Correlation of Transcriptional Repression by p21\textsuperscript{SNFT} with Changes in DNA-NF-AT Complex Interactions\textsuperscript{*}

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p21\textsuperscript{SNFT} (21-kDa small nuclear factor isolated from T cells) is a novel human protein of the basic leucine zipper family. The overexpression of p21\textsuperscript{SNFT} leads to the significant and specific repression of transcription from the interleukin-2 promoter as well as from several essential activator protein 1 (AP-1)-driven composite promoter elements. One example is the distal nuclear factor of activated T cells (NF-AT)/AP-1 element where the AP-1 (Fos/Jun) basic leucine zipper heterodimer interacts with members of the NF-AT family. p21\textsuperscript{SNFT} has been shown to replace Fos in dimerization with Jun on a consensus AP-1 binding site (12-O-tetradecanoylphorbol-13-acetate response element (TRE)) and to interact with Jun and NF-AT at the distal NF-AT/AP-1 enhancer element. A detailed biochemical analysis presented here compares interactions involving p21\textsuperscript{SNFT} with those involving Fos. The results demonstrate that a p21\textsuperscript{SNFT}/Jun dimer binds a TRE similarly to AP-1 and like AP-1 binds cooperatively with NF-AT at the NF-AT/AP-1 composite element. However, Fos interacts significantly more efficiently than p21\textsuperscript{SNFT} with Jun and NF-AT, and the replacement of Fos by p21\textsuperscript{SNFT} in the trimolecular complex drastically alters protein-DNA contacts. The data suggest that p21\textsuperscript{SNFT} may repress transcriptional activity by inducing a unique conformation in the transcription factor complex.

Combinatorial regulation is a powerful mechanism enabling transcription to be tightly controlled. The expression and induction levels of various transcription factors are cell and tissue type-dependent, leading to highly context-specific activities that are also coordinated by cooperativity between factors and sequence-specific DNA affinities (1–5). T lymphocytes represent a well characterized model for inducible combinatorial regulation, and activation of several cytokines and other essential genes in T cells is controlled by such transcription factor interactions (6–12). Differential stimulation through the T cell receptor and various co-receptors leads to stimulus-dependent activation of factors, which selectively converge at promoter and enhancer elements to modulate transcription (13, 14). Interactions between NF-AT\textsuperscript{1} and AP-1 family members at composite NF-AT/AP-1 binding elements are among the most common and highly studied examples of combinatorial regulation in T cells (15–19). NF-AT/AP-1 interactions, which are often cooperative, strongly influence the production of many cytokines required in T cells including IL-2, IL-3, IL-4, and granulocyte macrophage colony-stimulating factor (20–22).

IL-2 is the major mitogenic cytokine produced in T lymphocytes in response to antigenic stimulation through the T cell receptor and coreceptor CD28. This activation is an essential event in the T cell-mediated immune response, leading to both clonal expansion of the T cell and activation of other cell types involved in the response (7, 23–25). IL-2 activity is largely controlled at the level of transcription through convergence of transcription factors at the proximal 300-bp region of the IL-2 promoter (7, 8, 23–26). This region contains several AP-1 composite elements at which AP-1 proteins interact with transcription factors from other families (8, 27). The distal NF-AT/AP-1 enhancer element of the human promoter is regulated by highly cooperative interactions between AP-1 and NF-AT proteins (6, 8, 15, 22, 28–30). Although NF-AT is able to bind this element alone, the presence of AP-1 greatly stabilizes the interaction (31, 32). The AP-1 binding element is a low affinity site and neither Fos/Jun heterodimers nor Jun homodimers bind easily unless recruited by NF-AT (16). In contrast, Fos/Jun heterodimers and Jun homodimers readily bind a consensus TRE (12-O-tetradecanoylphorbol-13-acetate response element) in the absence of other proteins (33).

It has recently been shown that the overexpression of p21\textsuperscript{SNFT} leads to the specific repression of both human IL-2 promoter activity and the production of IL-2 by activated Jurkat cells (34). p21\textsuperscript{SNFT} is constitutively expressed, and several lines of evidence strongly indicate that it is able to replace Fos in dimerization with Jun on AP-1 binding sites (34). To develop an understanding of its mechanism of activity, heterodimeric complexes formed by p21\textsuperscript{SNFT} with Jun were compared in detail to c-Fos/c-Jun heterodimers. Like several known bZIP transcription factors including Fos and Jun family members (35–37), p21\textsuperscript{SNFT} localized to the nucleus. Additionally, interactions between a p21\textsuperscript{SNFT}/Jun dimer and NF-AT at the human distal NF-AT/AP-1 enhancer were shown to be highly cooperative similar to those of AP-1 proteins with NF-AT. However, a

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\textsuperscript{1} The abbreviations used are: NF-AT, nuclear factor of activated T cells; IL, interleukin; SNFT, small nuclear factor isolated from T cells; AP, activator protein; Ds, specific unbound DNA; Pd, specific bound DNA; Dn, nonspecific sites; bZIP, basic leucine zipper; TRE, 12-O-tetradecanoylphorbol-13-acetate response element; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; MMP-1, matrix metalloproteinase 1, MPE, medium propyl EDTA; GST, glutathione S-transferase.

