BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 126–132 (1997)
ARTICLE NO. RC976415

Binding Sequence of STAT4: STAT4 Complex Recognizes the IFN-γ Activation Site (GAS)-like Sequence (T/A)TTCC(C/G)GGAA(T/A)

Koh Yamamoto, Osamu Miura, Shinsaku Hirosawa, and Nobuyuki Miyasaka
The First Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan

Received March 3, 1997

Studies of transcriptional activation by interferons and various cytokines have led to the identification of a family of proteins that serve as signal transducers and activators of transcription (STAT). STAT4 is phosphorylated following interleukin (IL)-12 stimulation and is required for IL-12 signal transduction. By immunoprecipitation and PCR amplification, a specific consensus sequence for DNA binding of the STAT4 complex was determined. The binding sequence of the STAT4 complex, (T/A)TTCC(C/G)GGAA(T/A), proved to be palindromic and similar to the IFN-γ activated site (GAS)-like sequence. The first (T/A) and last (T/A) sites of the consensus sequence were critical for the binding affinity of the STAT4 complex.

Cytokines regulate various functions within the hematopoietic system, including proliferation, differentiation, apoptosis, and functional responses of cells. Many of these effects are mediated by cytokines interaction with receptors which constitute the cytokine receptor superfamily (1). Cytokines binding to their receptors leads to the tyrosine phosphorylation of associated cytoplasmic protein tyrosine kinases, *Janus* kinases (Jak) (2, 3). Following cytokine binding and activation of Jak(s), Signal Transducers and Activators of Transcription (STAT) proteins are phosphorylated. The phosphorylated STAT proteins form homo- or hetero- dimer complexes, and the complexes translocate into the nucleus where they bind to specific DNA sequences in promotor regions of responsive genes (4-5).

Six members of the mammalian STAT family have been cloned, and some of the cytokines that activate members of the STAT family have been studied (5, 6). STAT1α and STAT2 were identified as DNA-binding proteins in interferon (IFN)-regulated gene expression (7, 8). In response to IFN-α/β, a DNA-binding complex consisting of STAT1α, STAT2 and a DNA-binding protein termed p48 is rapidly formed, then the complex binds to IFN-stimulated response elements (ISRE). In contrast, in response to IFN-γ, a DNA-binding complex consisting of STAT1α homodimers that bind to a unique element termed the IFN-γ activated site (GAS) is formed. Later on, other STAT family members that serve as DNA-binding proteins were cloned. STAT3 is an IL-6 activated transcription factor (9), while STAT5 is a prolactin-activated transcription factor of sheep (10). Subsequently, it was found that in mice there are two highly related STAT5 genes, STAT5a and STAT5b (11). STAT6 was identified as an IL-4-activated DNA-binding protein (12).

STAT4 was cloned using PCR amplification (13) or low stringency screening method (14). In response to IL-12, STAT4 is inducibly tyrosine phosphorylated, and forms homo- or hetero-dimer complexes (15, 16, 17). Promotor sequences of the target genes that are regulated by binding of the STAT4 complex are still unknown. Here, we selected and characterized a specific consensus sequence for DNA binding of STAT4 complex by immunoprecipitation and PCR amplification (18).

MATERIALS AND METHODS

Cell cultures, expression vectors, and reagents. COS 7 cells were maintained in DMEM medium supplemented with 10% fetal calf serum. Peripheral blood cells (PBCs) from healthy donors were isolated with Lympholyte-H (Cosmo Bio, Tokyo, Japan) by gradient centrifugation. To induce IL-12 responsiveness, PBCs were cultured for 4 to 7 days in RPMI 1640 medium containing phytohemagglutinin (PHA) (Life Technologies, Gaithersburg, MD) (19, 20). Recombinant human IL-12 (rhIL-12) was purchased from R&D Systems (Minneapolis, MN). Expression vectors for STAT1α, STAT3, STAT4, STAT5a, STAT6 and Jak2 have been previously described (13, 21). For immunoprecipitation, Western blotting and supershift analysis in electrophoretic mobility shift assay (EMSA), FLAG or HA epitope was tagged as previously described (13). Anti-FLAG monoclonal antibody (M2) and agarose-conjugated anti-FLAG antibody (M2) were purchased from Kodak International (New Haven, CT). Anti-HA mono-

0006-291X/97 $25.00

clonal antibody (12CA5) was obtained from Boehringer Mannheim (Tokyo). Anti-phosphotyrosine monoclonal antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-STAT1, anti-STAT3, anti-STAT4, anti-STAT5 and anti-STAT6 antibodies have been previously described (21).

