interactions between such heterodimers and transcription factors that bind adjoining sequences are generally thought to require a specific orientation of heterodimer binding. For instance, cooperative DNA binding by Fos-Jun, Fos-Activating Transcription Factor 2 (ATF2),2 or ATF2-Jun heterodimers with Nuclear Factor of Activated T cells 1 (NFAT1) at composite regulatory elements imposes a fixed orientation of heterodimer binding (3–7). Differences in the preferred orientation of Fos-Jun heterodimer binding at different regulatory elements affect cooperative DNA binding and synergistic transcription activation with NFAT1 (6–8).

The interferon-β enhancer contains closely juxtaposed recognition sequences for ATF2-Jun heterodimers, IRF3 and HMGI (supplemental Fig. S1). Studies using different experimental approaches, including photo-crosslinking, x-ray crystallography, and equilibrium binding assays have produced contradictory interpretations concerning the effects of IRF3 on the orientation of ATF2-Jun heterodimer binding, as well as concerning the cooperativity of complex formation (9–11). Photo-crosslinking and x-ray crystallography were interpreted to show opposite orientations of ATF2-Jun heterodimer binding in the presence of IRF3, whereas no orientation preference was detected using fluorescence polarization (9–11). The results from x-ray crystallography and mutational analyses were interpreted to demonstrate that the cooperativity of ATF2-Jun-IRF3 binding was mediated by a change in DNA structure with no requirement for direct contacts between ATF2-Jun and IRF3 (10). In contrast, no binding cooperativity was detected by isothermal titration calorimetry (11). The effects of HMGI on the configuration and cooperativity of ATF2-Jun and IRF3 binding at the interferon-β enhancer have not been established.

1 This article contains supplemental Figs. S1–S14 and Table S1.
2 The abbreviations used are: ATF2, Activating Transcription Factor 2; Jun, c-Jun; IRF3, Interferon Regulatory Factor 3; bZIP, basic-region leucine zipper; gelFRET, gel-based fluorescence resonance energy transfer.

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The mechanisms of transcription activation by the complex assembled at the interferon-β enhancer are incompletely understood. Some of these mechanisms have been proposed to involve covalent protein modifications, whereas others invoke interactions between the complex assembled at the enhancer and the transcription initiation complex at the promoter (12–15). Many of these are thought to be mediated by co-factors that do not directly bind to the enhancer (12, 13, 15). A majority of previous studies investigating the mechanisms that regulate interferon-β transcription have been performed using in vitro assays or extrachromosomal reporter genes (12–14). Studies of endogenous gene regulation are necessary to establish the mechanisms that regulate genes in their normal context.

We investigated the effects of interactions with IRF3 and HMGI on the orientation of ATF2-Jun heterodimer binding at the interferon-β enhancer and the effects of heterodimer orientation on the cooperativity of complex formation in vitro and on endogenous interferon-β gene transcription in cells. Our results demonstrated that the ATF2-Jun-IRF3-HMGI complex assembled at the interferon-β enhancer in two different configurations that had distinct transcriptional activities.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—bATF2 (residues 321–397 of human ATF-2, T321C, C351S), ATF2 (full-length human ATF2 (residues 1–505)), bJun (residues 239–315 of human c-Jun, T239C, C269S), Jun (full-length human Jun (residues 1–331)), iIRF3 (resides 1–111 of human IRF-3), and HMGI (full-length human HMGI (residues 1–107)) were expressed in E. coli using plasmids pcDNA3.1 (Invitrogen, Grand Island, NY). ATF2, Jun, and full-length IRF3 were also expressed in Hela cells using pCDNA3.1+(Invitrogen, Grand Island, NY).

**Protein Purification and Labeling**—bATF2, ATF2, bJun, Jun, iIRF3, and HMGI were purified under the conditions described previously (16). The SUMO tag was cleaved using ULP1 protease, which were removed by nickel-chelate chromatography. bATF2 and bJun were labeled on N-terminal cysteine residues using Texas red C5 bromoacetamide (Molecular Probes, Eugene, OR). Protein labeling efficiency was determined by measuring the fluorescence intensity and Coomassie staining of proteins separated by SDS-PAGE.

**Oligonucleotide Labeling**—IFNb oligonucleotides (comprising 109 to –75 of the human interferon-β gene) labeled at their 5′-ends by 6-carboxyfluorescein (FAM) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Duplexes were annealed and purified by PAGE.

**gelFRET Analysis of Heterodimer Orientation**—Complexes were formed by incubation of 400–800 nM heterodimers containing one Texas red-labeled subunit with 500 nM IFNb oligonucleotides labeled at one end with FAM. 1.5 μM iIRF3 and 3 μM HMGI were included where indicated. The complexes were separated by native PAGE, and their fluorescence intensities were imaged using a 488-nm laser in a Typhoon scanner (GE Healthcare, Piscataway, NJ). Cross-talk between the donor and acceptor fluorescence signals was corrected by using standards.