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NF-AT/AP-1 complex formed more efficiently and footprinted DNA differently than a complex containing p21SNFT in place of Fos. The results suggest that a ternary complex containing p21SNFT may be conformationally distinct from one containing Fos and may explain the repression of NF-AT/AP-1 activity observed in the presence of this protein.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The IL-2 luciferase (38), pCI/SNFT (34), pNFATp5x (39), and GST-SNFT (34) constructs have been described previously. The GFP-SNFT construct was cloned by digesting pCI/SNFT with NotI/SallI and blunting the NotI site with T4 DNA Polymerase (New England Biolabs, Beverly, MA). The resulting fragment was ligated in the appropriate reading frame into a modified pEGFP-N2 vector (CLONTECH, Palo Alto, CA) digested with SallI/SallI. The His5-, His12-, and His15-Jun purification constructs pQE30-Fosdb and pQE32-Jundb contain the DNA binding domains of human c-Fos (amino acids 59–211) and human c-Jun (amino acids 223–233) cloned into the pQE His6 expression plasmids from Qiagen, Inc (Valencia, CA).

**Promoter Studies**—Transient transfection assays in Jurkat cells were performed as described previously (34) using 5 µg of IL-2 luciferase construct and the indicated amounts of pCI/SNFT, GFP-SNFT, or pEGFP expression constructs. Cells were stimulated with 10 ng/ml PMA and 50 ng/ml phorbol 12-myristate 13-acetate or left unstimulated for 15 min at 5000 rpm at 4 °C. Consequently, each sequence read was the reverse complement of the sequence with the 5′ end-labeled probe. In addition, 0.01 µg/ml trypsin, 0.01 µg/ml leupeptin, 0.01 µg/ml aprotinin, and 0.01 µg/ml poly(dI-dC) (Amersham Biosciences) was included in each reaction. The concentration of nonspecific sites (Dn) is 0.01 µg/ml/660 µM (15.1 µM) in each reaction (~140-fold greater than PDs + Dn). In EMSA analyses, the total concentration of specific sites (PDs + Dn) is equal to the input concentrations of labeled plus unlabeled specific DNA. EMSAs were exposed to PhosphorImager cassette. Free (unshifted) and bound (shifted) specific DNA were quantitated using ImageQuant software (Amersham Biosciences), and percent free versus percent bound was calculated. PDs and Ds were determined in Equations 1 and 2.

\[
Ds = \frac{<K>PDs}{<D>(Da)}
\]  

where \( K \) is identified as the negative slope of the line produced when these values are plotted.

**DNAse I Footprinting**—Footprinting probes were generated by cloning the matrix metalloproteinase 1 (MMP-1) or IL-2 promoter regions into the pCR2.1 TA cloning vector (Invitrogen). Single end-labeled probes were prepared by digesting to produce a 5′ overhang filling in with [γ-32P]dATP or dCTP and Klenow (New England Biolabs), and 3′ end-labeled double-stranded poly(dI-dC) (Amersham Biosciences) was included in each reaction. The concentration of nonspecific sites (Dn) is 0.01 µg/ml/660 µM (15.1 µM) in each reaction (~140-fold greater than PDs + Dn). In EMSA analyses, the total concentration of specific sites (PDs + Dn) is equal to the input concentrations of labeled plus unlabeled specific DNA. EMSAs were exposed to PhosphorImager cassette. Free (unshifted) and bound (shifted) specific DNA were quantitated using ImageQuant software (Amersham Biosciences), and percent free versus percent bound was calculated. PDs and Ds were determined in Equations 1 and 2.

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**Transcriptional Repression by p21SNFT**—To determine the ratios of specific unbound DNA (Ds) to specific bound DNA (PDs), EMSAs analyses were performed as described above using purified proteins. A protocol by Emerson et al. (40) was modified to calculate specific versus nonspecific DNA affinities for the conditions used. Labeled TRE or NF-AT/AP-1 site (specific DNA) was used at 216 µl, and proteins were used at the concentrations found for the conditions used. Labeled TRE or NF-AT/AP-1 site (specific DNA) was used at 216 µl, and proteins were used at the concentrations found for the conditions used. Labeled TRE or NF-AT/AP-1 site (specific DNA) was used at 216 µl, and proteins were used at the concentrations found for the conditions used. Labeled TRE or NF-AT/AP-1 site (specific DNA) was used at 216 µl, and proteins were used at the concentrations found for the conditions used. Labeled TRE or NF-AT/AP-1 site (specific DNA) was used at 216 µl, and proteins were used at the concentrations found for the conditions used. Labeled TRE or NF-AT/AP-1 site (specific DNA) was used at 216 µl, and proteins were used at the concentrations found for the conditions used.
subcellular localization of p21\textsuperscript{SNFT}, a p21\textsuperscript{SNFT} expression construct containing a N-terminal GFP tag, was generated and used in transient transfection assays in HeLa cells. Because HeLa cells are adherent and contain a relatively small nucleus, the nuclear and cytoplasmic areas can be easily distinguished. Cells transfected with either GFP (27 kDa) or GFP-SNFT (48 kDa) were examined under both phorbol 12-myristate 13-acetate-stimulated and unstimulated conditions, and fluorescence microscopy was used to locate GFP. Transfections were harvested, and the presence of GFP and GFP-SNFT was verified by Western blotting (Fig. 1A). In Western blots probed for GFP (Fig. 1A, top), both GFP (lanes 3 and 6) and GFP-SNFT (lanes 4 and 7) are constitutively expressed in the appropriate transfecants but not in untransfected cells (lanes 2 and 5). An identical blot probed for p21\textsuperscript{SNFT} (Fig. 1A, bottom) confirms the presence of GFP-SNFT in cells transfected with this construct (lanes 4 and 7). Fig. 1B demonstrates that GFP-SNFT localizes to the nucleus in transfected cells (right panels), whereas GFP remains cytoplasmic (left panels). These data demonstrate that the presence of the p21\textsuperscript{SNFT} protein causes translocation of GFP from the cytoplasm to the nucleus. The results suggest that like other bZIP transcription factors, endogenous p21\textsuperscript{SNFT} is probably a nuclear protein.