Preparation of proteins. To make STAT complex, COS 7 cells were transfected with Jak2 and/or the STAT expression vector (13, 21). PHA-activated PBCs were stimulated with rhIL-12 (4 ng/ml) without starvation. Before stimulation, PBCs were preincubated for 2 hours with protein kinase inhibitor, 50 μM of H7 (Seikagaku Co., Tokyo) or 0.5 μM of staurosporin (Sigma, St. Louis, MO). Whole cell extracts of COS 7 cells and PBCs were prepared by incubating the cells in lysis buffer as previously described (13).

Binding site selection. To determine the DNA sequences that are recognized by the STAT4 complex, two sets of double-stranded random oligonucleotides were synthesized as previously described (22). The random oligonucleotides which contained 15 and 35 bases were sandwiched between two constant flanking regions of 20 bases that could be used as primers for PCR amplification, and that also contained *Bam*HI and *Eco*RI restriction sites for subcloning. Whole cell extracts of COS 7 cells, containing approximately 200 μg of protein, were incubated with 2 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia, Tokyo) and 2 μg of double-stranded oligonucleotide for 1 h at 4°C, and then incubated with 10 μl of agarose-conjugated anti-FLAG antibody for 1 h at 4°C. The precipitated oligonucleotide-protein complex was washed four times with washing buffer (0.5% Nonidet P-40, 50 mM Tris-Cl (pH 8.0), 10% Glycerol, 0.1 mM EDTA, 150 mM NaCl, 0.1 mM Na_3VO_4, 50 mM NaF, 0.1 mM $ZnSO_4$). The selected oligonucleotides were eluted from the agarose in 10 μl of H_2O by boiling for 5min. The supernatant was harvested, and 1 μl was used for PCR amplification in a 100 μl mixture containing 30 pmoles of forward and reverse primers corresponding to the constant flanking regions of random oligonucleotides, and 2 U of Taq DNA polymerase (Takara, Kyoto, Japan). PCR was carried out as 25 cycles of 94°C for 1min in case of denaturation and 52°C for 1min in that of annealing. PCR products of 5 μl were used for the former cycle of binding reaction. After six rounds of binding and PCR amplification, PCR products were digested with *Eco*RI and *Bam*HI, and subcloned into *Eco*RI-*Bam*HI digested pBluescript, and sequenced using an autosequencer (Applied Biosystems, Chiba, Japan).

EMSA. EMSAs were carried out essentially as previously described (13) using [^{32}P]-Klenow labeled synthetic double-stranded oligonucleotide probes. The sequences of probes used in this study are shown in Fig. 1. Underlines indicate consensus sequences of the STAT bindings with a spacer, TTC(N)$_{2\text{-}4}$GAA (6), and letters in italics are mutated sequences.

Immunoprecipitation, DNA affinity precipitation, and Western blotting. Immunoprecipitation with anti-HA, anti-STAT4, anti-STAT5, and anti-STAT6 antibodies, and Western blotting with anti-phosphotyrosine, anti-HA, anti-STAT4, anti-STAT5, and anti-STAT6 antibodies, were carried out as previously described (13). To prepare the DNA affinity agarose, synthetic 5′-biotin labeled (biotin-N3) or non-biotin labeled oligonucleotide (N3), CTAGGCGTGGCTTTCCCGGAATCCTTGAG, was annealed with a complementary oligonucleotide, CTAGCTCAAGGATTCCGGGAAAGCCACGC. The double-stranded oligonucleotide was ligated by 5′ overhang, and recessed 3′ ends were filled by Klenow enzyme. The biotinylated DNAs were mixed with agarose-conjugated streptavidin (Sigma) for 30 min, washed with phosphate buffered saline (PBS), and stocked at 4°C as a 50% slurry DNA-agarose. Whole cell extracts containing approximately 800 μg of protein were incubated with 30 μl of DNA-agarose and 20 μg of poly(dI-dC)-poly(dI-dC) for 15 min at 4°C, washed with washing buffer, and eluted in SDS-PAGE loading buffer. The samples were analyzed by Western blotting.

