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IFP 35 Forms Complexes with B-ATF, a Member of the AP1 Family of Transcription Factors

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Multicellular organisms achieve the spatial and temporal regulation of genes during growth and development through the differential expression of transcription factors that associate in various combinations. In this paper, we report the physical association of B-ATF, a member of the AP1 family of basic leucine zipper transcription factors, with IFP 35, a leucine zipper protein that is translocated to the nucleus following the treatment of cells with interferons and for which no binding partners previously have been described. Our data suggest that the formation of B-ATF:IFP 35 heterodimers is an interferon-inducible event in specialized cell types expressing both proteins and that changes in AP1 mediated gene transcription likely play a role in the response of these cells to interferons.

The transcription of most developmentally regulated or growth responsive genes in eukaryotes is controlled by multiple protein complexes consisting of individual components that are represented differentially in cells. Often the interaction between the proteins in these transcription complexes is mediated by motifs which allow for multimerization and for the generation sequence-specific DNA binding domains. B-ATF (1) is a member of the AP1 transcription factor family, which is part of a larger superfamily of transcriptional regulators possessing basic leucine zipper (bZIP) domains that mediate protein-protein interactions and DNA binding capabilities (2). In previous studies, we demonstrated that B-ATF does not homodimerize, but heterodimerizes with the bZIP region of Jun to produce a complex with the same DNA binding specificity as a Fos:Jun heterodimer (1). However, since B-ATF lacks a transcription activation domain, the result of the B-ATF:Jun binding to DNA is not transcription activation, but rather the silencing of AP1 responsive genes in cells (H-J. Tae and E. Taparowsky, in preparation).

Unlike many AP1 family members, B-ATF displays a restricted expression pattern, with the highest levels noted primarily in human tissues of hematopoietic origin (1). Using the yeast two hybrid approach (3,4), we screened a human B cell cDNA library for proteins which interact with the bZIP of B-ATF and isolated a cDNA encoding IFP 35, a leucine zipper protein that is induced and is translocated to the nucleus upon exposure of cells to Type I and Type II interferons (IFNs) (5). Although the IFP 35 leucine zipper mediates homodimer formation, the dimer lacks the basic region required for DNA binding. Furthermore, attempts to show that IFP 35 dimerizes with other bZIP proteins possessing DNA binding domains, including Jun, Fos, CREB, C/EBP, LRF or the ATFs, were unsuccessful (5). In this paper, we show that IFP 35 forms heterodimers with B-ATF, suggesting that in human cells where both proteins are expressed, the IFN-induced nuclear localization of IFP 35 impacts the expression of

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Abbreviations used: GAL4DB, DNA binding domain of the yeast GAL4 activator; GAL4TAD, transcription activation domain of the yeast GAL4 activator; β-gal, β-galactosidase; MBP, maltose binding protein; GST, glutathione-s-transferase.

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AP1 responsive genes by disrupting B-ATF:Jun heterodimers and generating non-DNA-binding B-ATF:IFP 35 heterodimers.

MATERIALS AND METHODS

Yeast two hybrid screen and interaction assays. Identification of pMD41-14, expressing a partial IFP 35 cDNA, from a pACT cDNA library constructed from human B cell mRNA was performed as described in Dorsey *et al.* (1) using the GAL4 DB-B-ATF (28-125) protein as a bait. The details of the two hybrid interaction assays, as well as the structures of GAL4DB-B-ATF (1-125), GAL4DB-p53 and GAL4TAD-B-ATF (28-125) have been described previously (1). GAL4DB-IFP 35 was made by subcloning a 2.6 kb EcoR1 fragment from pMD41-14 into pPC62 (gift of P. Chevray, Johns Hopkins University). GAL4 DB-JunB was made in two steps by subcloning an EcoR1/XbaI fragment from pBS-JunB (1) into GAL4 (1-147) (6) and then subcloning the 2.2 kb HindIII/XbaI from this recombinant plasmid into pPC62. For detecting expression of GAL4DB fusion proteins, yeast cell extracts were prepared and analyzed by Western blot hybridization as described by Mak *et al.* (7) using a 1:500 dilution of an anti-GAL4(1-147) antibody (Santa Cruz) and the ECL detection system (Amersham).