The end preference values were calculated by dividing the acceptor-to-donor emissions ratio for left end-labeled complexes and right end-labeled complexes (TR<sub>α</sub>/FAM<sub>α</sub> and TR<sub>β</sub>/FAM<sub>β</sub>) by the sum of the acceptor-to-donor emission ratios for left end-labeled complexes and right end-labeled complexes (TR<sub>α</sub>/FAM<sub>α</sub> + TR<sub>β</sub>/FAM<sub>β</sub>) (17).

**Measurement of the Levels of Endogenous Transcripts in Cells Expressing Different Combinations of ATF2 and Jun Variants in Combination with IRF3—**HeLa cells were transfected with the indicated combinations of plasmids using FuGENE6 (Roche, Nutley, NJ). Cells were infected with 200 hemagglutinin units/ml of Sendai virus (Charles River Laboratories, Wilmington, MA). Six hours, or the indicated time, after infection, the cells were harvested and total mRNA was isolated, reverse transcribed, and quantified by RT-qPCR using the primers listed in supplemental Table S1.

**RESULTS**

**bATF2-bJun Heterodimers Bind the Interferon-β Enhancer in Both Orientations**—To establish whether ATF2-Jun heterodimers encompassing the bZIP dimerization and DNA binding domains (bATF2-bJun, supplemental Fig. S1A) bind the interferon-β enhancer (IFNb, supplemental Fig. S1B) in one or both orientations, we used gel-based fluorescence resonance energy transfer (gelFRET) analysis (2). In this approach, the efficiencies of energy transfer are compared among heterodimer-DNA complexes that are separated using gel electrophoresis. The complexes were labeled with a donor fluorophore at either end of the DNA and an acceptor fluorophore on either subunit of the heterodimer. The difference in energy transfer from opposite ends of the oligonucleotide to the labeled subunit was used to determine the end preference (Fig. 1A). The end preferences of complexes labeled on bATF2 (red bars) versus bJun (cyan bars) were compared to determine the preferred orientation of heterodimer binding at IFNb (Fig. 1C). bATF2 and bJun homodimers were used to estimate the end preference values of complexes with no orientation preference. Heterodimers labeled on bATF2 had a higher end preference than bATF2 or bJun homodimers, whereas heterodimers labeled on bJun had a lower end preference than those of the homodimers (Fig. 1C). This indicates that bATF2-bJun bound IFNb in a preferred orientation and that bATF2 favored the left half-site and bJun favored the right half-site.

We examined the effects of amino acid substitutions that were predicted to shift the orientation of bATF2-bJun heterodimer binding (18) (Fig. 1B). Substitution of R352 in bATF2, which can contact the asymmetric guanine in the central dinucleotide, by an alanine (bATF2XG) reversed the relative end preference values of heterodimers labeled on bATF2 versus bJun (Fig. 1C). Conversely, substitution of the homologous arginine (R270) in bJun by an alanine (bJunXG) caused the opposite shift in the end preference values of heterodimers labeled on bATF2 versus bJun. Thus, heterodimers in which these arginine residues that can contact the central guanine were substituted by alanines in different subunits (bATF2XG-bJun versus bATF2-bJunXG) bound IFNb in different orientations (Fig. 1C, p < 0.01). These amino acid substitutions had the converse effects on the orientation of heterodimer binding at sites in which the central base pair was inverted (supplemental Fig. S2). The large differences between the end preference values for bATF2- versus bJun-labeled heterodimers that con-
tained these amino acid substitutions than for wild type bATF2-bJun heterodimers. The data indicate that wild type bATF2-bJun heterodimers did not bind IFNb in only one orientation.

**iIRF3 Binding Does Not Alter the Orientation Preference of bATF2-bJun at IFNb**—We examined the effect of truncated IRF3 encompassing the minimal DNA binding domain (iIRF3:...}

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residues 1 to 111) on the orientation preference of bATF2-bJun binding at IFNβ. iIRF3 binding increased the absolute end preference values for all complexes, but did not change the relative end preference values of heterodimers labeled on bATF2 versus bJun, or of those of bATF2 or bJun homodimers (compare Fig. 1, C and D). The effects of iIRF3 binding on the absolute end preference values were likely caused by changes in the structures of these complexes that did not alter heterodimer orientation. Thus, bATF2-bJun heterodimers in which the arginine residues that can contact the central guanidine were substituted by alanines in different subunits (bATF2XG-bJun versus bATF2-bJunXG) bound IFNβ in different orientations in association with iIRF3 (Fig. 1D, p < 0.05). The absence of a change in bATF2-bJun orientation preference upon iIRF3 binding and the ability of iIRF3 to bind in association with bATF2 homodimers, bJun homodimers, as well as bATF2-bJun heterodimers bound in either orientation suggest that iIRF3 can interact equally with bATF2 and with bJun.