FIG. 1. Sequences of oligonucleotide probes used for EMSA. The consensus sequences (6) are underlined and mutations were introduced to the region of the consensus sequence (CA-TC, -TAA-, A---A, C---C, and G---G) and spacing sites (N2 and N4).

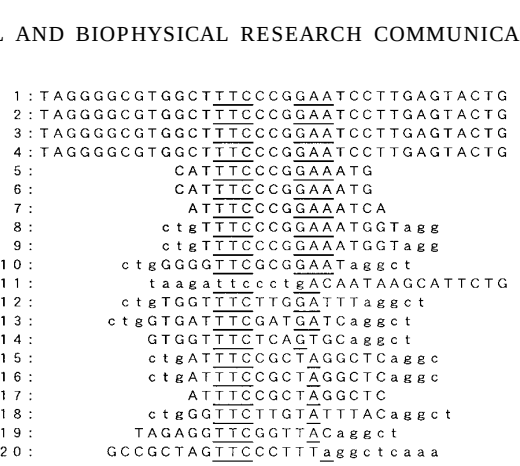

FIG. 2. Sequences of oligonucleotides obtained by binding and PCR amplification using the STAT4 complex. Identification of consensus sequences of clones obtained by immunoprecipitation and PCR amplification. Lowercase letters indicate nucleotides that were in the constant flank regions of the synthesized oligonucleotides. The obtained sequences were arranged from 5′ sites to obtain maximal homology. The sequences showing similarity with STAT binding sequences, TTC(N)$_{2\text{-}4}$GAA (6), were underlined.

RESULTS

In vitro binding sequence selection for STAT4. To select specific STAT4-DNA binding sequences, we employed a unique technique that involved immunoprecipitation of FLAG-tagged STAT4 and PCR amplification of bound oligonucleotides (18). We sequenced 20 independent clones prepared from STAT4-bound oligonucleotides (Fig. 2). A consensus binding sequence, (T/A)TTCC(C/G)GGAA(T/A) was readily found. The consensus sequence was palindromic and similar to the previously identified GAS-like elements, TTNCNNNAA (4).

STAT4 complex binding to the selected consensus sequence. To confirm binding of the STAT4 complex to the obtained consensus sequence, we performed EMSA. The double-stranded oligonucleotide N3, was synthesized according to the obtained consensus sequence,

