Molecular Cloning of a New Interferon-induced Factor That Represses Human Immunodeficiency Virus Type 1 Long Terminal Repeat Expression

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From the Institut de Genetique Moleculaire de Montpellier-UMR 9942, CNRS, BP 5051, 1919 Route de Mende, 34033 Montpellier Cedex 1, France

Transcriptional induction of genes is an essential part of the cellular response to interferons. To isolate yet unidentified IFN-regulated genes we have performed a differential screening on a cDNA library prepared from human lymphoblastoid Daudi cells treated for 16 h with human α/β interferon (Hu-α/βIFN). In the course of these studies we have isolated a human cDNA which codes for a protein sharing homology with the mouse Rpt-1 gene; it will be referred as Staf-50 for Stimulated Trans-Acting Factor of 50 kDa. Amino acid sequence analysis revealed that Staf-50 is a member of the Ring finger family and contains all the features of a transcriptional regulator able to initiate a second cascade of gene induction (secondary response). Staf-50 is induced by both type I and type II IFN in various cell lines and down-regulates the transcription directed by the long terminal repeat promoter region of human immunodeficiency virus type 1 in transfected cells. These data are consistent with a role of Staf-50 in the mechanism of transduction of the IFN antiviral action.

The interferons (IFNs)[1] are a family of secreted multifunctional proteins which exert a broad spectrum of biological activities. First characterized for their potent antiviral properties, it has now been established that they are involved in number of regulatory functions such as control of cell proliferation, differentiation, and regulation of the immune system (1). They are subdivided into two types that activate transduction pathways via different cell surface receptors (2, 3). Binding of both type I IFN (IFN-α/β) and type II IFN (IFN-γ) result in the differential activation of latent cytoplasmic transcription factors termed Stats (for Signal Transducer and Activator of Transcription) (4, 5) which act at different cis-acting DNA elements. Type I IFN promptly induces the phosphorylation of Stat-113 (p113 kDa), Stat-91 (p91 kDa), and Stat-84 (p84 kDa) (p91 and p84 are generated from the same gene by alternative splicing) proteins, by tyrosine phosphorylation involving the α/β IFN receptor-associated tyrosine kinases TYK2 and JAK1 (6–9). Following phosphorylation, Stat-113 and Stat-91 or Stat-84 form the transcriptionally active IFN-stimulated gene factor 3 by association with a 48-kDa subunit which binds DNA (10, 11). The specificity of the transcriptional activation by IFN-stimulated gene factor 3 is mediated by specific elements termed IFN-stimulatory element located in the promotor region of IFN-inducible genes (12, 13).

Gene induction by type II IFN involves solely the phosphorylation of Stat-91 by the JAK-2 kinase (a homolog of TYK2). This phosphorylation generates a homodimer of Stat-91 which is able to bind the IFN-γ-activated site (GAS element) to activate transcription (14–16).

Of the many IFN activities, the antiviral state has been best characterized at the biochemical level. The IFNs can act directly at various steps of the viral multiplication cycle including cell penetration, transcription, translation, and the assembly of viral particles (17, 18). Several IFN-induced proteins involved have been described such as the double stranded RNA-dependent p68 (human)/p65 (murine) protein kinase (double stranded-activated protein kinase) (19), the 2–5A synthetases (20–22), and the product of the Mx1 gene (23). In the presence of double stranded RNA, the phosphorylation on a serine residue activates the latent ribosome-associated double stranded-activated protein kinase which then phosphorylates the α-subunit of the eukaryotic initiation factor-2. The phosphorylated form of eukaryotic initiation factor-2α induces an inhibition of protein synthesis giving rise to the establishment of an antiviral state (24). It has been established that the replicating viral RNA of viruses, like encephalomyocarditis virus, is most probably responsible for the activation of double stranded-activated protein kinase during viral infection (22). The second of the two IFN-induced and double stranded RNA-activated enzymes is the 2–5A synthetase which catalyzes the synthesis of adenosine oligomers (2–5A). This 2–5A then activates the RNase L, an endoribonuclease latent in most mammalian cells (17). Various data suggest that the 2–5A synthetase/RNase L pathway inhibits the replication of picornaviruses such as encephalomyocarditis virus (20, 25) and mengovirus (26). The human and mouse Mx1 gene have been shown to confer selective innate resistance to influenza virus in cultured cells as well as in mice without affecting the development of many other viruses (18).

IFNs may also act indirectly on viral replication, by favoring the recognition of infected cells by the immune system. For example, IFN-γ can control cytomegalovirus (CMV) infection by favoring presentation of viral antigen by the major histocompatibility class I molecules of CMV infected cells to the immune system (27).

[1] The abbreviations used are: IFN, interferon; Stat, signal transducer and activator of transcription; CMV, cytomegalovirus; LTR, long terminal repeat; HIV-1, human immunodeficiency virus type 1; kb, kilobase pair(s); bp, base pair(s); IM, intermediate motif.

Since viruses have developed various strategies to circumvent the antiviral activities of IFNs (28), mammalian cells usually make use of several strategies that act in cooperation to interfere with the viral multiplication cycle. The mechanisms of the IFN-induced antiviral state are still far from being understood, and the molecular characterization of the IFN-induced proteins remains a main challenge for the comprehension of the molecular mechanism of IFN action.

We have therefore established a cDNA library from IFN-treated Daudi cells and made use of differential screening to search for yet unidentified IFN-regulated genes. In the course of these studies we have isolated a human cDNA with homologies to the mouse Rpt-1 gene (29) which will be referred as Staf-50 for Stimulated Trans-Acting Factor of 50 kDa. We demonstrate that Staf-50 is induced by both type I and type II IFN and that its gene product down-regulates transcription directed by the long terminal repeat (LTR) promoter region of human immunodeficiency virus type 1 (HIV-1). The potential role of Staf-50 in the mechanism of antiviral action of IFNs is discussed.