**HMG1 and iIRF3 Form Complexes with Both Orientations of bATF2-bJun Heterodimers at IFNβ**—We investigated if concerted interactions with HMG1 and iIRF3 affected the orientation of bATF2-bJun binding at IFNβ. HMG1 and iIRF3 binding to IFNβ in association with bATF2-bJun reduced the difference in end preference values of heterodimers labeled on bATF2 versus bJun compared with their end preference values alone or in association with iIRF3 (compare Fig. 1E with Fig. 1, C and D). HMG1 and iIRF3 could also bind IFNβ in association with bATF2 and bJun homodimers, as well as with bATF2-bJun heterodimers bound in either orientation. Thus, heterodimers in which the arginine residues that can contact the central guanidine were substituted by alanines in different subunits (bATF2XG-bJun versus bATF2-bJunXG) bound IFNβ in different orientations also in association with iIRF3 and HMG1 (Fig. 1E, p < 0.05). Thus, HMG1 and iIRF3 could interact with both orientations of bATF2-bJun heterodimers, and HMG1 binding reduced the intrinsic orientation preference of bATF2-bJun at IFNβ.

**iIRF3 and HMG1 Have Similar Effects on the Orientations of Heterodimers Containing Full-length ATF2 or Jun as on bATF2-bJun**—We investigated whether iIRF3 and HMG1 affected the orientations of heterodimers formed by full-length ATF2 and Jun with labeled bJun and labeled bATF2, respectively, at IFNβ. We compared the end preferences of these heterodimers alone, in association with iIRF3, and in association with iIRF3 and HMG1. iIRF3 binding had little effect on the relative end preferences of heterodimers containing full-length ATF2 or Jun at IFNβ (supplemental Fig. S3). HMG1 binding reduced the difference in the end preference values of heterodimers containing full-length ATF2 or Jun at IFNβ. Thus, heterodimers formed by full-length and truncated ATF2 and Jun bound to IFNβ in both orientations in association with iIRF3 and HMG1.

iIRF3 and HMG1 also had similar effects on the orientation of bATF2-bJun heterodimer binding when IFNβ oligonucleotides of different lengths and bATF2 and bJun labeled with different acceptor fluorophores were used (supplemental Fig. S4). Taken together, these results suggest that the labeled bATF2 and bJun proteins and the IFNβ oligonucleotide were valid models for studies of the interactions of ATF2 and Jun with iIRF3 and HMG1 at the interferon-β enhancer.

**iIRF3 Interactions with Symmetry-related Amino Acid Residues in bATF2 and bJun Facilitate Opposite Orientations of Heterodimer Binding at IFNβ**—The simplest interpretation of the observation that both orientations of bATF2-bJun heterodimers facilitated iIRF3 binding to IFNβ is that iIRF3 can make equivalent contacts with bATF2 and bJun when the heterodimer binds in opposite orientations. D45 of iIRF3 contacts R345 of ATF2 in the ATF2-Jun-IFNβ crystal in which ATF2 binds to the left half-site (Fig. 2A, upper left quadrant) (10). R345 of ATF2 and R263 of Jun are symmetry-related by a 180° rotation about the dimer axis. We predicted that D45 of iIRF3 contacted R263 of bJun when the heterodimer bound IFNβ in the opposite orientation (Fig. 2A, lower left quadrant).

We tested the effects of substitutions at these symmetry-related arginine residues in bATF2 and bJun on the orientation of heterodimer binding at IFNβ. In the absence of iIRF3, the R345A substitution in bATF2 (bATF2XDG-bJun) and the R263A substitution in bJun (bATF2-bJunXD) had small effects on heterodimer orientation (Fig. 2B). In contrast to the absence of an effect of iIRF3 binding on the orientation of wild type bATF2-bJun heterodimers, iIRF3 binding had dramatic and opposite effects on the orientations of heterodimers containing these substitutions at IFNβ (compare Fig. 2B and Fig. 2C). Thus, heterodimers in which the symmetry-related arginine residues that can contact D45 were substituted by alanines in different subunits (bATF2XG-bJun versus bATF2-bJunXG) bound IFNβ with different orientation preferences in association with iIRF3 (Fig. 2C, p < 0.05). In both complexes, the shift in heterodimer orientation caused by iIRF3 binding reflected a decrease in binding by the mutated (XD) subunit to the left IFNβ half-site proximal to D45 of iIRF3 and a corresponding increase in binding by the wild type subunit to the left IFNβ half-site (Fig. 2E).