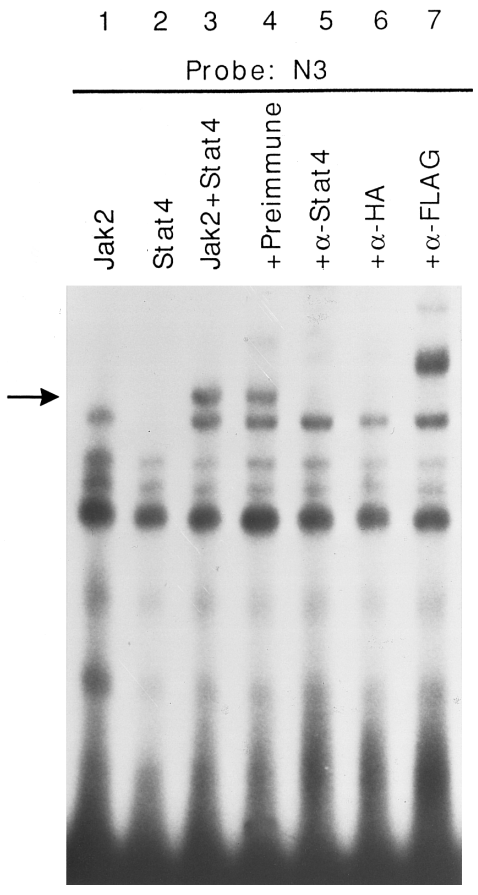

FIG. 3. EMSA with a labeled oligonucleotide of the consensus sequence. A radiolabeled probe (N3) that corresponded to the STAT4 consensus sequence was incubated with extracts of COS 7 cells that were transfected with Jak2 (lane 1), STAT4 (lane 2), Jak2 and STAT4 (lanes 3-5), Jak2 and STAT4-HA (lane 6), and Jak2 and STAT4-FLAG (lane 7). Preimmune serum, anti-STAT4 serum, anti-HA antibody, and anti-FLAG antibodies were added (lanes 4-7). The arrow indicates the STAT4-DNA complex.

and used as a probe (Fig. 1). As shown in Fig. 3, a STAT4 DNA-binding complex was observed only when COS 7 cells were transfected with Jak2 and STAT4 expression vectors (lane 3). The complex was not affected by the addition of pre-immune serum (lane 4), but the band disappeared when anti-STAT4 serum or anti-HA antibody was added (lanes 5 and 6), and shifted upward when anti-FLAG antibody was added to the reaction mixture (lane 7).

Next, DNA affinity precipitation was carried out to determine whether the STAT4 directly binds to the consensus sequence. As shown in Fig. 4, phosphorylated STAT4 was precipitated with the oligonucleotide-agarose that corresponded to the consensus sequence (lane 4). Without Jak2 transfection, STAT4 was not phosphorylated and not precipitated (data not shown).

Analysis of STAT4 binding sequence using mutated oligonucleotide probes. The specificity of DNA sequence for STAT4-DNA binding was further characterized by competitive assay of EMSA using the N3 probe. To confirm similarity with STAT binding sequence, TTCNNNGAA (6), unlabeled oligonucleotide probes N3, mIRF-1, N2, N4, CA-TC and -TAA-, were used in the assay as competitors (Fig. 1). As shown in Fig. 5A, the STAT4-DNA binding complex was competed not only by the corresponding unlabeled N3 probe in an excess amount, but also by the unlabeled mIRF-1 probe (lanes 3-8). No competition was observed with unlabeled N2, N4, CA-TC probes (lanes 9-11) and unlabeled -TAA- probe (data not shown). No binding was observed using labeled CA-TC and -TAA- probes (data not shown).

To investigate the effect of the flanking sequence of NTTCNNNGAAN on binding affinity, unlabeled oligonucleotides, A---A, C---C, G---G were employed in the competition assay (Fig. 1). As shown in Fig. 5B, the STAT4-DNA binding complex was completely inhibited by the A---A probe (lanes 5 and 6), weakly inhibited the C---C

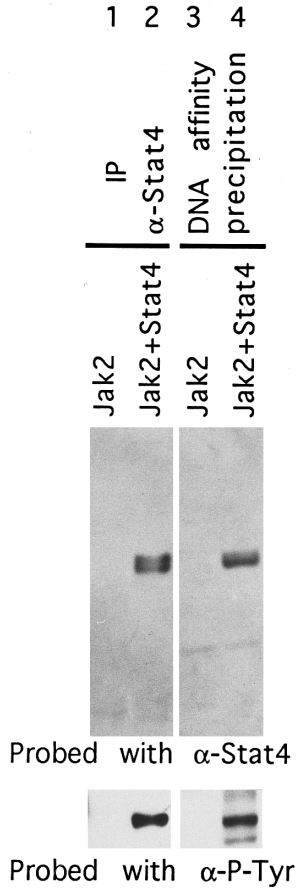

FIG. 4. DNA affinity precipitation and immunoprecipitation of STAT4. Whole cell extracts of COS 7 cells transfected with Jak2 (lanes 1 and 3) or Jak2 and STAT4 (lanes 2 and 4) were immunoprecipitated with anti-STAT4 antibody (lanes 1 and 2) or DNA affinity precipitated (lanes 3 and 4) and subjected to anti-STAT4 blotting (α-Stat4) followed by reprobing with anti-phosphotyrosine (α-P-Tyr).