DNA sequencing. Dideoxy chain terminating sequencing was performed with the Taquence 1.0 kit (United States Biochemical). The complete sequence of the partial IFP 35 cDNA contained in pMD41-14 was determined for both strands and is reported in the databases under accession number U72882. Nucleotides encoding the first 7 AA of IFP 35 were added to the IFP 35 cDNA by standard PCR using information from the published IFP 35 AA sequence (5) to design a 5′ primer. To confirm discrepancies in the amino acid sequence encoded by our cDNA and the cDNA of Bange *et al.* (5), selected regions were sequenced from 3 additional IFP 35 cDNAs cloned from human B cell mRNA using RT-PCR. Briefly, the RT reaction was performed using reagents from Promega, a poly T primer and 1 μg total RNA. PCR was performed with primers internal to the IFP 35 coding region (5′GCTGGACAAGCTAGAGATC; 3′GCTCTAGAACAGACCTCCT) and the following conditions: 30 cycles of denaturation for 1 min at 94°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C, followed by a final extension at 72°C for 5 min. The PCR products were digested with BglII and XbaI, gel purified and ligated into pBluescript KS+.

Northern blot hybridization. Poly A^+ mRNA was prepared from human B cell lines using the PolyA Tract Isolation System (Promega). 2.5 μg of each poly A^+ mRNA was analyzed by Northern blot hybridization as previously described (8). Sequential hybridization of the filter was performed as described (8) using as probes a 0.6 kb BglII fragment from pMD41-14, a 0.4 kb EcoR1/XbaI fragment from pBS-B-ATF (1) and a β-actin cDNA fragment (Clonetech) radiolabeled with [α^{32}P]dCTP (6000 Ci/mmol) (Amersham) and reagents supplied by the Oligo-labeling Kit (Pharmacia). The human multiple tissue blot was purchased from Clonetech and was hybridized sequentially with IFP 35 and β-actin probes as described above.

β-Galactosidase assay. Quantitative detection of β-gal activity using liquid yeast cultures was performed as described by Miller (9) and calculated using the following equation: $1.7 \times [A_{420}/\text{min}]/[4.5 \times 10^{-3}] \times$ [ml protein extract] $\times$ [mg/ml of protein]. Each value in Table 1 represents the average ($\pm$ standard deviation) of β-gal activities measured for at least 3 independently isolated transformants.

Intracellular localization of IFP 35 and B-ATF in HeLa cells. pDCR and pDCR-B-ATF were described previously (1). pDCR-IFP 35 was constructed by inserting the PCR generated IFP 35 coding sequence into the SalI site of the pDCR vector, in-frame with the sequence encoding the hemagglutinin antigen (HA) as an epitope tag. HeLa cells were transiently transfected as described (10) and 24 hr after transfection treated with 100U/ml human IFN-γ (Boehringer Mannheim) for an additional 24 hr. Cultures were lysed in 20 mM HEPES, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton-100, 1 mM PMSF, 10 μg/ml leupeptin and pepstatin and 0.1 mg/ml aprotinin and an aliquot of the whole cell lysate saved for Western analysis. The remainder of the lysate was used to prepare nuclear extract as described (10). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and hybridized as described (10) using a 1:700 dilution of an anti-HA monoclonal antibody (Boehringer Mannheim) and the ECL detection system (Amersham).

In vitro binding studies. pMBP-B-ATF was described previously (1) and pGST-IFP 35 was constructed by ligating a PCR-generated IFP 35 cDNA fragment into the EcoR1/XbaI sites of pGEX 2T-1 (gift of S. Konieczny, Purdue University). Bacterial expression of GST or MBP fusion proteins was performed as described (11) and quantitated following SDS-PAGE and Commassie blue staining. *In vitro* interaction studies were performed by resuspending amylose beads coated with $\sim$2 μg of MBP-B-ATF in 100 μl of binding buffer (0.25% NP40, 50 mM NaCl, 100 μM Tris, pH 7.4, 1 mM EDTA, 1 mg/ml BSA, 1 mM PMSF and 2 μg/ml leupeptin and aprotinin) and mixing the slurry with purified GST-IFP 35 ($\sim$4 μg) for 1 hr at 4°C. After washing the beads several times with binding buffer minus BSA and protease inhibitors, complexes were eluted with SDS sample buffer and analyzed by SDS-PAGE. GST fusion proteins were visualized by Western blot hybridization as described above using a 1:1000 dilution of an anti-GST monoclonal antibody (Santa Cruz).