To determine the response of IL-2 promoter activity to GFP-tagged p21\textsuperscript{SNFT} relative to untagged p21\textsuperscript{SNFT}, transient transfection assays were performed in the Jurkat transformed T cell line. Cells were transfected with a luciferase construct driven by the proximal 300-bp region of the IL-2 promoter in the presence or absence of p21\textsuperscript{SNFT} expression constructs. As shown in Fig. 1C, IL-2 promoter activity is 70–85% reduced in response to p21\textsuperscript{SNFT} expression; the GFP-SNFT expression construct represses the IL-2 promoter comparably to the pCI/SNFT construct. In contrast, the GFP protein alone produces no significant repression. The data demonstrate that the repression observed is specifically attributed to the presence of p21\textsuperscript{SNFT} and suggest that the 27-kDa N-terminal GFP tag does not affect the localization or activity of the p21\textsuperscript{SNFT} protein.

p21\textsuperscript{SNFT} Dimerizes with Jun on a Consensus TRE and p21\textsuperscript{SNFT}/Jun Dimers Cooperate with NF-AT on the IL-2 Promoter Distal NF-AT/AP-1 Enhancer—EMSA analyses were performed to demonstrate the in vitro interactions of p21\textsuperscript{SNFT} with DNA and with other proteins. Bacterially expressed c-Fos and c-Jun bZIP domains and full-length p21\textsuperscript{SNFT} were purified and examined for binding to a consensus TRE (Fig. 2B). Lane 1 shows the migration of TRE probe in the absence of proteins, and lanes 2–4 demonstrate that all three proteins were used at concentrations insufficient to form homodimers under the given conditions. This is particularly important for Jun, which homodimerizes on a consensus TRE at higher concentrations. When Fos and Jun are present together, an AP-1 heterodimer
A. The IL-2 Promoter

![Diagram of the IL-2 promoter region with NFAT/AP-1 and other binding sites highlighted.]

B. EMSA analysis was performed on oligonucleotides containing the NF-AT/AP-1 distal enhancer element. Binding reactions were performed on 10,000 cpm of TRE oligonucleotide using bacterially expressed proteins. Purified His-Fos (F), His-Jun (J), and GST-SNFT (S) were used at 60, 160, and 120 nM, respectively, as indicated. GST-SNFT was titrated from 120 to 480 nM in lanes 6–8. EMSA analysis was performed on oligonucleotide containing the NF-AT/AP-1 enhancer region from the IL-2 promoter. Binding reactions were performed as in A with 240 nM His-NF-AT (N) added where indicated.

C. EMSA analysis was performed using purified bacterially expressed proteins as indicated. Fos, Jun, and p21SNFT were used at concentrations that do not form homodimers or heterodimers in the absence of NF-AT (lanes 2–7). In contrast, NF-AT binds alone (lane 8). Lanes 9–12 demonstrate that at the concentrations used, NF-AT does not interact with Fos, Jun, or p21SNFT homodimers or with Fos/p21SNFT heterodimers. This is particularly relevant for Jun (lane 10), because at higher Jun concentrations, a NF-AT/Jun/Jun complex forms at this site (Figs. 4, 6, and 7) (16). On the other hand, the same concentrations of NF-AT, Fos, and Jun incubated together produce a NF-AT/AP-1 complex (lane 13), whereas NF-AT, p21SNFT, and Jun produce a higher molecular weight complex (lane 14). If all four proteins are incubated together, the NF-AT/Fos/Jun and NF-AT/p21SNFT/Jun complexes are both apparent, but no new complex forms (lane 15), indicating that only one complex or the other can form on a single piece of DNA. These data support previous results suggesting that p21SNFT competes with Fos on the NF-AT/AP-1 distal enhancer for dimerization with Jun (34).

It is particularly significant that a p21SNFT/Jun dimer does not bind DNA in the absence of NF-AT at this site (lane 7) despite the fact p21SNFT and Jun will readily dimerize on a consensus TRE using proteins from the same preparations at these concentrations (Fig. 2B). This finding demonstrates that interactions between the p21SNFT/Jun dimer and NF-AT are highly cooperative as has been shown for NF-AT and AP-1 (16).