MATERIALS AND METHODS

Cell Cultures—Human lymphoblastoid Daudi cells were grown in suspension in RPMI 1640 medium, supplemented with 10% (v/v) fetal calf serum. HeLa cells were grown in monolayer cultures in Dulbecco's medium containing 10% (v/v) fetal calf serum. For IFN induction, exponentially growing cells were exposed 16 h to 500 international units/ml of human lymphoblastoid IFN (Hu-IFNα/β obtained from Hayashibara Biochemical Laboratories Inc.) or 500 units/ml of γ-IFN (a gift of Roussel Uclaf, France). SV40-transformed monkey kidney epithelial cells (COS-7 m6) were grown in monolayer cultures in Dulbecco's medium supplemented with 10% (v/v) fetal calf serum.

RNA Purification and Northern Blot Analysis—For RNA purification the cells were pelleted, washed in phosphate-buffer saline, and total mRNAs were isolated by the guanidine thiocyanate method, as described previously (30). RNAs were fractionated by electrophoresis on a 10% (v/v) formaldehyde-containing 1.2% (w/v) agarose gel and transferred to nylon membranes (Hybond N, Amersham). The multiple tissues Northern blot membrane (Clontech) was a gift of Dr. P. Fort. Prehybridizations were performed at 42 °C for 12 h, in a mixture containing 50% (v/v) formamide, 0.75 M NaCl, 50 mM sodium phosphate buffer, pH 7, 1 mM EDTA, 0.2% (w/v) sodium dodecyl sulfate (SDS), 5 × Denhardt's, 10% (w/v) dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. An additional 12-h hybridization was performed in the presence of 10^6 cpm/ml of the ^{32}P random primed cDNA probe. Stringent washings were then conducted at 65 °C in 0.1 × SSC buffer (0.15 M NaCl, 0.015 M sodium citrate) before autoradiography.

Construction of cDNA Library and Isolation of cDNA Clones—Poly(A$^+$) RNAs were isolated from total mRNAs using the Dynabeads biomagnetic separation system (Biosys S.A) and the cDNA library was constructed in the λ ZAP-cDNA synthesis system (Stratagene). The library was plated at low density in order to obtain individual plaques and transferred to nylon membranes (Hybon N, Amersham). A single round screening was performed by successive hybridization of a single filter using ^{32}P-labeled cDNA probes (2 × 10^6 cpm/ml) obtained from poly(A$^+$) RNAs of untreated or IFN-treated Daudi cells. Prehybridization, hybridization, and washing of the filter were performed as described for Northern blot analysis. Clones exhibiting a variation in signal intensity were isolated and the pBluescript phagemid vectors containing inserts were excised using the ExAssit-SORL system (Stratagene). Phagemid DNAs were then extracted and used to probe Northern blot membranes.

Sequence Determination and Characterization of cDNA Clones—Plasmid DNA of individual clones were prepared and their sequences were determined by the Sanger dideoxy sequencing method (T7 sequencing kit from Pharmacia). The complete sequence of Staf-50 cDNA was obtained on both strands by overlap of sequenced fragments of the original clone after subcloning in the pBluescript II KS vector. Search for sequence homologies in the EMBL and GenBank data bases, as well as sequence analyses were performed by using the BISANCE facilities (31).

In Vitro Translation—In vitro transcription-translation of the Staf-50 containing vector was performed in the transcription/translation T-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. The [^{35}S]methionine-labeled proteins were

fractionated by SDS-polyacrylamide gel electrophoresis before autoradiography.

Plasmid Constructions—The pJ-Staf50 and pJ-Staf50as were generated by cloning the *XbaI-XbaI* fragment, in the sense or the antisense orientation, respectively (see restriction map in Fig. 1D), downstream from the CMV promoter in the pJ7Ω vector (32). The pLTR-luc and the pAcβ-gal vectors were a generous gift of I. Barlat and the pSVβ-gal (33) was a gift of Dr. J. M. Blanchard. The pCMVβ-gal vector expressing the β-galactosidase gene under the dependence of the CMV promoter was provided by Stratagene.

Transient Transfection Experiments—For transfection experiments, 1–5 × 10^5 exponentially growing COS-7 m6 and HeLa cells were inoculated in 60-mm culture dishes. The following day, the cells were washed twice with phosphate-buffered saline and transfected with 9 μg of the appropriate mixture of vectors using the modified bovine serum mammalian transfection kit of Stratagene. The cells were then incubated 48 h at 37 °C, washed twice with phosphate-buffered saline, and the luciferase activities were determined using the Luciferase Assay system (Promega) in a Berthold Luminometer counter (Lumat LB 9501). The β-galactosidase activities were measured as described previously (34).

RESULTS

Construction and Screening of a cDNA Library from Hu-α/βIFN-treated Daudi Cells—Total RNAs were extracted from human lymphoblastoid Daudi cells treated for 16 h with 500 international units of Hu-α/βIFN. These conditions were previously described to induce strong antiviral and antiproliferative action in this cell line (35). An oriented cDNA library was constructed using the λ ZAP-cDNA synthesis kit (Stratagene). 5000 primary recombinant clones were screened successively with single stranded ^{32}P-labeled cDNA derived from exponentially growing untreated cells and with cDNA from IFN-treated cells. A single filter was probed sequentially with both cDNA preparations in order to avoid false-positive clones (36). Since a limited number of clones were screened, the comparative analysis of autoradiographic data was performed manually. 105 spots exhibiting a variation in signal intensity were selected and pBluescript phagemid vectors containing inserts were excised using the Stratagen ExAssit-SORL system. DNA were prepared and used to probe a Northern blot containing total RNA extracted from Daudi cells treated for various times with Hu-α/βIFN. Clones exhibiting differential expression upon Northern blotting analysis by comparison with an invariant glyceraldehyde-3-phosphate dehydrogenase (37) probe were selected. Half of the 105 clones were false-positives and partial sequence examination of the others revealed four unknown IFN-regulated genes.