Simultaneous substitution of both amino acid residues in bATF2 and bJun (bATF2XDG-bJunXD) eliminated the effect of iIRF3 on the orientation of heterodimer binding. These results indicate that the absence of an effect of iIRF3 binding on wild type bATF2-bJun orientation was due to counterbalancing effects of interactions with symmetry-related residues in bATF2 versus bJun.

We examined the effects of other symmetry-related amino acid residues in ATF2 and Jun that could influence the orientation of heterodimer binding through interactions with iIRF3. Both ATF2 and Jun contain a cluster of negatively charged residues adjacent to their basic regions (E331, D332, D334, E335 in bATF2 and D246, E248, E251 in bJun). These residues are predicted to be in the vicinity of K70 of IRF3 in the heterodimer subunit bound to the right half-site (Fig. 2A, upper and lower right quadrants). In the absence of iIRF3, substitution of these residues in bATF2 (bATF2XK-Jun) or in bJun (bATF2-bJunXK) caused a moderate decrease and increase in heterodimer orientation preference, respectively (Fig. 2B). As in the case above, iIRF3 binding had dramatic and opposite effects on the orientations of these heterodimers at IFNβ (compare Fig. 2B and Fig. 2C). Thus, heterodimers in which these clusters of charged residues in the vicinity of K70 were substituted by alanines in different subunits (bATF2XK-Jun versus bATF2-bJunXK) bound IFNβ with different orientation preferences in association with iIRF3 (p < 0.05). In both complexes, the shift
in heterodimer orientation reflected a decrease in binding by the mutated (XK) subunit to the right IFNβ half-site proximal to K70 of iIRF3, and a corresponding increase in binding by the wild type subunit to the right IFNβ half-site (Fig. 2E). Simultaneous substitution of both clusters of negatively charged residues in bATF2 and bJun (bATF2XK-bJunXK) eliminated the effect of iIRF3 binding on heterodimer orientation, indicating that interactions with the clusters of negatively charged residues counterbalanced the effects of each other on the orientation of heterodimer binding.

FIGURE 2. Substitution of symmetry-related amino acid residues in bATF2 versus bJun shifts the orientation of heterodimer binding in opposite directions in complexes formed with iIRF3 at IFNβ. A, molecular models depicting potential interactions between iIRF3 (green) and the bATF2(bJun(cyan) heterodimer. The orientation of bATF2-bJun observed in the x-ray crystal structure (10) is shown in the upper panels. The opposite orientation of bATF2-bJun is shown in the lower panels. The structure of the region on the N-terminal side of the basic region is not known, and was modeled in an α-helical conformation (right panels). The diagrams between the models depict the predicted interactions between amino acid residues in iIRF3 and those in bATF2 and bJun when heterodimers bind in opposite orientations. The amino substitutions in bATF2XD, bJunXD, and bATF2XK are indicated on either side of the models.

B, gelFRET analysis of the effects of symmetry-related amino acid substitutions in bATF2 and bJun on the orientation of heterodimer binding at IFNβ. The end preference values of complexes formed by the proteins indicated above the bars at IFNβ were determined as described in Fig. 1C. The amino acid substitutions in the proteins were as follows: bATF2XD (R345A), bJunXD (R263A), bATF2XK (E331A, D332A, D334A, E335A), and bJunXK (D246A, E248A, E251A). The orientations of heterodimer binding were analyzed for complexes formed by the same proteins analyzed in part B with iIRF3 (C) and with iIRF3K70E (D). The diagrams below the bars reflect the preferred orientations of heterodimer binding. The data shown represent the mean values and standard deviations from two independent experiments (*, p < 0.05; **, p < 0.01). E, diagrams summarizing the effects of amino acids substitutions in bATF2, bJun, and iIRF3 on the orientation of heterodimer binding at IFNβ.
We tested the effects of the K70E substitution in iIRF3 on the orientation preferences of heterodimers containing amino acid substitutions that affected interactions with iIRF3. iIRF3K70E binding caused equivalent shifts in the orientation preferences of wild type bATF2-bJun as well as of bATF2XD-bJun and bATF2-bJunXD heterodimers as were caused by wild type iIRF3 binding at IFNb (compare Fig. 2D and Fig. 2C). In contrast, iIRF3K70E binding did not shift the orientation preferences of bATF2XK-bJun or bATF2-bJunXK heterodimers compared with their orientation preferences in the absence of iIRF3 (compare Fig. 2D and Fig. 2B).