A p21SNFT/Jun Dimer Forms Less Readily Than an AP-1 Heterodimer but More Readily than a Jun Homodimer—To compare the ability of Jun to dimerize with p21SNFT relative to Fos or with itself, purified proteins were used in quantitative EMSA analyses. The absolute amounts of each protein used in these studies are dependent on the percent of each individual preparation that is active. The studies are based on the assumption that if more Jun from a single protein preparation is required to optimally dimerize with p21SNFT than with Fos, the overall ability of Jun to interact with p21SNFT is lower than its ability to interact with Fos. The first study compared the formation of p21SNFT/Jun heterodimers, AP-1 (Fos/Jun) heterodimers, and Jun homodimers on a consensus TRE (Fig. 3 and Table I). To identify the maximal shift that could be produced for a given amount of labeled probe, both dimer partners were initially titrated together over a series of several binding reactions beginning with concentrations that did not homodimerize (Fig. 3A). The concentration of Jun that produced the maximal shift on this gel with Fos and with p21SNFT was then held constant while Fos and p21SNFT were titrated (Fig. 3B). From the second gel, the concentrations of Fos and p21SNFT that maximally shifted the probe were found.

Finally, Fos and p21SNFT were held constant while Jun was titrated alone (Fig. 3C). The data show that optimal amounts of p21SNFT and Jun can only shift ~71% of the probe (lane 13). Increasing Jun above this concentration results in the appearance of a Jun homodimer, evident as a complex of lower molecular weight that begins to form at 2 μM Jun (lane 14), whereas increasing the concentration of p21SNFT has no further effect (as determined in Fig. 3B). Therefore, the concentration of Jun required for optimal dimer formation with p21SNFT is 1.2 μM. On the other hand, <160 nM Jun is required with optimal Fos

...strates that the higher molecular weight complex is formed by a p21SNFT/Jun dimer. The data are consistent with previous observations (34) that p21SNFT can bind a TRE with Jun and strongly suggest that it can physically replace Fos in the AP-1 complex.

In vitro interactions of p21SNFT were also assessed on the distal NF-AT/AP-1 enhancer element from the IL-2 promoter (Fig. 2C). EMSA analysis was performed using purified bacterially expressed proteins as indicated. Fos, Jun, and p21SNFT were used at concentrations that do not form homodimers or heterodimers in the absence of NF-AT (lanes 2–7). In contrast, NF-AT binds alone (lane 8). Lanes 9–12 demonstrate that at the concentrations used, NF-AT does not interact with Fos, Jun, or p21SNFT homodimers or with Fos/p21SNFT heterodimers. This is particularly relevant for Jun (lane 10), because at higher Jun concentrations, a NF-AT/Jun/Jun complex forms at this site (Figs. 4, 6, and 7) (16). On the other hand, the same concentrations of NF-AT, Fos, and Jun incubated together produce a NF-AT/AP-1 complex (lane 13), whereas NF-AT, p21SNFT, and Jun produce a higher molecular weight complex (lane 14). If all four proteins are incubated together, the NF-AT/Fos/Jun and NF-AT/p21SNFT/Jun complexes are both apparent, but no new complex forms (lane 15), indicating that only one complex or the other can form on a single piece of DNA. These data support previous results suggesting that p21SNFT competes with Fos on the NF-AT/AP-1 distal enhancer for dimerization with Jun (34).

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Finally, Fos and p21SNFT were held constant while Jun was titrated alone (Fig. 3C). The data show that optimal amounts of p21SNFT and Jun can only shift ~71% of the probe (lane 13). Increasing Jun above this concentration results in the appearance of a Jun homodimer, evident as a complex of lower molecular weight that begins to form at 2 μM Jun (lane 14), whereas increasing the concentration of p21SNFT has no further effect (as determined in Fig. 3B). Therefore, the concentration of Jun required for optimal dimer formation with p21SNFT is 1.2 μM. On the other hand, <160 nM Jun is required with optimal Fos
to shift 71% of the probe (Fig. 3C, lane 3). The results demonstrate that from the same preparation, at least an 8-fold greater concentration of Jun is required to produce a p21SNFT/Jun heterodimer than a Fos/Jun heterodimer as summarized in Table I. Therefore, the ability of p21SNFT to dimerize with Jun is 8-fold lower than that of Fos with Jun.