Analysis and Specificity of the Expression of a New IFN-induced RNA—We have focused our interest on a strongly IFN-induced gene which contains a 2.8-kb insert and which will be referred to as Staf-50. The kinetic of expression of the RNA hybridizing to this cDNA probe was analyzed by probing a Northern blot of total RNAs isolated from Daudi cells treated for various times with Hu-α/βIFN. As shown in Fig. 1A, the 2.8-kb probe hybridized strongly to a RNA species of the same size which accumulated rapidly after the onset of IFN treatment (2-fold induction after 2 h). A 9-fold increase in its steady state level was reached after 16 h of exposure to IFN. Hybridization to a glyceraldehyde-3-phosphate dehydrogenase probe used as unvariant control confirmed that each lane of the blot contained an equal amount of total RNA (Fig. 1A).

The specificity of induction of the 2.8-kb RNA in response to treatment with the various types of IFN was then analyzed. Since Daudi cells failed to respond to Hu-γIFN for the lack of functional receptors, HeLa cells were treated with 500 units/ml of Hu-α/βIFN or Hu-γIFN and total RNA were extracted and analyzed as described above. The kinetics of induction of Staf-50 mRNA was found to be similar with the two types of IFN (Fig. 1B), although Hu-α/βIFN was revealed to be a

FIG. 1. **Northern analysis of IFN-induced Staf-50 mRNA and restriction map of Staf-50 cDNA.** Total RNAs (20 μg/lane) were separated on 1.2% formaldehyde-agarose gel, transferred to nylon membrane, and hybridized to a ^{32}P-labeled Staf-50 cDNA probe. The same blots were reprobed with a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe to ensure that equal amounts of RNA were loaded in each lane. *A*, time course of Staf-50 mRNA induction with Hu-α/βIFN. Daudi cells were treated by Hu-α/βIFN (500 units/ml) for the indicated times. The location of the 18 S and 28 S rRNAs are indicated. *B*, specificity of Staf-50 mRNA induction by Hu-α/βIFN and Hu-γIFN. HeLa cells were treated or not by Hu-α/βIFN (500 units/ml) or Hu-γIFN (500 units/ml) for the indicated times. *C*, induction of Staf-50 mRNA during protein synthesis inhibition. HeLa cells were incubated with cycloheximide (20 μg/ml) for 30 min and then treated (*CHX+IFN*) or not (*CHX*) by Hu-α/βIFN (500 units/ml) for 6 h. *D*, partial restriction map of Staf-50 cDNA. Restriction enzyme sites *Eco*RI, *Xba*I, *Xho*I, and their nucleotide location are indicated. The *Xba*I and *Xho*I sites at the extremities are linker-derived sites. The open reading frame is represented by the *solid box* (position +123 to +1448).
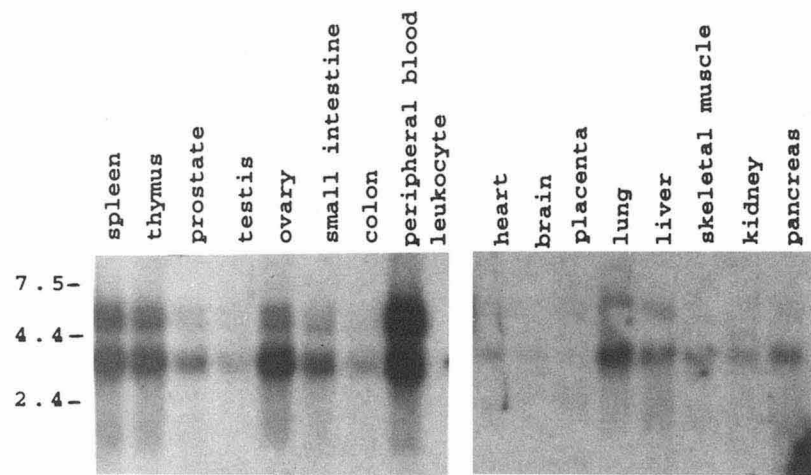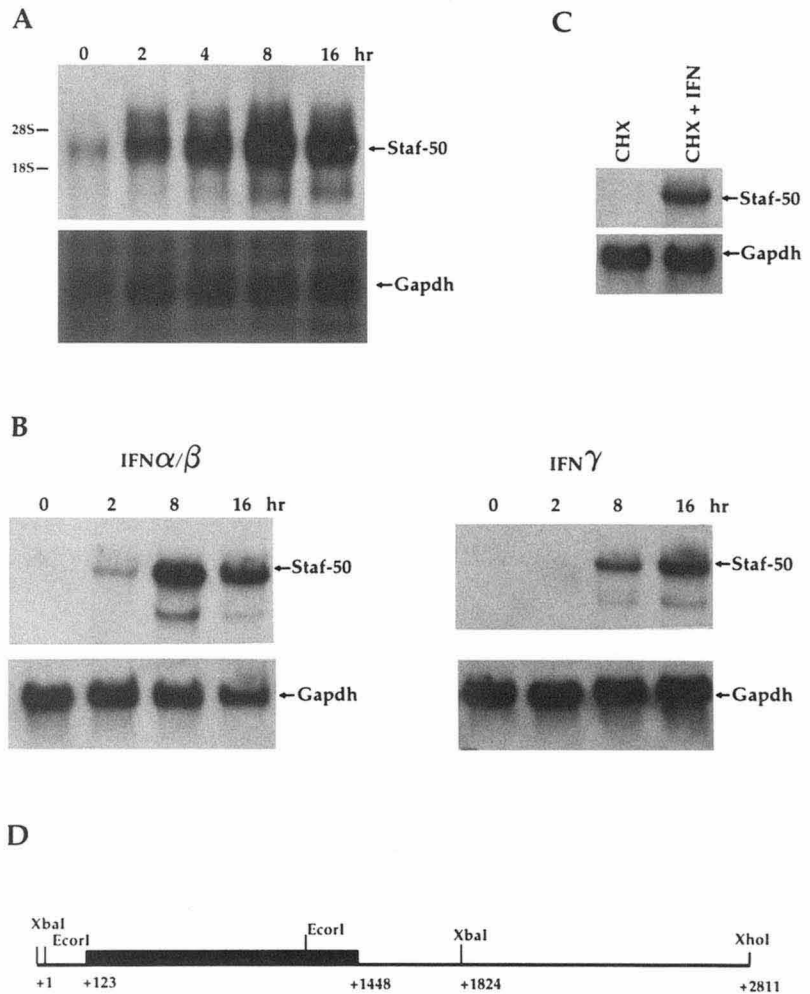
FIG. 2. **Tissue specificity of Staf-50 expression.** A multiple tissues Northern blot membrane (Clontech) was hybridized to the ^{32}P-labeled Staf-50 cDNA probe. The tissue source of the mRNA sample in each lane is indicated at the *top* of the figure. The position of the molecular weight markers is indicated at the *left*.