These results indicate that the negatively charged residues adjacent to the basic regions of bATF2 and bJun affected heterodimer orientation through mutually exclusive interactions with K70 of iIRF3 at the right half-site of IFNb. The amino acid substitutions in bATF2-bJun and in iIRF3 had qualitatively similar effects on heterodimer orientation in complexes containing HMGI, indicating that HMGI did not displace iIRF3 from bATF2-bJun-IFNb complexes and that interactions between ATF2-Jun and iIRF3 are likely to be similar in the absence versus the presence of HMGI (supplemental Fig. S5).

**Residues in bATF2 and in bJun Have Concerted Effects on the Orientation of Heterodimer Binding in association with iIRF3 and HMGI**—Simultaneous substitution of the arginine in the basic region of one subunit (XD) and the negatively charged residues adjacent to the basic region in the other subunit (XK) was predicted to eliminate interactions with both K70 and D45 in iIRF3 when the heterodimer bound in one orientation but not to affect these interactions when the heterodimer bound in the opposite orientation. In the absence of iIRF3, bATF2XD-bJunXK heterodimers bound IFNb with a higher orientation preference than wild type bATF2-bJun (Fig. 3, left panel). iIRF3 binding reversed the orientation of bATF2XD-bJunXK binding at IFNb (Fig. 3, middle panel). Conversely, iIRF3 binding caused the opposite shift in the orientation of bATF2XD-bJunXK binding at IFNb. Similar shifts in heterodimer orientation preferences were observed in complexes formed with iIRF3 and HMGI (Fig. 3, right panel). Thus, heterodimers in which the
reciprocal combinations of amino acid residues were substituted (bATF2XD-bJunXK versus bATF2XK-bJunXD) bound IFNb in different orientations in association with iIRF3 as well as in association with HMGI (p < 0.05). The amino acid substitutions in bATF2 and bJun that affected heterodimer interactions with IRF3 at the left and the right IFNb half-sites had cumulative effects on heterodimer orientation preference.

Amino Acid Residues That Influence the Orientation of Heterodimer Binding Also Affect Cooperative iIRF3 and bATF2-bJun Binding at IFNb—We investigated if the amino acid residues in bATF2, bJun, and iIRF3 that affected heterodimer orientation also affected cooperative DNA binding at IFNb. We compared the effects of substitution of both symmetry-related residues in bATF2 and bJun on the concentration dependence of wild type iIRF3, iIRF3K70A, and iIRF3D45A binding at IFNb. Similar concentrations of wild type iIRF3 and iIRF3K70A were required for half-maximal binding of IFNb in association with wild type bATF2-bJun (Fig. 4Ai). In contrast, a much higher concentration of iIRF3K70A than of wild type iIRF3 was required for half-maximal binding of IFNb in association with bATF2XD-bJunXD (Fig. 4Aii). Conversely, a slightly lower concentration of iIRF3K70A than of wild type iIRF3 was required for half-maximal binding of IFNb in association with bATF2XK-bJunXK (Fig. 4Aiii).

Higher concentrations of iIRF3D45A than of wild type iIRF3 were required for half-maximal binding of IFNb in association with all the bATF2-bJun variants tested (Fig. 4). The difference between wild type iIRF3 and iIRF3D45A binding at IFNb was slightly smaller in association with bATF2XD-bJunXD, but larger in association with bATF2XK-bJunXK than it was in association with wild-type bATF2-bJun (Fig. 4A). The effects of the K70A and D45A substitutions in iIRF3 were therefore reversed or reduced by substitutions in bATF2-bJun that were predicted to disrupt interactions with the substituted residues, but their effects were accentuated by substitutions in bATF2-bJun that were predicted to disrupt interactions involving other residues.
iIRF3, iIRF3D45A, and iIRF3K70A bound to IFNb that was occupied by bATF2-bJun much more efficiently than to free IFNb, indicating that their binding was enhanced by their interaction with bATF2-bJun (supplemental Fig. S6B). Combinations of substitutions that were predicted to disrupt both sets of interactions between iIRF3 and bATF2-bJun (iIRF3D45AK70A and bATF2XD-bJunXDK) had more severe effects than the individual substitutions (supplemental Fig. S6). In aggregate, the results indicate that the amino acid residues in bATF2, bJun and iIRF3 that affected the orientation of heterodimer binding at IFNb also affected the cooperativity of iIRF3 binding in association with bATF2-bJun at IFNb.

iIRF3 and HMGI Bind Cooperatively with Both Orientations of bATF2-bJun Heterodimers at IFNb—We measured the cooperativity of iIRF3 and HMGI binding at IFNb in association with bATF2 and bJun variants that favored opposite orientations of heterodimer binding (bJun-bATF2XG versus bJunXG-bATF2 and bATF2XD-bJunXK versus bATF2XXK-bJunX). There was no detectable difference in the concentrations of iIRF3 and HMGI required for half-maximal binding of IFNb in association with bATF2-bJun heterodimers bound in opposite orientations (Fig. 4B). Thus, iIRF3 and HMGI bound with indistinguishable cooperativity in association with both orientations of bATF2-bJun heterodimers at IFNb.