To compare the data with that previously shown for AP-1 (3), the homodimerization of Jun was also analyzed. Fig. 3C, lanes 17–19, shows a titration of Jun on a consensus TRE. It is evident from this figure that 2 μM Jun only shifts 54% of the probe (lane 19). In contrast, 320 nM Jun (8-fold less) shifts 65% of the probe with p21SNFT (lane 11). Correcting for the fact that twice the amount of Jun is required to form a homodimer than a heterodimer, a conservative estimate for the ability of Jun to

**Fig. 3.** A p21SNFT/Jun complex forms more efficiently than a Jun homodimer but less efficiently than an AP-1 heterodimer. EMSA analyses were performed using a 216-pm labeled probe with purified His-Fos, His-Jun, GST-SNFT, and His-NF-AT. A, to determine the maximal shift of TRE probe by Fos/Jun (F/J) or p21SNFT/Jun (S/J) dimers, Fos was used at 0.05 μM in lanes 2 and 5 and titrated from 0.05 to 1.6 μM in lanes 6–11. p21SNFT was used at 0.12 μM in lanes 4 and 5 and titrated from 0.12 to 2 μM in lanes 12–16. Jun was used at 0.16 μM in lane 3 and titrated from 0.16 to 4 μM in lanes 6–11 and from 0.16 to 2 μM in lanes 12–16. B, to determine the optimal concentrations of Fos and p21SNFT needed to maximally shift TRE with excess Jun (determined in A to be 2 μM with Fos and 1.2 μM with p21SNFT), Fos was titrated from 0.2 to 1.3 μM in lanes 2–7, whereas Jun was held constant at 2 μM. p21SNFT was titrated from 0.03 to 0.73 μM in lanes 8–14, whereas Jun was held constant at 1.2 μM. C, to determine the concentrations of Jun needed to maximally shift TRE with optimized Fos (0.7 μM) and p21SNFT (0.5 μM) as shown in B, Fos and p21SNFT were held constant at these concentrations, whereas Jun was titrated from 0.08 to 8 μM (lanes 2–10) or 0.32 to 8 μM (lanes 11–16). To demonstrate the formation efficiency of a Jun homodimer (J/J), Jun was titrated from 0.64 to 2 μM (lanes 17–19). D, to determine the concentrations of NF-AT needed to optimally produce an NF-AT/Fos/Jun (N/F/J) or NF-AT/p21SNFT/Jun (N/S/J) ternary complex on an NF-AT/AP-1 site, Fos, Jun, and p21SNFT were held constant at the optimal concentrations as determined in C, whereas NF-AT was titrated from 0.03 to 2.4 μM with Fos and Jun (lanes 2–8) or p21SNFT and Jun (lanes 9–15). Shown below are percentages of labeled probe bound to each ternary complex (3°) to Fos/Jun (F/J), p21SNFT/Jun (S/J), or Jun/Jun (J/J) dimers or to NF-AT alone (N/P) as well as the percent that is not bound (Free).
homodimerize is at least 4-fold lower than the ability of a p21SNFT/Jun heterodimer to form. Together, the data estimate that the AP-1 heterodimer interacts approximately 30-fold more readily than the Jun homodimer (8-fold more readily than the p21SNFT/Jun dimer, which interacts 4-fold more readily than the Jun homodimer). The results summarized in Table I are consistent with a previous report that estimated an approximate 25-fold difference between the formations of AP-1 heterodimers versus Jun homodimers (3).

NF-AT Interacts as Readily with a p21SNFT/Jun Heterodimer as with an AP-1 Heterodimer.—To determine the ability of NF-AT to form trimolecular complexes with Fos/Jun and p21SNFT/Jun dimers, ternary interactions on an NF-AT/AP-1 site were compared (Fig. 3D). To ensure optimal stoichiometry of the bZIP dimer partners, the concentrations of Fos, Jun, and p21SNFT that maximally shifted the TRE (Fig. 3, B and C, and Table I) were used in this experiment. NF-AT was titrated into the complex to determine the amount required for maximal ternary complex formation with each optimized dimer. Fig. 3D shows that as more NF-AT is titrated into a complex with Fos and Jun, the ternary complex becomes increasingly predominant. At 0.26 μM NF-AT (lane 5), the probe is completely shifted and ~82% of the total is shifted into the trimolecular complex. When p21SNFT/Jun dimers are analyzed with NF-AT, ~1 μM NF-AT is required to optimally shift the probe (lane 14). However, this result is attributed not to an increase in NF-AT/p21SNFT/Jun interactions, which are optimal at 0.26 μM NF-AT, but rather to an increase in NF-AT binding DNA alone. At the higher concentrations of NF-AT used, NF-AT binding alone has increased to account for roughly 35% of the total probe (lanes 13–15). In summary, approximately equal amounts of NF-AT (~0.26 μM) are required for optimal levels of ternary complex with Fos/Jun or p21SNFT/Jun dimers, indicating that the abilities of Fos/Jun versus p21SNFT/Jun dimers to interact with NF-AT are roughly equivalent. However, the NF-AT/Fos/Jun complex interacts with DNA more efficiently (~85% shifted) than the NF-AT/p21SNFT/Jun complex (~55% shifted). This may reflect a difference in DNA affinity, a difference in active p21SNFT/Jun versus Fos/Jun dimer, or a difference in specific activities between the Fos and p21SNFT protein preparations.

The results shown in Figs. 3 and 4 are summarized in Table I.