stronger inducer. This induction was not dependent on continuous protein synthesis since it was unaffected by cycloheximide treatment (Fig. 1*C*). These data demonstrated that Staf-50 participates in the primary response of IFN action and was not the consequence of a second set of gene induction. Comparison between untreated Daudi and HeLa cells showed that a basal level in the expression of Staf-50 is easily detectable in Daudi cells but not in HeLa cells. In order to determine the tissue specificity of Staf-50 expression, the 2.8-kb insert was used to probe a set of RNAs isolated from several tissues (Multiple Tissue Northern from Clontech). As shown in Fig. 2, Staf-50 is strongly expressed, in the absence of exogenous IFN treatment,

in peripheral blood leukocytes, in lymphoid tissues, such as spleen or thymus, and in ovary. Various basal levels were detected in other tissues. In contrast with the data observed in Daudi and HeLa cells, two major RNA species were detected specially in peripheral blood leukocytes. These results will be discussed later on the basis of nucleotide sequence analysis.

Sequence Analysis of Staf-50 cDNA—The complete nucleotide and amino acid sequences of the 2.8-kb insert are presented in Fig. 3. Computer search in the EMBL and GenBank data bases reveal homologies with the nucleotidic sequences of the mouse Rpt-1 gene (for regulatory protein, T-lymphocyte 1) (29) and the human SS-A/RO autoantigen (38). The Rpt-1 gene

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GAATTCGGCACGAGCTCTTCTCCCCTGATTCAAGACTCCTCTGCTTTGGACTGAAGCACTGCAGGAGTTTG    71

TGACCAAGAACTTCAAGAGTCAAGACAGAAGGAAGCCAAGGGAGCAGTGCA ATG GAT TTC TCA GTA   137
                                                     M   D   F   S   V

AAG GTA GAC ATA GAG AAG GAG GTG ACC TGC CCC ATC TGC CTG GAG CTC CTG ACA   191
 K   V   D   I   E   K   E   V   T   C   P   I   C   L   E   L   L   T

GAA CCT CTG AGC CTA GAT TGT GGC CAC AGC TTC TGC CAA GCC TGC ATC ACT GCA   245
 E   P   L   S   L   D   C   G   H   S   F   C   Q   A   C   I   T   A

AAG ATC AAG GAG TCA GTG ATC ATC TCA AGA GGG GAA AGC AGC TGT CCT GTG TGT   299
 K   I   K   E   S   V   I   I   S   R   G   E   S   S   C   P   V   C

CAG ACC AGA TTC CAG CCT GGG AAC CTC CGA CCT AAT CGG CAT CTG GCC AAC ATA   353
 Q   T   R   F   Q   P   G   N   L   R   P   N   R   H   L   A   N   I

GTT GAG AGA GTC AAA GAG GTC AAG ATG AGC CCA CAG GAG GGG CAG AAG AGA GAT   407
 V   E   R   V   K   E   V   K   M   S   P   Q   E   G   Q   K   R   D

GTC TGT GAG CAC CAT GGA AAA AAA CTC CAG ATC TTC TGT AAG GAG GAT GGA AAA   461
 V   C   E   H   H   G   K   K   L   Q   I   F   C   K   E   D   G   K

GTC ATT TGC TGG GTT TGT GAA CTG TCT CAG GAA CAC CAA GGT CAC CAA ACA TTC   515
 V   I   C   W   V   C   E   L   S   Q   E   H   Q   G   H   Q   T   F

CGC ATA AAC GAG GTG GTC AAG GAA TGT CAG GAA AAG CTG CAG GTA GCC CTG CAG   569
 R   I   N   E   V   V   K   E   C   Q   E   K   L   Q   V   A   L   Q

AGG CTG ATA AAG GAG GAT CAA GAG GCT GAG AAG CTG GAA GAT GAC ATC AGA CAA   623
 R   L   I   K   E   D   Q   E   A   E   K   L   E   D   D   I   R   Q

GAG AGA ACC GCC TGG AAG ATC GAG AGA CAG AAG ATT CTG AAA GGG TTC AAT GAA   677
 E   R   T   A   W   K   I   E   R   Q   K   I   L   K   G   F   N   E

ATG AGA GTC ATC TTG GAC AAT GAG GAG CAG AGA GAG CTG CAA AAG CTG GAG GAA   731
 M   R   V   I   L   D   N   E   E   Q   R   E   L   Q   K   L   E   E