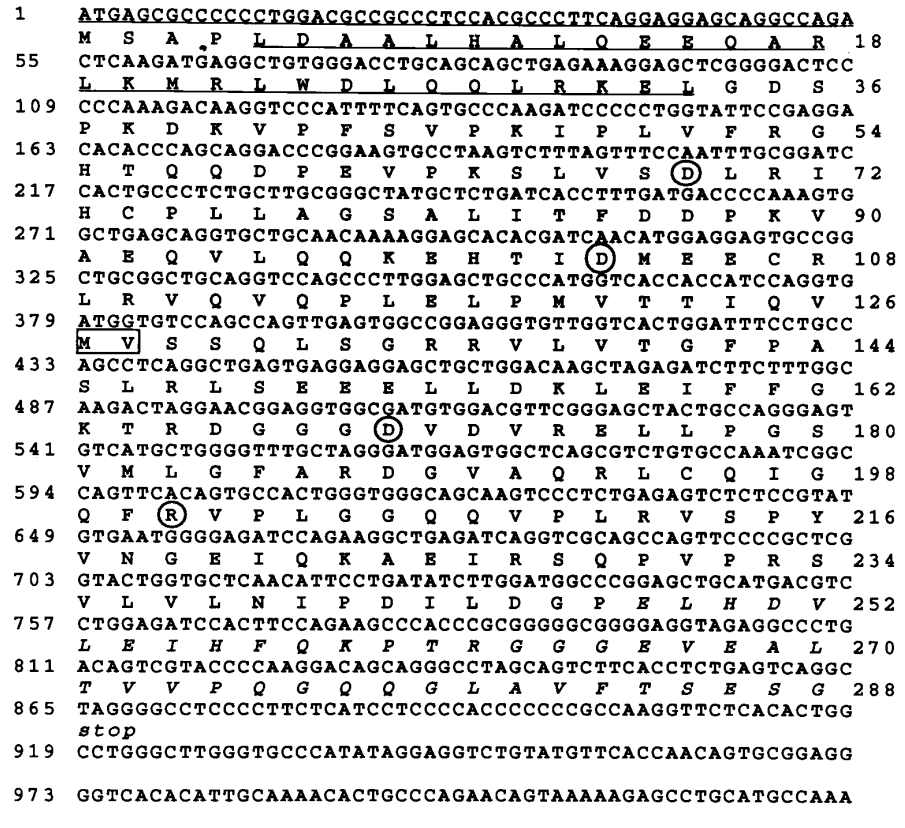

FIG. 1. Nucleotide sequence of the partial IFP 35 cDNA expressed from pMD41-14 (numbered on left) and the predicted AA sequence of the encoded protein (numbered on right). The underlined nucleotides were amended to the cDNA as described under Materials and Methods to complete the open reading frame. The underlined AA sequence is the leucine zipper motif. Discrepancies between this sequence and the information published by Bange *et al.* (5) include four AA substitutions (circled), a single 2 AA insertion (boxed), and the presence of two G nucleotides at positions 794 and 795 which shifts the open reading frame and produces a distinct COOH terminus (italicized). The IFP 35 cDNA sequence has been entered into the databases under the accession number U72882.

RESULTS

Isolation of IFP 35 as a binding partner of B-ATF. A yeast two hybrid screening strategy using the GAL4DB B-ATF (28-125) fusion protein as a bait was used as described previously (1) to isolate B-ATF interacting proteins from a pACT cDNA library generated from human B cell mRNA (12). Analysis of 2×10^6 trp^+, leu^+ transformants generated 20 his^+, $lacZ^+$ positives, with one positive colony expressing a 1.0 kb partial cDNA encoding AA 8-288 of the human IFP 35 protein (5). The complete nucleotide sequence of the IFP 35 cDNA isolated from the B cell library is presented in **Figure 1.** The sequence encoding the first 7 AA of IFP 35 was amended to the cDNA using PCR as described in Methods. The IFP 35 cDNA encodes a protein with ~98% AA identity to the published sequence over the first 265 residues, but diverges after AA 265 due to the inclusion of two G residues at nucleotide positions 794 and 795 that shifts the reading frame of the cDNA. We have confirmed the accuracy of the IFP 35 cDNA sequence in **Figure 1** by independently isolating and determining the nucleotide sequence of three, additional IFP 35 cDNA clones.