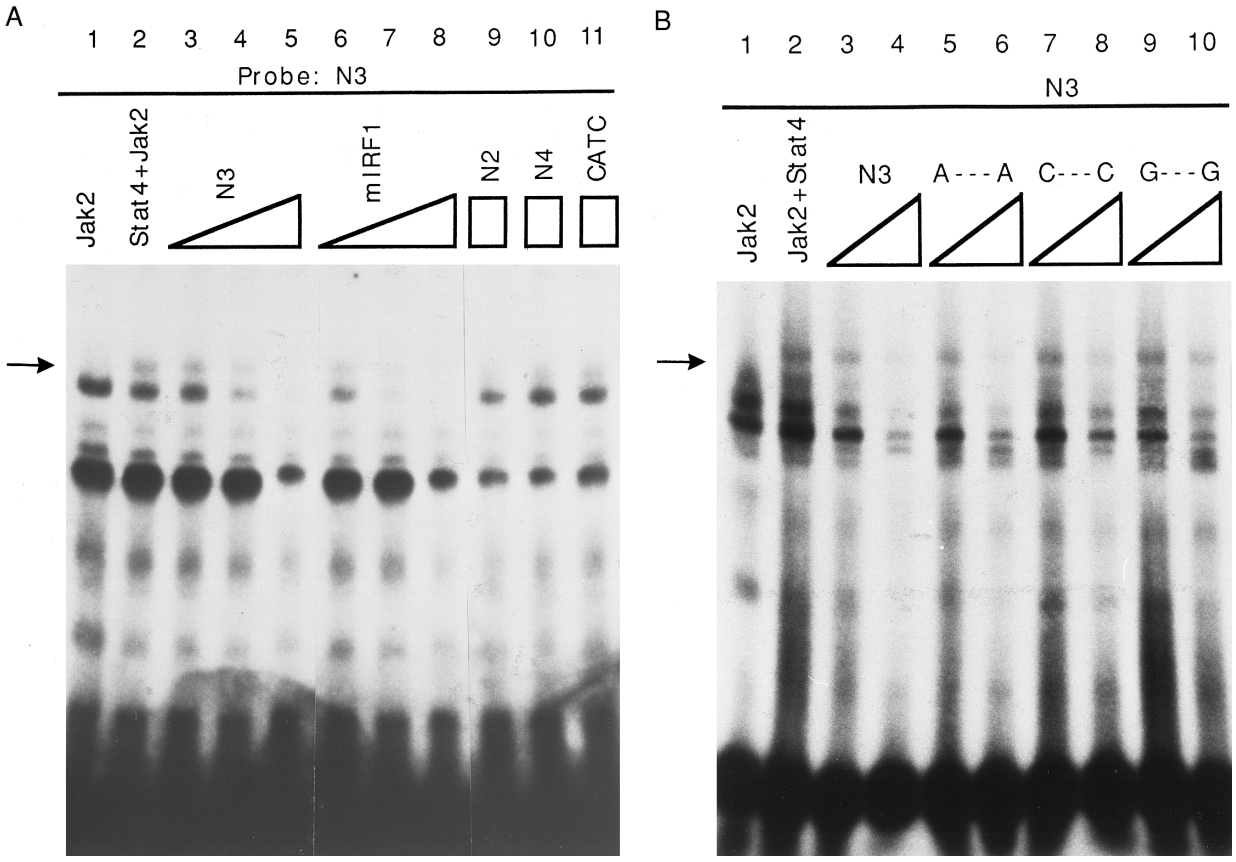

FIG. 5. Competition analysis of EMSA. The radiolabeled N3 probe was incubated with extracts of COS 7 cells that were transfected with Jak2 (A, lane 1, and B, lane 1), and Jak2 and STAT4 (A, lanes 2-11, and B, lanes 2-10) with 1, 10, or 100-fold excess of unlabeled oligonucleotides, N3, mIRF-1, N2, N4, CA-TC, A---A, C---C, G---G.

probe (lanes 7 and 8), and not inhibited by the G---G probe (lanes 9 and 10). The labeled A---A oligonucleotide probe bound to the STAT4-DNA complex, but labeled C---C and G---G probes did not bind (data not shown).