GGT GAG GTG AAT GTG CTG GAC AAC CTG GCA GCA GCT ACA GAC CAG CTG GTC CAG   785
 G   E   V   N   V   L   D   N   L   A   A   A   T   D   Q   L   V   Q

CAG AGG CAG GAT GCC AGC ACG CTC ATC TCA GAT CTC CAG CGG AGG TTG ACG GGA   839
 Q   R   Q   D   A   S   T   L   I   S   D   L   Q   R   R   L   T   G

TCG TCA GTA GAG ATG CTG CAG GAT GTG ATT GAC GTC ATG AAA AGG AGT GAA AGC   893
 S   S   V   E   M   L   Q   D   V   I   D   V   M   K   R   S   E   S

TGG ACA TTG AAG AAG CCA AAA TCT GTT TCC AAG AAA CTA AAG AGT GTA TTC CGA   947
 W   T   L   K   K   P   K   S   V   S   K   K   L   K   S   V   F   R

GTA CCA GAT CTG AGT GGG ATG CTG CAA GTT CTT AAA GAG CTG ACA GAT GTC CAG  1001
 V   P   D   L   S   G   M   L   Q   V   L   K   E   L   T   D   V   Q

TAC TAC TGG GTG GAC GTG ATG CTG AAT CCA GGC AGT GCC ACT TCG AAT GTT GCT  1055
 Y   Y   W   V   D   V   M   L   N   P   G   S   A   T   S   N   V   A

ATT TCT GTG GAT CAG AGA CAA GTG AAA ACT GTA CGC ACC TGC ACA TTT AAG AAT  1109
 I   S   V   D   Q   R   Q   V   K   T   V   R   T   C   T   F   K   N

TCA AAT CCA TGT GAT TTT TCT GCT TTT GGT GTC TTC GGC TGC CAA TAT TTC TCT  1163
 S   N   P   C   D   F   S   A   F   G   V   F   G   C   Q   Y   F   S

TCG GGG AAA TAT TAC TGG GAA GTA GAT GTG TCT GGA AAG ATT GCC TGG ATC CTG  1217
 S   G   K   Y   Y   W   E   V   D   V   S   G   K   I   A   W   I   L

GGC GTA CAC AGT AAA ATA AGT AGT CTG AAT AAA AGG AAG AGC TCT GGG TTT GCT  1271
 G   V   H   S   K   I   S   S   L   N   K   R   K   S   S   G   F   A

TTT GAT CCA AGT GTA AAT TAT TCA AAA GTT TAC TCC AGA TAT AGA CCT CAA TAT  1325
 F   D   P   S   V   N   Y   S   K   V   Y   S   R   Y   R   P   Q   Y

GGC TAC TGG GTT ATA GGA TTA CAG AAT ACA TGT GAA TAT AAT GCT TTT GAG GAC  1379
 G   Y   W   V   I   G   L   Q   N   T   C   E   Y   N   A   F   E   D

TCC TCC TCT TCT GAT CCC AAG GTT TTG ACT CTC TTT ATG GCT GTG CTC CCT GTC  1433
 S   S   S   S   D   P   K   V   L   T   L   F   M   A   V   L   P   V
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FIG. 3. **Nucleotide sequence and predicted amino acid sequence of Staf-50 cDNA.** The complete nucleotide sequence of Staf-50 cDNA (*top line*) and the predicted amino acid sequence (*bottom line*) are shown. The nucleotides are numbered at the *right* of the sequence. The two zinc finger motif are *double underlined*. The IM motif is represented in a *box*. The bi-partite nuclear location signal is *underlined* and the potential polyadenylation signals founded in the 3'-noncoding region are indicated with *dotted lines*.

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GTA TTG GGG TTT TCC TAG  ACTATGAGGCAGGCATTGTCTCATTTTTCAATGTCACAAACCACGG  1497
 V   L   G   F   S

ACGACTCATCTACAAGTTCTCTGGATGTCGCTTTTCTCGACCTGCTTATCCGTATTTCAATCCTTGGAACT  1568

GCCTAGTCCCCATGACTGTGTGCCCACCGAGCTCCTGAGTGTTCTCATTCCTTTACCCACTTCTGCATAGT  1639

AGCCCTTCTGTGAGACTCAGATTCTGCACCTGAGTTCATCTCTACTGAGACCATCTCTTCCTTTCTTTCCC  1710

CTTCTTTTTACTTAGAATGTCTTTGTATTCATTTGCTAGGGCTTCCATAGCAAAGCATCATAGATTGCTGAT  1781

TTAAACTGTAATTGTATTGCCGTACTGTGGGCTGAAATCCCAAATCTAGATTCCAGCAGAGTTGGTTCTTT  1852

CTGAGGTCTGCAAGGAAGGGCTCTGTTCCATGCCTCTCTCCTTGGCTTGTAGAAGGCATCTTGTCCCTATG  1923

ACTCTTCACATTGTCTTTATGTACATCTCTGTGCCCAAGTTTTCCCTTTTTATTAAGACACCAGTCATACT  1994

GGCCTCAGGGCCCACCGCTAATGCCTTAATGAAATCATTTTAACATTATATTGTGTACAAAGACCTTATTT  2065

CCAAATAAGATAATATTTGGAGGTATTGGGAATAAAATTTGAGGAAGGCGATTTCACTCATAACAATCTTA  2136
                                      ••••••
CCCTTTCTTGCAAGAGATGCTTGTACATTATTTTCCTAATACCTTGGTTTCACTAGTAGTAAACATTATTA  2207