ATF2-Jun Heterodimers That Have Opposite Orientation Preferences Differ in Their Effects on Endogenous Interferon-β Transcription—To investigate the role of the orientation of ATF2-Jun heterodimer binding in transcription regulation, we examined endogenous interferon-β gene transcription in cells that expressed ATF2 and Jun containing the amino acid substitutions that altered the orientation of bATF2-bJun binding at IFNb in vitro. In the absence of exogenous IRF3, these amino acid substitutions in ATF2 and Jun had small effects on the level of interferon-β transcripts (Fig. 5). In contrast, in cells that expressed exogenous IRF3, the co-expression of ATF2XK-JunXK or ATF2-JunXG heterodimers in which Jun was predicted to favor binding to the right half-site produced higher levels of interferon-β transcripts than the co-expression of ATF2XD-JunXK or ATF2XG-Jun heterodimers in which Jun was predicted to favor binding to the left half-site (Fig. 5, p < 0.05). The ectopic ATF2 and Jun transcripts were expressed at levels that were 20–300-fold higher than the levels of the cor-

**FIGURE 5.** ATF2-Jun heterodimers that bind IFNb in opposite orientations in vitro have distinct effects on transcription of different endogenous genes. The levels of interferon-β, LEPREL1-T1, RANTES, TPA, and UPA transcripts were measured in cells that expressed the ATF2 and Jun variants indicated below the bar graphs in combination with or without IRF3 (+ indicates wild type protein, the letters indicate the amino acid substitutions described in Fig. 1 and 2, indicates that the expression vector encoding the protein was not transfected). The diagrams above the bar graphs indicate the orientations of binding by bATF2-bJun heterodimers containing the same amino acid substitutions in association with IRF3 at IFNb. The levels of all transcripts were measured in the same cells and were normalized by the level of GAPDH transcripts. The levels of transcripts in cells that expressed heterodimers containing amino acid substitutions that favor Jun binding to the left versus the right half-site are shown by red versus green bars, respectively. The levels of interferon-β transcripts in cells that expressed wild-type ATF2-Jun are shown by white bars, and those in cells that expressed IRF3 alone, or no exogenous proteins are shown by black bars. The graphs show the means and standard deviations of replicate qPCR reactions, and are representative of between two and four separate transfection experiments (*, p < 0.05; **, p < 0.01).
Heterodimer Binding Have Consistent Effects on Transcription of Different Endogenous Genes.—We investigated if the amino acid substitutions that affected bATF2-bJun heterodimer orientation at IFNb and the level of interferon-β transcription also affected transcription of other genes. The LEPREL1-T1 transcript (ENST00000437063) is transcribed downstream of an element in which the ATF2, Jun and IRF3 recognition sequences are identical to those found in the IFNβ enhancer (supplemental Fig. S9). Co-expression of ATF2-JunXG with IRF3 resulted reproducibly in a higher level of LEPREL1-T1 transcripts than co-expression of ATF2XG-Jun (Fig. 6). Co-expression of the ATF2 and Jun variants containing amino acid substitutions that altered the intrinsic orientation preference of heterodimer binding at IFNβ with IRF3 (Fig. 5). Likewise, co-expression of ATF2XG-JunXG with IRF3 resulted reproducibly in a higher level of LEPREL1-T1 transcripts than co-expression of ATF2XG-JunXK with IRF3. Thus, heterodimers that were predicted to favor Jun binding to the right half-site resulted in higher levels of LEPREL1-T1 transcripts than heterodimers predicted to favor Jun binding to the left half-site.

The RANTES chemokine gene contains ATF2-Jun and IRF3 recognition sequences that are separated by a longer distance than those of the interferon-β enhancer (supplemental Fig. S9). Co-expression of the ATF2 and Jun variants containing amino acid substitutions that affected the orientation of heterodimer binding at IFNβ with IRF3 had similar effects on the level of RANTES transcripts as they had on the levels of interferon-β and LEPREL1-T1 transcripts (Fig. 5). The UPA and TPA genes contain ATF2-Jun recognition sequences, but no known IRF3 binding sites (supplemental Fig. S9). ATF2 and Jun variants containing substitutions that altered the intrinsic orientation preference of heterodimer binding at IFNβ (ATF2-JunXG versus ATF2XG-Jun) had opposite effects on the levels of UPA and TPA transcripts compared with their effects on the levels of interferon-β, LEPREL1-T1 and RANTES transcripts in the presence of IRF3 (Fig. 5). The amino acid substitutions in ATF2 and Jun that altered the orientation of heterodimer binding at IFNβ in complexes with IRF3 (ATF2XD-JunXX and ATF2XX-JunXD) had smaller and more variable effects on the levels of UPA and TPA transcripts in the absence and presence of IRF3. Taken together, these results demonstrate that the amino acid substitutions in ATF2 and Jun that affected the orientation of heterodimer binding at IFNβ in vitro had distinct effects on transcription of different endogenous genes.