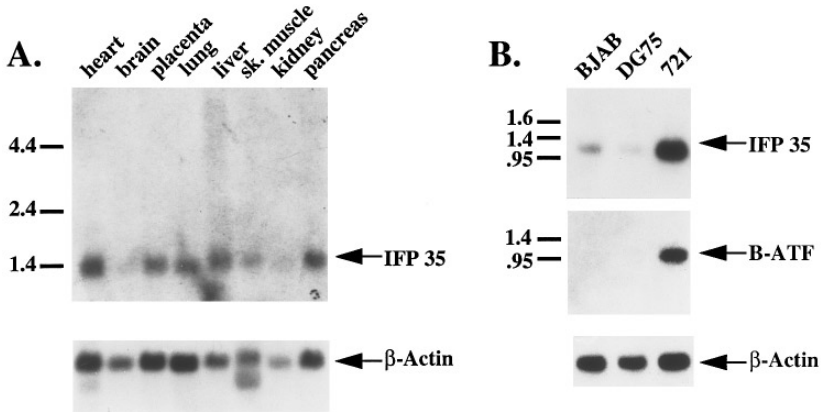

FIG. 2. (**A**) Expression of IFP 35 mRNA in human tissues. Poly A^+ mRNA from the indicated tissues was analyzed by Northern blot hybridization as described under Materials and Methods. Migration of the ~1.4 kb IFP 35 mRNA and control β-actin mRNA is indicated on the right of the blots and RNA size markers (kb) are indicated on the left. (**B**) Co-expression of IFP 35 and B-ATF mRNA in the Epstein Barr virus immortalized human B cell line, 721. Poly A^+ mRNA from the indicated human B cell lines was analyzed by Northern blot hybridization as described under Materials and Methods. Migration of the ~1.4 kb IFP 35 mRNA, the ~1.0 kb B-ATF mRNA and control β-actin mRNA is indicated on the right and RNA size markers (kb) are indicated on the left.

Tissue distribution and nuclear localization of IFP 35. Northern blot analysis was used to establish the widespread expression of IFP 35 mRNA in human tissues (**Figure 2A**). This differs from the highly restricted expression of B-ATF mRNA observed previously in these same tissues (1). Interestingly, B-ATF and IFP 35 are co-expressed in several human B cell lines (**Figure 2B** and data not shown) as well as in the human Jurkat T cell line (data not shown) demonstrating that these proteins have the potential to interact. B-ATF is exclusively a nuclear protein (1) and IFP 35 has been shown to translocate to the nucleus following treatment of HeLa cells with IFN-α, -β or -γ (5). To demonstrate the co-localization of B-ATF and IFP 35 in the nucleus of IFN-γ treated cells, HeLa cells were transiently transfected with pDCR-B-ATF and pDCR-IFP 35 to generate HA-tagged fusion proteins *in vivo.* Whole cell lysates and nuclear extracts were prepared from groups treated, or not treated, with IFN-γ and were analyzed by Western blot hybridization with an anti-HA antibody (**Figure 3**). Results show the presence of both B-ATF and IFP 35 fusion proteins in the nuclear fraction from the IFN-γ treated HeLa cells.

B-ATF interacts with IFP 35 in yeast. A yeast two hybrid assay was used to assess the specificity and the relative strength of the interaction between B-ATF and IFP 35 *in vivo.* As presented in **Table 1,** the indicated GAL4DB and GAL4TAD constructs were used to co-transform yeast possessing an integrated *lacZ* gene regulated by GAL4 protein binding sites (1,4) and the β-gal activity resulting from fusion protein interaction measured using liquid assays as described in Methods. GAL4DB-B-ATF, -JunB and -IFP-35 proteins are unable to activate transcription of the *lacZ* gene on their own in yeast, while the interaction between GAL4DB-B-ATF and GAL4TAD-IFP 35, or between GAL4DB-B-ATF and GAL4TAD-JunB, results in significant levels of β-gal activity. Consistent with a previous report (5), the GAL4DB-JunB protein does not interact with GAL4TAD-IFP 35 while in contrast to that report, dimerization of IFP 35 was not detected using the two hybrid assay. We attribute this latter result to the low level of GAL4DB-IFP 35 expressed in the cells as determined by Western blot hybridization using an anti-GAL4 antibody (**Figure 4**). Studies in which we