TTTTTTTTTATATTTGCAAAGGAAACATATCTAATCCTTCCTATAGAAAGAACAGTATTGCTGTAATTCCTT  2278

TTCTTTTCTTCCTCATTTCCTCTGCCCCTTAAAAGATTGAAGAAAGAGAAACTTGTCAACTCATATCCACG  2349

TTATCTAGCAAAGTCATAAGAATCTATCACTAAGTAATGTATCCTTCAGAATGTGTTGGTTTACCAGTGAC  2420

ACCCCATATTCATCACAAAATTAAAGCAAGAAGTCCATAGTAATTTATTTGCTAATAGTGGATTTTTAATG  2491

CTCAGAGTTTCTGAGGTCAAATTTTATCTTTTCACTTACAAGCTCTATGATCTTAAATAATTTACTTAATG  2562

TATTTTGGTGTATTTTCCTCAAATTAATATTGGTGTTCAAGACTATATCTAATTCCTCTGATCACTTTGAG  2633

AAACAAACTTTTATTAAATGTAAGGCACTTTTCTATGAATTTTAAATATAAAAATAAATATTGTTCTGATT  2704
                                          ••••••
ATTACTGAAAAGATGTCAGCCATTTCAATGTCTTGGGAAACAATTTTTTGTTTTTGTTCTGTTTTCTTTTT  2775

GCTTCAATAAAACAATAGCTGGCTCTAAAAAAAAAA                                    2811
•••••••
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FIG. 3.—*continued.*

was selectively expressed in quiescent helper/inducer T-cells and was shown to down-regulate gene expression directed by the interleukin 2 receptor-α chain promoter region (CD25) and by the LTR promoter region of HIV-1. In contrast, the function of the SS-A/RO gene, described in the Sjorgren type A syndrome, remains unknown. The patterns of expression of Staf-50 and Rpt-1 are rather similar with a preferential expression in quiescent T-lymphocytes (data not shown), peripheral blood leukocytes, and in the lymphoid tissues (Fig. 2). However, such correlations do not provide, at the present time, definitive proof that Staf-50 is the human homolog of the Rpt-1 gene or a member of a same family of genes, including the human SS-A/RO gene.

The full-length cDNA (2811 bp) of Staf-50 contains an open reading frame encoding 442 amino acids (nucleotide 123-1451; Fig. 3), followed by a very long 3'-untranslated region (1360 bp). Analysis of the nucleotide sequence of this 1360-bp region revealed the presence of several potential polyadenylation signals (Fig. 3). The presence of additional more distant polyadenylation signals in the 3' part of the gene may explain the occurrence of an additional RNA species, with a longer 3'-noncoding region, in peripheral blood leukocytes and in lymphoid tissues (Fig. 2). The predicted molecular mass of the Staf-50 protein (50,123 daltons) was verified by transcription-translation of a pBluescript phagemid containing the 2.8-kDa insert in the transcription/translation coupled reticulocyte lysate system (Promega). Proteins synthesized in the presence of [35S]methionine were fractionated in a 10% (w/v) SDS-polyacrylamide gel and the labeled proteins were visualized by autoradiography. The Staf-50 clone directs the synthesis of a

major polypeptide with an apparent molecular mass of 54,000 daltons (Fig. 4). Differences between the electrophoretic mobilities of proteins and their calculated molecular mass can be attributed either to post-transcriptional modifications of the translated products or to specific amino acid regions (like an arginine-rich polypeptide) which lead to abnormal migration in SDS-polyacrylamide gel (39).

Staf-50 Is a Member of the Ring Finger Family—A search and analysis for amino acid sequence homologies in the GenBank data base revealed that the complete Staf-50 protein shares 44% amino acid homology with the mouse Rpt-1 protein and 40.5% with the human SS-A/RO gene product. Some important characteristics of these three proteins can immediately be drawn from the comparison of their amino-terminal sequences whose alignment is presented in Fig. 5. Strong amino acid cluster homology is found in the 130 first amino acids although these proteins exhibit a relatively weak global homology. The strict conservation of motifs between human and mouse proteins is in favor of their role in the biochemical properties of these proteins. A second round of data base analysis using the PROSITE software was then performed in order to identify specific peptide motifs. The analysis revealed the presence of a C3HC4 zinc finger motif (Fig. 5) characteristic of the Ring finger family of proteins, whose functions are known to be mediated through DNA binding (40). Many of them are viral and cellular proteins involved in some aspect of the gene regulation. In particular, the immediate early genes of herpes simplex virus type 1 are implicated in the reactivation of latent virus in herpes simplex virus type 1 infection (41). Others are involved in activation of DNA recombination and DNA repair
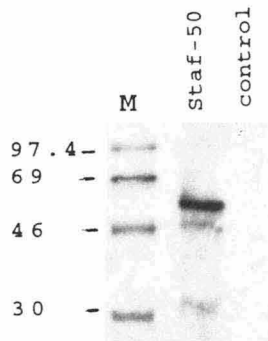
FIG. 4. *In vitro* **expression of the Staf-50-encoded protein.** Transcription-translation of the pBluescript Staf-50-containing vector was performed in the transcription/translation-coupled reticulocyte lysate system in the presence of [^{35}S]methionine. The labeled proteins were fractionated by SDS-polyacrylamide gel electrophoresis before autoradiography. Size molecular weight marker (*M*) are [^{14}C]methylated proteins (Amersham). *Staf-50 lane*, translational products of Staf-50; and *control lane*, translation products obtained with a pBluescript vector without insert.

(for review, see Ref. 40). These results would be consistent with a role of Staf-50 in the mechanism of signal transduction by cytokines like IFN or in gene expression regulation by IFNs.