DNA binding complex in IL-12-stimulated PBCs. To further characterize DNA-binding complexes in IL-12-stimulated cells, PHA-activated PBCs were stimulated with rhIL-12 for various periods of time, and whole cell extracts were analyzed by EMSA and Western blotting. Phosphorylated STAT4 was detected after 2 min of rhIL-12 stimulation and persistently found for 120 min in phosphotyrosine blot (data not shown). As shown in Fig. 6A, a slowly migrating DNA-binding complex (upper arrow in Fig. 6A) was observed after 5 min of rhIL-12 stimulation and a faster migrating complex (lower arrow in Fig. 6A) was similarly detected after 60 min. These bands completely disappeared when anti-STAT4 antibody, but not anti-STAT3 or anti-STAT1 antibody was added (Fig. 6B). These bands were not affected by preincubation with H7, but, were abolished by preincubation with staurosporin (Fig. 6A, lanes 10-14). The slowly migrating DNA-binding complex had strongest affinity at 30 min and high affinity was found in the faster migrating complex at 90 min.

Specificity of other STAT complexes for N3 and mutated probes. The specificity of other STAT complexes for N3, N2 and N4 probes were investigated by EMSA. COS 7 cells were transfected with STAT1α, STAT3, STAT5a and STAT6 expression vectors. All STAT complexes were similarly tyrosine-phosphorylated with Jak2 cotransfection in a phosphotyrosine blot (data not shown). As shown by the arrowheads in Fig. 7, STAT1α- and STAT5-DNA complexes could bind to N2, N3, and N4 (lanes 3 and 9), while STAT3 and STAT6 bound to N4 (lanes 5, 11), and STAT4 complex bound only to N3.

DISCUSSION

By immunoprecipitation and PCR amplification, we determined the STAT4-binding sequence (T/A)TTCC(C/G)GGAA(T/A), which is palindromic and very similar to the previously identified GAS-like element TTNCNNNAA (4). Recently, the optimal sequence for STAT4 binding,