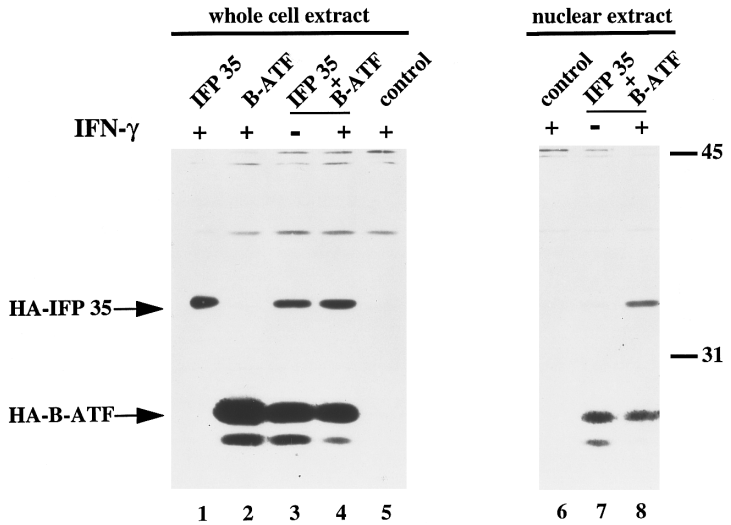

FIG. 3. Intracellular location of HA-B-ATF and HA-IFP 35 fusion proteins in INF-γ treated cells. HeLa cells were transfected with expression vectors for HA-B-ATF and HA-IFP 35 and treated with INF-γ as described under Materials and Methods. Whole cell and nuclear extracts were analyzed by Western blot hybridization with an anti-HA antibody. Lanes 1 and 2 show the migration of HA-B-ATF and HA-IFP 35, respectively. Lanes 3 and 4 show the levels of both proteins in whole cell extracts [(+) and (−) IFN-γ] and Lanes 7 and 8 show the presence of both proteins in nuclear extracts prepared from IFN-γ treated cells (+) only. Lanes 5 and 6 are extracts from IFN-γ treated cells transfected with the pDCR vector. The migration of protein standards (kD) is indicated on the right.

examined the interaction between *in vitro* translated IFP 35 and GST-IFP 35 proteins (data not shown) clearly demonstrate that the IFP 35 protein does dimerize.

B-ATF and IFP 35 form a complex in vitro. We have observed the co-expression of B-ATF and IFP 35 mRNA in human cells (**Figure 2B**) and have experimentally demonstrated that both proteins localize to the nucleus following the treatment of interferon-responsive cells

TABLE 1
Two Hybrid Interaction Assays in Yeast

GAL4 DB	GAL4 TAD	β-gal activity nmol/min/mg
B-ATF (1-125)	—	7 ± 1
B-ATF (1-125)	IFP 35	1372 ± 190
B-ATF (28-125)	IFP 35	1067 ± 197
Jun B	—	9 ± 3
Jun B	B-ATF (28-125)	545 ± 4
Jun B	IFP 35	8 ± 2
IFP 35	—	5 ± 1
IFP 35	IFP 35	11 ± 2
p53	IFP 35	5 ± 2

Note. Yeast were co-transformed with the indicated plasmids described under Materials and Methods and individual colonies assayed for β-gal activity. Each number represents the average activity (± standard deviation) for a minimum of three independent determinations.