Recent findings report that a synthetic peptide corresponding to the C3HC4 domain of the Ring1 gene product binds to DNA, in a zinc dependent manner, although weakly and non-specifically (40). These results strongly suggest that other peptide motifs are responsible for the specificity of DNA binding activity. The alignment of the amino acid sequences presented in Fig. 5 reveals the presence near the C3HC4 zinc finger motif, of a second putative zinc finger structure with a CHC3H2-type signature. This motif has already been identified in three other members of the Ring family (42), the human and mouse Rfp tyrosine kinase gene products (43), the T18 transforming mouse fusion protein (44), and the protein encoded by the human promyelocytic leukemia gene (42). For the latter, the last histidine residue is not present. Interestingly, in Staf-50, Rpt-1, SSA-/RO, and Rfp proteins, the two zinc finger structure are separated by 40 amino acid residues. In these regions we have identified a conserved basic motif (the relative basicity: H+K+R residues/D+E residues = .5) that we have termed IM (for intermediate motif) (Fig. 5). Such a basic motif is known to increase the affinity of DNA-binding protein to the DNA. The presence of two zinc fingers and the IM motif in the same configuration in these four proteins (Fig. 5) suggests that they act in synergy to bind DNA targets.

The Staf-50 amino acid sequence also encloses a **KR**SESWTL**KKPK**SVS**KKLK**SV bi-partite motif (see Fig. 3) similar to the nuclear location signal present in most nuclear proteins (45). This observation is consistent with the proposed DNA binding activity of Staf-50.

Trans-acting Function of the Staf-50 Protein—In order to determine, by analogy with Rpt-1, the ability of Staf-50 to affect transcription directed by the LTR promoter region of HIV-1, cotransfection experiments were performed with COS-7 m6 and HeLa cells. To this aim, the 1826-bp *XbaI-XbaI* fragment of the pBluescript-Staf-50 cDNA (Fig. 1*D*) was cloned under the transcriptional dependence of the CMV promoter in the pJ7Ω vector. The Staf-50 cDNA was positioned in sense (pJ-Staf-50) or antisense orientation as a negative control (pJ-Staf-50as). A luciferase gene under the control of the LTR promoter region of HIV-1 (pLTR-luc) was used as a reporter gene. Transfections were performed by calcium phosphate precipitation or by a lipofectamine procedure (Life Technologies, Inc.). The cells were collected 72 h later, and cellular extracts were prepared to determine the luciferase activities as described under "Materials and Methods."

Cotransfection of pLTR-luc with pJ-Staf-50 resulted in a 60–90% inhibition of the luciferase activity as compared with pJ-Staf-50as and pJ7Ω (Fig. 6), or with pCMV-β-gal (data not shown). The experiment was repeated several times in the linear range of the assay and with different batches of DNA. Identical data were obtained with both transfection procedures. In contrast, pJ-Staf-50 had no effect on β-galactosidase expression directed by the SV40 promoter (pSVβ-gal) or by the actin promoter (pAcβ-gal) (Fig. 6).

DISCUSSION

Binding of IFNs to their specific cell surface receptors triggers the rapid nuclear translocation of a complex formed by association between the various phosphorylated Stats proteins (see Introduction). This mechanism is not dependent on continuous protein synthesis and results in a first set of genes induction or, primary IFN response. Some of these genes, as 2–5A synthetases (17), Mx1 gene (23), double stranded-activated p68 protein kinase (19), major histocompatibility complex class I and class II, or tryptophanyl tRNA synthetase (46) are known mediators of the biological functions of IFNs. Other genes code for nuclear proteins which share all the features of transcription factors and are able to initiate a second cascade of gene induction (secondary response) requiring continued protein synthesis. As an example, the IRF-1 and IRF-2 genes act, respectively, as transcriptional activator and repressor of the Hu-βIFN gene (47). However, the function of most of IFN-induced genes remains unknown. In this report, we describe the cloning and the partial characterization of a new IFN-induced gene, designated as Staf-50 and exhibiting properties of transcriptional regulator.

The comparison of the nucleotide sequence of Staf-50 with all the sequences of EMBL and GenBank data bases revealed significant homologies between Staf-50, mouse Rpt-1, and human SS-A/RO autoantigen cDNA sequences. The best score determined was between Staf-50 and Rpt-1 with 64% homology in the coding region. The three proteins share a weak similar homology at the amino acid level (44% between Staf-50 and Rpt-1, and 40.5% between Staf-50 and SS-A/RO). In order to establish the family relationship between Staf-50 and the mouse Rpt-1 gene we have compared their tissue specificity of mRNA expression. As described previously for Rpt-1 (29), Staf-50 mRNA is constitutively expressed in peripheral blood leukocytes and in lymphoid tissues, such as spleen or thymus (Fig. 2). Although such data do not prove that Staf-50 is the human homolog of Rpt-1 or a member of a gene family. Staf-50 is also expressed in non-lymphoid HeLa cells after treatment with type I or type II IFN (Fig. 1). Further studies on the modulation of the mouse Rpt-1 as well as the human SS-A/RO genes by the IFNs might be worth performing.

Sequence analysis indicated that Staf-50 is a new member of the Ring finger superfamily of proteins involved in gene regulation, DNA recombination, and DNA repair (40). Interestingly, the alignment of the amino acid sequence of Staf-50 with several members of the Ring family, such as the Rpt-1 protein, the human and mouse Rfp tyrosine kinases, and the human SS-A/RO autoantigen (Fig. 5) revealed the presence of two zinc finger motifs. This tandem zinc finger domain contains a C3HC4 conserved motif described in the Ring finger family (40) and a putative zinc finger motif with a CHC3H2 type signature. The C3HC4 motif is unable to confer high specificity and affinity for DNA binding (40). It is likely that other motifs, like CHC3H2, might be required to generate a high affinity complex. However, the contribution of the CHC3H2 finger to DNA binding has not been demonstrated. The two zinc finger motifs are joined by a basic domain (36 ± 1 amino acids with an isoelectric pH = 12) which is also characteristic of DNA-binding proteins. We have identified, in this region, a conserved