Fos/Jun and p21SNFT/Jun Dimers Exhibit Similar Relative Specific DNA Affinities for a Consensus TRE.—The studies presented in Fig. 3 raised the question of whether Fos-containing complexes may bind DNA with a higher affinity than p21SNFT-containing complexes. Therefore, the relative specific affinities of Fos/Jun and p21SNFT/Jun heterodimers and Jun homodimers for a consensus TRE were determined using purified proteins in quantitative EMSA analyses (Fig. 4A). The concentrations of Dn, proteins, and labeled specific DNA were held constant as described under “Experimental Procedures.” The concentration of nonspecific sites was at least 140-fold greater than that of specific sites in each reaction to ensure that the amount of unbound protein was negligible. Bound versus free probe was quantitated using ImageQuant software, enabling the calculation of PDs and free Ds concentrations. A plot of the titration curves (PDs versus Ds) is shown in Fig. 4B. As shown in Fig. 4C and summarized in Table I, the relative affinity constants ($K_r$) of Fos/Jun and p21SNFT/Jun dimers for a TRE are roughly equivalent (~6000). In contrast, $K_r$ for a Jun homodimer is ~3-fold lower (~2000).

The experiments in Fig. 4 can be used to estimate the amount of active dimers able to form from each protein preparation. Because $(PDs)(Dn)/Ds = P_r(K_r)$ at $PDs = 0$ (40), the y intercept values ($P_r$) represent the relative amounts of active dimer in these equations. As shown in Fig. 4C, the amount of active dimer in the Jun homodimer reactions is ~2-fold lower than in the p21SNFT/Jun heterodimer reactions. Because twice the Jun is required for heterodimerization than for homodimerization, the data suggest that the specific activities of the p21SNFT and Jun protein preparations are roughly equivalent. In contrast, the amount of active dimer in the Fos/Jun reactions is ~1.75-fold greater than that in the p21SNFT/Jun reactions. This finding suggests that in Fig. 3, p21SNFT interacts less efficiently in complexes with NF-AT and Jun than Fos because of a difference in specific activities between the Fos and p21SNFT protein preparations rather than inefficient protein-protein interaction or a significantly lower affinity for DNA with Jun. Collectively, the data presented in Figs. 3 and 4 indicate that p21SNFT/Jun dimers form complexes with NF-AT and Jun with an intermediate efficiency between that of AP-1 heterodimers and Jun homodimers (Table I).

A p21SNFT/Jun Dimer Footprints a Consensus TRE Similarly to AP-1.—To determine whether complexes containing p21SNFT interact with DNA similarly to those containing Fos, footprinting analyses were performed using bacterially purified proteins. The footprints of Fos/Jun, Jun/Jun, and p21SNFT/Jun dimers were compared on a consensus TRE (Fig. 5). Both strands of the MMP-1 promoter were digested with DNase I in the presence or absence of proteins. To identify the consensus TRE at ~73, ddNTP-sequencing reactions were run alongside as shown for antisense strand in Fig. 5A. Lane 1 shows the digestion pattern of the probe in the absence of proteins. In lanes 2–4, His$_6$-Jun was titrated into the reaction, resulting in a gradual protection of the TRE by Jun homodimer formation. To footprint the appropriate heterodimers, Jun was held constant at a concentration insufficient to footprint alone (80 nM from lane 2), whereas His$_6$-Fos or GST-SNFT was titrated into the reaction (lanes 5–9 and 10–14, respectively). As with the Jun homodimer, the consensus TRE is completely protected by both an AP-1 heterodimer and a p21SNFT/Jun heterodimer, indicating that the p21SNFT complex interacts with the TRE similarly to AP-1. Interestingly, the sequences flanking the core consensus TRE appear to be protected somewhat differently by all three complexes. These data show that p21SNFT forms a dimer with Jun that is structurally similar to AP-1 at the DNA binding level.

An NF-AT/p21SNFT/Jun Complex Footprints the IL-2 Promotor Distal NF-AT/AP-1 Enhancer Region Differently than an NF-AT/AP-1 Complex.—To analyze the DNA interactions of a ternary complex containing p21SNFT footprints of the NF-AT/p21SNFT/Jun complex on a NF-AT/AP-1 site were compared with those of NF-AT/AP-1 complexes. The IL-2 promoter region was used in DNase I protection assays with purified His$_6$-NFAT, His$_6$-Fos, His$_6$-Jun and GST-SNFT (Fig. 6). Footprinting patterns on both strands of DNA are shown, and sequencing

| ![Table I](image) | Summary of protein:protein interactions and DNA binding of p21SNFT | |  |  |  
|---|---|---|---|---|--- |
| ![Column 1](image) | ![Column 2](image) | ![Column 3](image) | ![Column 4](image) |  |  |
| Fos/Jun complexes | p21SNFT/Jun complexes | Jun/Jun complexes |
| ![Entry 1](image) | ![Entry 2](image) | ![Entry 3](image) | ![Entry 4](image) | ![Entry 5](image) | ![Entry 6](image) |
| ![Entry 7](image) | ![Entry 8](image) | ![Entry 9](image) | ![Entry 10](image) | ![Entry 11](image) | ![Entry 12](image) |


reactions run alongside (data not shown) were used to identify the distal NF-AT/AP-1 enhancer region as indicated. *Lane 1* shows the digestion pattern of this probe in the absence of proteins, and *lane 2* shows protection of the NF-AT binding site by NF-AT alone. Because NF-AT is required to recruit Fos, Jun, and p21SNFT to this site, NF-AT was included in all subsequent reactions. *Lanes 3–5* show that a Jun homodimer extends the footprint over the AP-1 binding region of the probe, as Jun is titrated into the reaction. When Jun is held constant at the concentration that does not completely footprint the AP-1 site (320 nM from *lane 3*) and Fos is titrated into the complex, the footprint again extends over the entire AP-1 binding region as AP-1 heterodimers are formed (*lanes 6–9*). However, a titration of GST-SNFT into the same reaction fails to protect much of the AP-1 binding site (*lanes 10–13*). This result is most obvious on the antisense strand (Fig. 6A), because the AP-1 binding region is much better probed with DNase I on this strand but it can be seen on the sense strand as well (Fig. 6B).