The ATF2 and Jun Variants That Affected the Orientation of Heterodimer Binding Have Consistent Effects on Transcription at Different Levels of Expression.—We investigated if differences in the levels of expression of the ATF2 and Jun variants contributed to their distinct effects on the transcription of endogenous genes. When different relative amounts of plasmids that encoded ATF2 and Jun variants were transfected, the relative levels of ATF2 and Jun variant expression varied over a 25-fold range (supplemental Fig. S8). Under all of these conditions, expression ATF2 and Jun variants that were predicted to favor Jun binding to the right half-site in combination with IRF3 (ATF2-JunXG and ATF2XX-JunXD) produced higher levels of interferon-β transcripts that the expression of variants that were predicted to favor Jun binding to the left half-site (ATF2XG-Jun and ATF2XD-JunXK) (Fig. 6, supplemental Fig. S10; ANOVA $p < 10^{-3}$ for both combinations). Moreover, consistent differences in the levels of LEPREL1-T1, RANTES, UPA, and TPA transcripts were observed when ATF2 and Jun variants that favored opposite orientations of heterodimer binding were expressed (Fig. 6, supplemental Fig. S10; ANOVA $p < 10^{-3}$ for both combinations at each gene). These results indicate that the differences in the transcriptional activities of ATF2-Jun heterodimers that favored opposite orientations of binding at IFNβ were not due to differences in their relative levels of expression.

The ATF2 and Jun Variants That Favor Opposite Orientations of Heterodimer Binding Have Consistent Effects on Interferon-β Transcription at Different Times After Viral Infection.—We investigated the effects of the ATF2 and Jun variants that favored opposite orientations of heterodimer binding on interferon-β transcription at different stages of viral infection. Whereas the absolute level of interferon-β transcripts varied more than 40-fold at different times after infection and between different experiments, there was a consistent difference between the levels of interferon-β transcripts in cells that expressed ATF2 and Jun variants that favored opposite orientations of heterodimer binding (Fig. 7; supplemental Fig. S11; ANOVA $p < 0.05$).

Different Regions of ATF2 and Jun Mediate the Effects of the Orientation of Heterodimer Binding on Transcription of Different Genes.—We investigated if the amino acid substitutions in ATF2 and Jun that altered the orientation of heterodimer binding at IFNβ affected transcription of different genes through similar or distinct mechanisms. We compared the effects of the amino acid substitutions on the transcriptional activities of the intact proteins described above with their effects on the transcriptional activities of chimeric proteins in which the amino- and carboxyl-terminal regions of ATF2 and Jun were exchanged. At the interferon-β and LEPREL1-T1 genes, these amino acid substitutions had similar effects on the transcriptional activities of the intact and chimeric proteins that contained the same bZIP and C-terminal regions (Fig. 8). In contrast, at the RANTES, TPA, and UPA genes, these amino acid substitutions had similar effects on the transcriptional activities of the intact and chimeric proteins that contained the same N-terminal regions (Fig. 8). Comparison of the levels of transcripts produced in cells that expressed different relative amounts of the chimeric proteins using two-way ANOVA were consistent with the hypothesis that different levels of these transcripts were produced in cells that expressed chimeric proteins that were predicted to favor opposite orientations of heterodimer binding (supplemental Fig. S13; ANOVA $p < 10^{-3}$ for both combinations at each gene). These results indicate that
different regions of ATF2 and Jun determined the effects of the orientation of heterodimer binding on transcription at different genes.

DISCUSSION

The gelFRET analysis of the configurations of bATF2-bJun-iIRF3 and bATF2XG-bJun-iIRF3-HMG-I complexes challenges the conventional view that multi-protein complexes bind composite regulatory elements in a unique and fixed configuration. It is generally recognized that the conformations of nucleoprotein complexes undergo thermal fluctuations. However, these

indicated by the step pattern below the graphs) of plasmids encoding the proteins indicated by the key above the graphs together with IRF3 as described in Fig. 5. The levels of all transcripts were measured in the same cells and were normalized by the level of RPL9 transcripts. The diagrams show the means and standard deviations of replicate qPCR reactions, and are representative of two separate transfection experiments. The diagrams in the upper right corner of each graph indicate the results of ANOVA analysis of the effects either of bATF2XG-bJunXK versus bATF2XG-bJun or of bATF2XG-bJunXK versus bATF2XG-bJunXK on transcript levels (*, p < 0.05; **, p < 0.01). The complete ANOVA results are shown in supplemental Fig. S11.