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Rpt-1     MAS-SVLEMIKEEVTCPICLELLKEPVSADCNHSFCRACITLNY-ESNRNTDGKGNCPVCRVPYPF   64
SS-A/RO   MASAARLTMMWEEVTCPICLDPFVEPVSIECGHSFCQECIS----QV-GKGGGSV-CPVCRQRFLL   60
Staf-50   MDF-SVKVDIEKEVTCPICLELLTEPLSLDCGHSFCQACITAKIKESVIISRGESSCPVCQTRFQP   65
RFP       MASGSVAECLQQETTCPVCLQYFAEPMMLDCGHNICCACLA----RCWGTAETNVSCPQCRETFPQ   62
          *    º    º   * **º*º**º  º  **º   º*  *ºº*   *ºº              **º*º   º
                                    RING Finger
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Rpt-1     GNLRPNLHVANIVERLKGFKSIPEEEQK--VNICAQHGEKLRLFCRKDMMVICWLCERSQEHRGHQ   128
SS-A/RO   KNLRPNRQLANMVNNLKEISQEAREGTQGER--CAVHGERLHLFCEKDGKALCWVCAQSRKHRDHA   124
Staf-50   GNLRPNRHLANIVERVKEVKMSPQEGQK--RDVCEHHGKKLQIFCKEDGKVICWVCELSQEHQGHQ   129
RFP       RHMRPNRHLANVTQLVKQLRTERPSGPGGEMGVCEKHREPLKLYCEEDQMPICVVCDRSREHRGHS   128
          ºº***  ºº**º º  º*              *  *    *  ºº*   *    º*  º*   *º  *ºº*
                IM motif                          CHC3H2 zinc finger
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FIG. 5. **Alignment of the amino-terminal sequences of Staf-50, Rpt-1, SS-A/RO, and Rfp proteins.** The alignment of the predicted amino-terminal sequence of Staf-50 with several members of the Ring finger family is shown. Protein names are indicated at the *left* and the position of amino acids at the *right* of the sequences. The two zinc finger motifs and the IM motif are identified. The position of the conserved amino acids is indicated by an *asterisk* (*) and the conserved hydrophobic residues by a ○. Cys and His residues in the zinc finger motifs are shown in *boldface*.

FIG. 6. **Inhibition of LTR-directed luciferase expression by Staf-50 in transfected COS-7 m6 cells.** The relative luciferase activities were calculated by dividing the values measured after cotransfection of LTR-luc reporter and the indicated constructions (pJ-Staf-50as or pJ-Staf-50) by the values measured after cotransfection of LTR-luc and pJ7Ω. Values of less than 1 indicate inhibition of LTR-luc and represent the mean and standard deviation for several distinct experiments. pSVβ-gal and pAcβ-gal activities were determined the same way.

basic motif that we have termed IM (for intermediate motif) (Fig. 5). Interestingly, these three motifs are rigorously positioned in the same configuration in Staf-50, Rpt-1, Rfp tyrosine kinase, and SS-A/RO autoantigen. Although it is tempting to speculate that they could act via common mechanisms we have no direct evidence to accredit this hypothesis at the present time.

Knowing the antiviral properties of IFNs, we have analyzed the capacity of Staf-50 expression to induce an antiviral state in various cell lines. Based on the homology with the mouse Rpt-1 gene product we first examined the ability of Staf-50 to down-regulate the transcription directed by the LTR promoter region of HIV-1. Cotransfection experiments performed in different cell lineages showed a significant and reproducible 60–90% inhibition of the luciferase activity used as a reporter gene (Fig. 6). These results strongly suggest that Staf-50 may be involved in the antiviral process of IFNs against retroviral infections. Experiments are underway to determine whether the constitutive Staf-50 expression is able to confer a partial or total protection against HIV-1 infection in various cell lines. In a more general way, it will be important to determine whether

the constitutive expression of Staf-50 interferes with the multiplication cycle of other virus types.

The transcriptional activity of the LTR-HIV-1 promoter region is controlled by several regulatory elements acting in either positive or negative fashion (for review, see Ref. 48). The most influential DNA elements, identified as positive regulator of basal transcription, include the TATA box element recognized by TFIID (49), three SP1 binding sites (49, 50), and a tandemly repeated enhancer region recognized by the cellular NF-κB (51) and EBP (52) transcription factors. The transactivation response element responsive for the viral trans-activator protein Tat (53) controls the LTR transcription at the RNA level. The main functional region with the potential to decrease the synthesis of viral RNA is composed by the negative regulatory element (54). This region is recognized by cellular factors including AP-1 and NF-AT-1 (55).

The two consensus NF-κB binding motifs are very important for the expression of HIV-1 at high level in activated CD4⁺ T lymphocytes (56). The NF-κB-mediated transactivation of the HIV-1 LTR promoter is inhibited in IFN-producing cells. This down-regulation is associated with an alteration of the binding pattern of NF-κB-specific nuclear proteins to the core enhancer element of the HIV-1 LTR (57). The induction of HIV-1 provirus by herpes simplex virus-1 infection involves cooperation between NF-κB and the virus-encoded transactivator ICP0 (58). These data suggest that Staf-50 could act as a repressor of the NF-κB activation and interact either directly with NF-κB-binding proteins, thereby altering their affinity for DNA, or indirectly with its DNA target, to modulate HIV-1 LTR expression. Staf-50 could also act as an activator of negative regulatory element. However, our results do not provide direct evidence for specific Staf-50-DNA interaction and furthermore, do not exclude the possibility that Staf-50 protein binds to a RNA structure. Sequence homology with the 52-kDa component of the SS-A/RO ribonucleoparticle suggests that Staf-50 may interact with the the transcription response element of LTR promoter to regulate transcription. Experiments are underway to delineate the target of Staf-50 protein. The availability of specific antibodies against this protein is essential to determine its function and their preparation is now in progress.

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