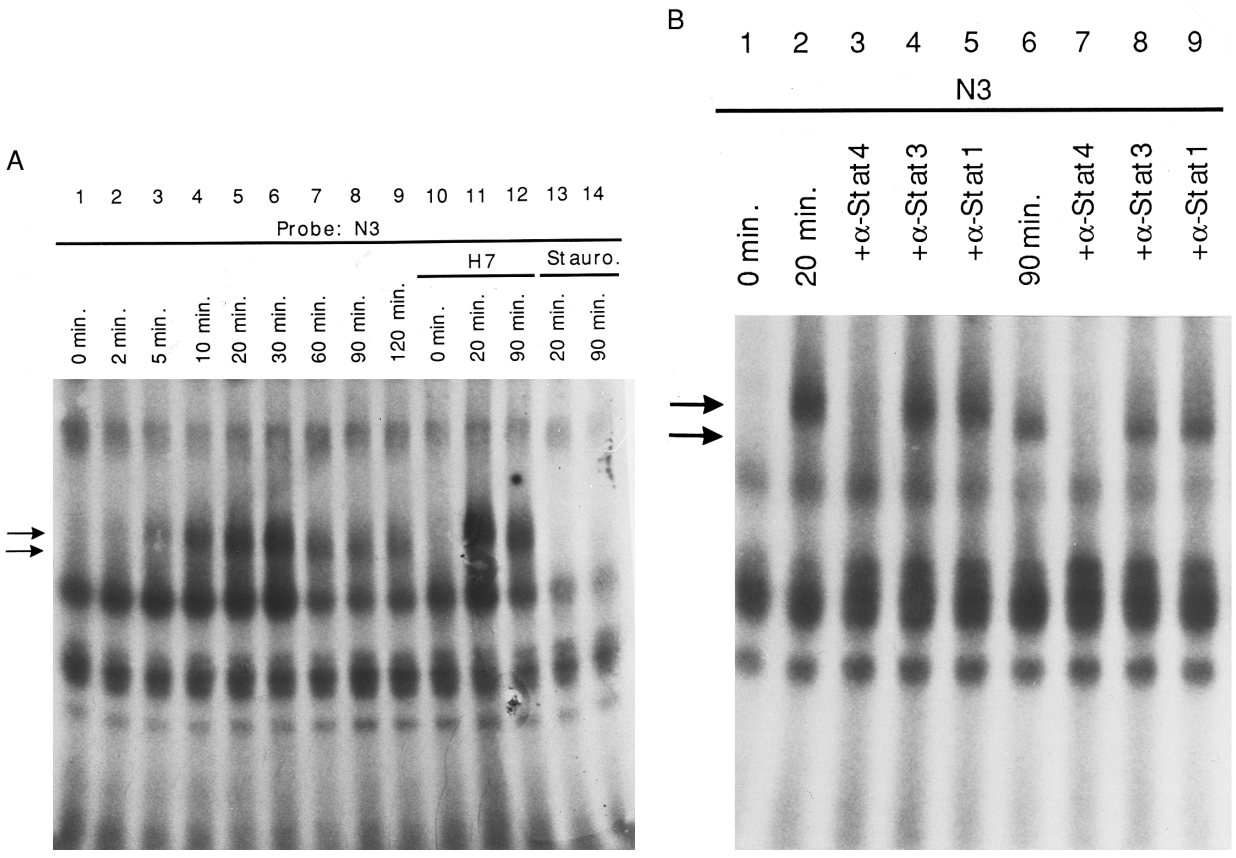

FIG. 6. EMSA of PHA-activated PBCs stimulated with rhIL-12. PHA-activated PBCs were stimulated with rhIL-12 for indicated periods of time. Following stimulation, cells were lysed, and EMSA was carried out with the N3 probe. Before stimulation, PBCs were preincubated with H7 (A, lanes 10-12) or staurosporin (A, lanes 13 and 14). Whole cell extracts were incubated with anti-STAT4, anti-STAT3, or anti-STAT1 antibody (B, lanes 3-5 and 7-9).

TTCCGGGAA, was reported (23), and its sequence is quite similar to the STAT4 binding consensus sequence obtained in this study. As previously reported, the STAT4 complex can bind to GAS-like elements, such as mIRF-1 (AGCCTGATTTCCCCGAAATGATGAG) (13), IFN-γ response element (GRR) of the human Fcγ receptor I (FcγRI) gene (AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG) (15, 16, 17), and m67 (GTCGACATTTCCCGTAAATCGTCGA) (15). These sequences contain sequences very similar to the obtained STAT4-binding sequence (underlined).

The binding consensus sequences of other STATs have been reported (6). STATs recognize a palindromic sequence characterized by half-sites bearing the trinucleotide sequence 5′-TTC-3′, with 2- to 4- base spacing between dyad half sites (24). STAT1α and STAT5 complexes favor a 3-bp spacing (TTCNNNGAA), STAT3 a 2- or 3-bp spacing (TTCNNGAA or TTCNNNGAA), and STAT6 a 3- or 4-bp spacing (TTCNNNGAA or TTCNNNNGAA) (24, 25). The GAS sequence shows a 3-bp spacing. In this report, the STAT4 complex bound to only consensus sequences with 3-bp spacing, and did not bind to those with a 2-bp or 4-bp spacing. Also half-sites bearing the trinucleotide sequence 5′-TTC-3′, are critical for STAT4 binding.

Because mIRF-1, GRR, and m67 contain either T or A in the flanking positions of NTTCNNNGAAN, and 90% of the obtained sequence of clones had either T or A in one of the flanking positions, we determined the effect of the flanking sequence of TTCNNNGAA by introducing mutations at both ends of the consensus sequence. The mutations of flanking T/A nucleotides abolished STAT4-DNA binding. The first and the last flanking bases of T/A must be prerequisites to the formation of the STAT4-DNA complex.