FIGURE 6. Amino acid substitutions in ATF2 and Jun that affect the orientation of heterodimer binding in vitro have consistent effects on transcription of endogenous genes over a wide range of plasmid ratios. The levels of interferon-β, LEPREL1-T1, RANTES, TPA, and UPA transcripts were measured in cells transfected with different ratios (5:1, 3:1, 1:1, 1:3, and 1:5 as indicated by the step pattern below the graphs) of plasmids encoding the proteins indicated by the key above the graphs together with IRF3 as described in Fig. 5. The levels of all transcripts were measured in the same cells and were normalized by the level of RPL9 transcripts. The diagrams show the means and standard deviations of replicate qPCR reactions, and are representative of two separate transfection experiments. The diagrams in the upper right corner of each graph indicate the results of ANOVA analysis of the effects either of bATF2XG-bJunXK versus bATF2XG-bJun (solid green and red bars) or of bATF2XG-bJunXK versus bATF2XG-bJunXK (striped green and red bars) on transcript levels (*, p < 0.05; **, p < 0.01). The complete ANOVA results are shown in supplemental Fig. S11.
formations are often assumed to be distributed around a single configuration, and this “average” configuration is thought to be representative of the entire population.

The discovery that the bATF2-bJun-iIRF3 and bATF2-bJun-iIRF3-HMGI complexes bind the interferon-β enhancer in two stable configurations extends previous observations that heterodimeric transcription factors can bind pseudo-symmetrical recognition sequences in two opposite orientations (1, 2). The alternative configurations of these nucleoprotein complexes involve large-scale rearrangements of the protein-protein and protein-nucleic acid interfaces in the complex. The large differences between the structures of complexes in which heterodimers bind in opposite orientations predict that they have distinct functions.

Two independent lines of investigation corroborate the interpretation that bATF2-bJun-iIRF3-HMGI complexes assemble in two distinct configurations at the interferon-β enhancer. First, bATF2-bJun heterodimers bound the interferon-β enhancer in two opposite orientations in association with iIRF3 alone or with iIRF3 and HMGI in the gelFRET assay. Second, iIRF3 and HMGI bound the interferon-β enhancer with same cooperativity in association with bATF2-bJun variants that favor opposite orientations of binding. The two configurations of bATF2-bJun-iIRF3-HMGI complexes that can bind cooperatively to the interferon-β enhancer represent a new mode of cooperative DNA binding at a composite regulatory element that does not require a fixed configuration of protein interactions.

The phenomenon of dual contact interfaces on opposite faces of heterodimers may be widespread since the residues that mediate protein interactions are often conserved among different members of a protein family (supplemental Fig. S14). We propose to designate interfaces that enable alternative configurations of protein interactions “Janus” interfaces as they provide multiple faces for engagement with interaction partners.

Different configurations of the same transcription factors can have distinct transcriptional activities at different composite regulatory elements (19–21). Our results extend this principle by demonstrating that transcription factor variants that bind in different configurations can have distinct transcriptional activities at the same composite regulatory element. The mechanisms whereby the orientation of ATF2-Jun heterodimer binding affected interferon-β transcription are likely to reflect orientation-dependent interactions with other factors at the interferon-β gene.

The amino acid substitutions in ATF2 and Jun that affected the orientation of heterodimer binding at IFNb had opposite effects on the transcription of different endogenous genes. These differences could be due to opposite effects of the substitutions on the orientations of heterodimer binding at different genes, or they could indicate that the mechanisms whereby heterodimer orientation affects transcription vary among different genes. The observation that different regions of ATF2 and Jun...
determined the effect of the orientation of heterodimer binding on transcription at different genes is consistent with the latter interpretation.

The consequences of the multiple configurations of transcription factor binding at the interferon-β enhancer and their distinct transcriptional activities for cellular and organismal phenotypes remain unknown. Different cellular processes require either unidirectional or bidirectional action. Some functions long thought to be unidirectional, such as promoter-directed gene transcription, have been recently found to be bidirectional (22, 23). The structural basis for this bidirectional transcription remains to be established, but could be rooted in the bidirectional binding of transcription regulatory proteins as well as the TATA box-binding protein at their recognition sites (24).

REFERENCES


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