Because a difference in footprinting ability between the NF-AT/AP-1 complexes and the NF-AT/p21 SNFT/Jun complex was evident using DNase I, MPE:Fe footprinting was employed to further examine the footprints using a more sensitive technique (Fig. 7, A and B). Because virtually every nucleotide is probed using the MPE:Fe technique (41, 42), a more complete visualization of the DNA contacts made by the three complexes is possible with this reagent than with DNase I. The results using this technique demonstrate even more clearly that the NF-AT/p21SNFT/Jun complex fails to footprint much of the NF-AT/AP-1 binding site. Indeed, with the more efficient probe it appears that the entire AP-1 binding region is left unprotected by the p21SNFT complex while it remains completely protected by the NF-AT/AP-1 complexes.

The lack of footprint in this area by the NF-AT/p21SNFT/Jun complex occurs despite the fact that this complex forms at least four times more readily than the NF-AT/Jun complex as shown in Figs. 3 and 4. Moreover, the efficient formation of this complex on the IL-2 promoter under identical binding conditions to those footprinted is directly demonstrated in EMSA analysis (Fig. 7C). The EMSA also demonstrates that higher concentrations of p21SNFT result in less binding of the NF-AT/p21SNFT/Jun ternary complex. The optimal concentration of p21SNFT is consistently 244 nM (*lane 12*) as evident in the EMSA and all four footprints. The high molecular weight complex produced by NF-AT alone (*lane 2*) is formed because seven times the NF-AT is required to footprint alone than with other proteins due to stabilization of NF-AT at this site that is conferred by

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**Fig. 4.** The affinity of a p21SNFT/Jun dimer is similar to that of AP-1 for a consensus TRE. A, p21SNFT/Jun dimers were gradually competed off of labeled consensus TRE with unlabeled TRE. Fold excesses of labeled relative to unlabeled TRE are indicated above. B, gels similar to A were done for Fos/Jun heterodimers and Jun homodimers, and the PDs and Ds were calculated and plotted to ensure that titrations were complete. C, PDs/Dn/Ds versus PDs were plotted to determine the affinity constants K_r for each complex on a consensus TRE.
other proteins (as evident in Fig. 2). Several NF-AT binding sites are present on this probe, all of which become occupied at this high concentration of NF-AT. At lower concentrations of NF-AT, the laddering pattern shown in lanes 3 and 10 occurs as different numbers of sites are occupied. As evident in lane 12, with this concentration of NF-AT, optimal NF-AT/p21SNFT/Jun complexes form despite the fact that no footprinting of the AP-1 site is observed (Fig. 7B).

The Difference in Footprint Observed between NF-AT/p21SNFT/Jun and NF-AT/AP-1 Complexes Is Attributed to the p21 SNFT Protein and Not the N-terminal GST Tag of GST-SNFT—Because the large N-terminal GFP tag did not significantly affect the activity of p21 SNFT (Fig. 1) and purified GST was previously eliminated as a factor influencing the formation of these complexes (34), it was hypothesized that the N-terminal GST tag would not influence the binding of purified p21 SNFT. To test this
hypothesis, the affinity tag was cleaved with thrombin, and GST was removed using column purification. After confirming via EMSA analyses that the untagged p21SNFT was active in binding DNA with Jun and NF-AT, the identical footprinting reactions shown in Fig. 7 were repeated. As shown in Fig. 8, the footprinting pattern produced using thrombin-cleaved p21SNFT was identical to that produced using GST-SNFT. Indeed, in the absence of the GST tag, the NF-AT/p21SNFT/Jun complex failed to completely protect the AP-1-binding region from MPE:Fe. This result demonstrates that the p21SNFT protein rather than the GST tag was responsible for the difference in footprint observed with p21SNFT relative to that with Fos. The footprinting data in Figs. 6–8 show that a ternary complex containing p21SNFT, Jun, and NF-AT interacts quite differently than the well characterized NF-AT/AP-1 complexes with DNA. The results suggest that the NF-AT/AP-1 and NF-AT/p21SNFT/Jun complexes are conformationally distinct.