Interestingly, IL-12 stimulation induced a decrease in the mobility of STAT4 protein in Western blotting (16). Approximately half of the STAT4 protein migrated slowly after stimulation for 20 min, and most of the STAT4-DNA complex migrated as the upper band after stimulation for 90 min. The shift of the upper band was inhibited by preincubation with H7 (data not shown), and the shift of the upper band was thought to be due to phosphorylation of the serine resi-

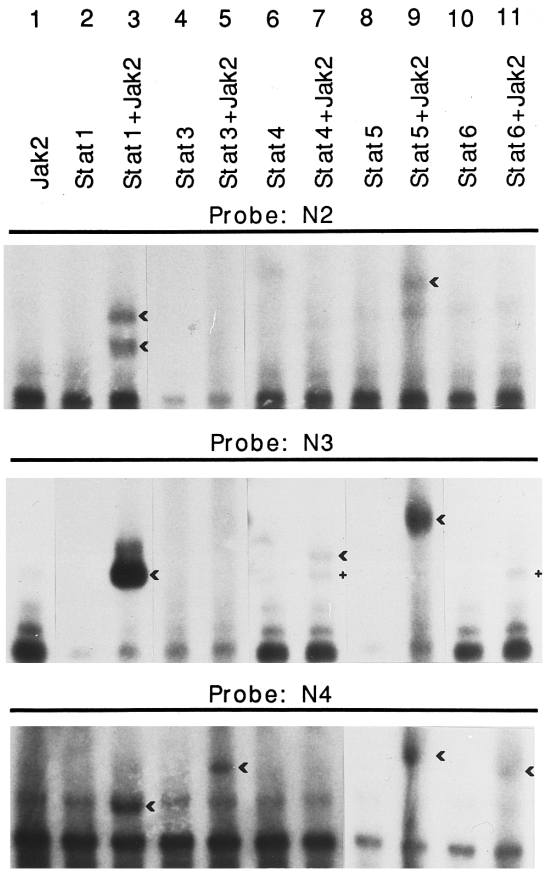

FIG. 7. EMSA of STAT1α, STAT3, STAT4, STAT5a, and STAT6 with N2, N3, and N4 probes. Arrowheads indicate STAT-DNA complexes. The weak bands indicated by "+" (lanes 7 and 11) were due to activation of endogenous STAT1.

due of STAT4 (16, 26, 27). As for the serine residue, we introduced a point mutation at the phosphorylation site of the putative serine residue for STAT4. This mutant was tyrosine-phosphorylated with cotransfection of Jak2, and retained DNA binding activity in EMSA (data not shown).

In EMSA, STAT4 complex migrated faster after stimulation for 90 min than that when stimulated for 20min, and this faster band was not affected by preincubation with H7. In response to IL-6 and CNTF, a heterodimer of STAT1α and STAT3 appeared later and migrated faster than the STAT3 homodimer in EMSA (26). The difference in the migration pattern of the STAT4 complex may be due to phosphorylation of the serine residue and binding of other protein(s). The ability of various STATs to heterodimerize may also influence their sequence specificity, and the binding sequence of STAT heterodimer should be further studied.

IL-12 is required for T cell-independent induction of IFN-γ, a critical step in the initial suppression of bacterial and parasitic infections (28). IL-12 is also important for the development of Th1 response which is critical for an effective host defense against intracellular pathogens (28, 29). Recently, gene targeting of STAT4 have been reported by two groups (30, 31). STAT4-deficient mice lack IL-12 mediated responses of NK and T cells, and STAT4 is required for IL-12 signal transduction. Although STAT4 can bind to GAS-like sequences in vitro, IL-12 did not induce m-IRF gene expression (data not shown). The target genes of IL-12-receptor-Jak-STAT4 signal transduction pathway should be further determined.

ACKNOWLEDGMENT

We thank Ms. K. Okada for helping us with the experiments.

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