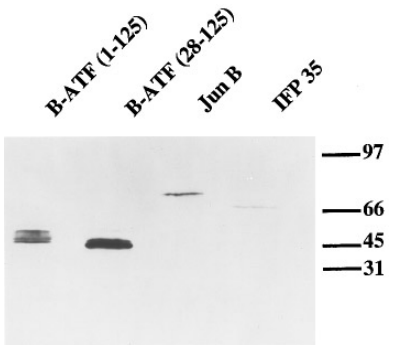

FIG. 4. Expression of GAL4 DB fusion proteins in yeast. Cell extracts from yeast transformed with expression vectors for the indicated GAL4 DB fusion proteins were prepared as described under Materials and Methods and analyzed by Western blot hybridization with an anti-GAL4 antibody. The migration of protein standards (kD) is indicated on the right.

with IFN-γ (**Figure 3**). To rule out the possibility that the B-ATF:IFP 35 interaction detected in the two hybrid assay relies on a protein that is present in yeast, but perhaps not in other cell types, *in vitro* binding studies using purified MBP-B-ATF and GST-IFP 35 fusion proteins were performed as described in Methods. **Figure 5** shows the results of a Western blot hybridization using an anti-GST antibody to identify proteins bound to amylose-MBP or amylose-MBP-B-ATF resin. While the GST moiety has no affinity for MBP-B-ATF, GST-IFP 35 interacts with MBP-B-ATF and not with MBP, indicating that B-ATF associates directly with IFP 35 *in vitro.*

DISCUSSION

Type I and Type II IFNs bind cell surface receptors to stimulate intracellular tyrosine kinase activity and a signal transduction cascade that culminates in the differential expression of

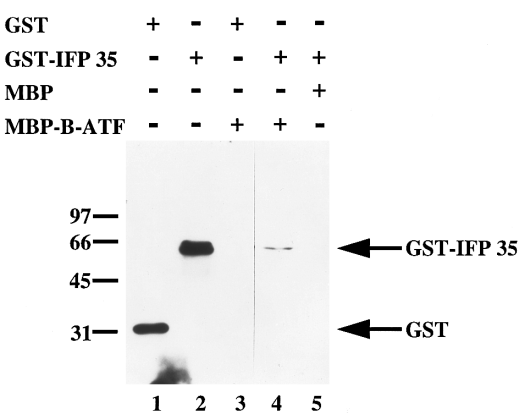

FIG. 5. Interaction of bacterial expressed MBP-B-ATF and GST-IFP 35 fusion proteins *in vitro.* Fusion proteins were incubated in the indicated combinations, purified on amylose resin as described under Materials and Methods, and detected by Western blot hybridization with an anti-GST antibody. Lanes 1 and 2 contain control GST and the GST-IFP 35 fusion protein, respectively, as marked on the right of the blot. Lanes 3 and 4 show the levels of control GST and GST-IFP 35, respectively, that are co-purified with MBP-B-ATF. Lane 5 shows that the binding detected in Lane 4 is not due to interaction of GST-IFP 35 with the MBP moiety. The migration of protein standards (kD) is indicated on the left.

target genes possessing interferon-responsive DNA elements (13). The biological response to IFN treatment varies with the cell type but, in general, causes anti-proliferative effects in target cells (13). The recent description of IFP 35 (5), a ubiquitously expressed, predominantly cytoplasmic leucine zipper protein that is induced and then translocated to the nucleus following the treatment of cells with IFNs, implicates IFP 35 as a new mediator of IFN triggered responses in cells. The primary structure of the IFP 35 protein has been reported previously, along with the results of unsuccessful attempts to identify a nuclear bZIP protein with which IFP 35 interacts (5). In this paper, we report the nucleotide sequence of a human IFP 35 cDNA and identify B-ATF as a specific heterodimerization partner for IFP 35. B-ATF is a tissue-specific member of the AP1 family of bZIP transcription factors and forms heterodimers with Jun to bind AP1 DNA sites. Using a reporter gene regulated by tandem AP1 binding sites, we recently have demonstrated that B-ATF:Jun heterodimers function as negative regulators of AP1 transcriptional activity (H-J. Tae and E. J. Taparowsky, in preparation). However, in the context of a complex promoter, where AP1 synergizes with other transcription factors to regulate target gene expression, the impact of B-ATF is not yet known. Heterodimer formation between B-ATF and IFP 35, a leucine zipper protein that does not possess a DNA-binding domain, is likely to reverse the influence of B-ATF:Jun complexes on AP1 target gene transcription. Using human B and T cell lines which naturally express both B-ATF and IFP 35, future experiments examining the impact of INF-induced B-ATF:IFP 35 heterodimer formation on the expression of AP1 reporter genes will define a role for AP1 in cell type specific responses to IFNs.

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