**DISCUSSION**

The studies presented here demonstrate that a p21SNFT/Jun complex interacts cooperatively with NF-AT at the distal NF-AT/AP-1 enhancer of the IL-2 promoter, as AP-1 complexes are well known to do (8, 16, 22). Although NF-AT binds the site alone as previously reported, the p21SNFT/Jun dimer requires recruitment by NF-AT to interact. This cooperativity with NF-AT may be significant in the mechanism by which p21SNFT is able to repress transcription from this element. For instance, although cooperative interactions between NF-AT and AP-1...
are required for synergistic transactivation, NF-AT alone is able to partially activate the site (22). Indeed, site-specific NF-AT mutants (e.g. R468E) that fail to interact cooperatively with AP-1 can retain a greater than 50% activation of the element (22). In contrast, the overexpression of p21SNFT leads to repression of transcription from the NF-AT/AP-1 enhancer by an additional 20% or greater repression (34). Therefore, it is a viable hypothesis that cooperativity between p21SNFT/Jun dimers and NF-AT may be required in the formation of a stable repressive complex. This notion is particularly interesting given that very few proteins other than AP-1 family members have been reported to bind and even fewer proteins reported to cooperate with NF-AT at this element. It is reasonable to speculate that the presence of p21SNFT in the complex may eliminate the ability of NF-AT to activate DNA, even partially, by imposing an inactive conformation upon NF-AT.

The hypothesis that a ternary complex containing p21SNFT is conformationally distinct from one containing Fos is supported by the footprinting data presented in Figs. 5–8. These figures show that although a p21SNFT/Jun dimer binds a TRE similarly to AP-1, a NF-AT/p21SNFT/Jun complex interacts differently at the IL-2 promoter distal enhancer element than the NF-AT/AP-1 complex. Specifically, the data show that several nucleotides within the AP-1 binding region of the site are not protected by the p21SNFT complex. This indicates that although the essential NF-AT/DNA interactions remain, p21SNFT may either fail to contact DNA or prevent Jun from doing so, or its presence may lead to both of these effects. Moreover, the fact that the p21SNFT/Jun dimer requires recruitment by NF-AT to interact at the NF-AT/AP-1 element suggests that extensive protein-protein interactions exist between NF-AT and the p21SNFT/Jun dimer, similarly to those between NF-AT and AP-1 (17). Therefore, it is probable that the drastic difference in footprint also reflects a larger structural change in protein-protein interactions between the two complexes.

The differences in protein-protein interactions between Fos/Jun and p21SNFT/Jun dimers may reflect the significant differences in leucine zipper composition between Fos and p21SNFT as well. Although Fos dimerizes more readily with Jun than p21SNFT dimerizes with Jun (Fig. 3), the relative affinity constants of the two dimers are almost identical (Fig. 4). This finding suggests that Fos is the preferred dimer partner for Jun in binding a TRE, but once formed, a p21SNFT/Jun dimer has an equal affinity for DNA to an AP-1 heterodimer. In contrast, both p21SNFT/Jun and Fos/Jun heterodimers preferentially interact with a TRE to Jun homodimers. These results are consistent with previous data using murine c-Fos with JunB, JunD, and c-Jun, which reported an approximate 8-fold increase in the affinity of all three heterodimers over the corresponding Jun homodimers (43). Although p21SNFT and Fos exhibit different abilities to dimerize with Jun, p21SNFT/Jun dimers seem to interact with NF-AT as readily as AP-1 heterodimers on the distal NF-AT/AP-1 enhancer of the IL-2 promoter. Therefore, it is reasonable to hypothesize that the relative concentrations of Fos versus p21SNFT may influence which complex is formed preferentially. However, it is unlikely that competition between p21SNFT and Fos is the sole mechanism leading to their different effects on transcription as p21SNFT represses transcription, whereas NF-AT mutants that fail to recruit Jun and retain transcriptional activation (22). In light of the footprinting data, the complexes formed by p21SNFT appear to change the interactions of NF-AT and Jun with DNA, an effect that may lead to the observed transcriptional repression.

If the in vitro data presented here reflect the natural combinatorial tendencies of the proteins, one may speculate as to the cellular role of p21SNFT. Fig. 1 demonstrates that the presence of p21SNFT causes translocation of the GFP protein from the cytoplasm to the nucleus under both stimulated and unstimulated conditions. The results indicate that endogenous p21SNFT is probably a constitutively nuclear protein. Because known bZIP transcription factors including Fos, Jun, and ATF family members localize to the nucleus (35–37), the data support the putative role of p21SNFT as a bona fide transcription factor. However, in contrast to many of the well characterized bZIP transcription factors including Fos and Jun, endogenous p21SNFT expression is not apparently regulated by stimulation (34). Additionally, although no regulation by phosphorylation or any other means has been reported for p21SNFT, both the abundance and activity of Fos are regulated by stimulation (33). Thus, it is possible that competition between Fos and p21SNFT in dimerization with Jun is regulated in part by Fos levels and activity in the cell. The relative efficiency and affinity data suggest that p21SNFT may interact preferentially during conditions when Fos levels are low because of suboptimal stimulation of the T cell. Hence, cell and tissue specificity as well as stimulation may tightly control the availabilities of dimer partners for Jun and of other proteins that cooperate with AP-1. In this model, one would anticipate that Fos forms an activating complex with Jun and other proteins such as NF-AT under stimulating conditions, whereas p21SNFT interacts under conditions that favor transcriptional repression.

The data presented here suggest that p21SNFT, which lacks a transactivation domain and therefore cannot transactivate DNA, may also repress transcription by altering the interactions of NF-AT and Jun in the complex.

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