MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7515–7521
0270-7306/93/127515-07$02.00/0

Vol. 13, No. 12

A Nuclear Tyrosine Phosphatase Downregulates Interferon-Induced Gene Expression

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Received 19 July 1993/Returned for modification 20 August 1993/Accepted 3 September 1993

Alpha and gamma interferons rapidly induce several early response genes in primary human diploid fibroblasts. The transcription rates of these genes are maximal after 1 h of interferon treatment and return to basal levels within 8 h. Three different interferon-activated DNA-binding complexes (ISGF3, GAF, and FcRFγ) that are responsible for transcriptional activation of cellular genes have been characterized. Assembly of these complexes requires tyrosine phosphorylation of one or more of the protein components. In this report, we demonstrate that a nuclear tyrosine phosphatase is responsible for the deactivation of these interferon-regulated transcription factors and the subsequent transcriptional downregulation of the corresponding genes. Furthermore, tyrosine phosphorylation is required for nuclear localization of the 91-kDa protein that is part of all three interferon-induced transcription complexes. These results provide the first evidence for a nuclear tyrosine phosphatase activity as a mechanism of transcriptional regulation.

The rapid transcriptional activation of cellular genes by either alpha interferon (IFN-α) or IFN-γ is responsible for many of the pleotropic actions of these cytokines. IFN-activated gene expression is mediated by the formation of protein complexes whose preexisting components assemble as a result of ligand-induced tyrosine phosphorylation. These complexes then translocate to the nucleus, where they bind to enhancers within IFN-activated genes (3, 5, 7, 12, 15, 18, 28). Although the signals required for IFN induction of gene expression are becoming better understood, considerably less is known regarding how transcription of these genes becomes downregulated. In primary diploid fibroblasts, 6 to 8 h of continuous treatment with either IFN-α or IFN-γ shuts off the transcription of early response genes and desensitizes cells such that reexposure to the cytokine is ineffective in reinducing the expression of IFN-stimulated genes (ISGs) (16). As downregulation of ISGs in fibroblasts can be abrogated by inhibitors of protein synthesis, IFN-induced proteins were thought to be responsible for shutting off the transcriptional response (6, 9, 17). A likely candidate to mediate this downmodulation would be a nuclear tyrosine phosphatase, because tyrosine phosphorylation is required for the integrity of the IFN-activated complexes ISG factor 3 (ISGF3), IFN-γ-activated factor (GAF), and FcRI-binding factor, IFN-γ induced (FcRFγ) (4, 5, 10, 14, 15). We therefore initiated experiments to explore whether dephosphorylation of IFN-induced transcription factors correlated with their nuclear localization and a decline in the rates of transcription of ISGs.

MATERIALS AND METHODS

Cells and culture. Human foreskin diploid fibroblasts (GM00468) from the National Institute of General Medical Science were grown and passaged in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

IFNs and reagents. Recombinant human IFN-α was a generous gift from Hoffmann LaRoche. Recombinant human IFN-γ was provided by Genentech Corp.

Preparation of nuclear cell extracts. Cells were collected after being washed with phosphate-buffered saline and homogenized by a douncing in buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 10 mM KCl, 10 mM $MgCl_2$, 1 mM orthovanadate, 0.5 mM dithiothreitol (DTT), 0.25 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.1% Nonidet P-40). The lysate was layered on a sucrose cushion (35% sucrose in 100 mM HEPES [pH 7.0]–20 mM $MgCl_2$), and the nuclei were isolated by centrifugation at 3,000 × *g* for 15 min. Nuclei were resuspended in buffer A with 0.3 M NaCl and extracted by vortexing.

Selected extracts were treated with 10 mM *N*-ethylmaleimide (NEM) for 20 min at room temperature and then quenched with 20 mM DTT on ice for 10 min.

Antibodies. ISGF3 is composed of three ISGF3α proteins (p84, p91, and p113) and a 48-kDa DNA-binding component, ISGF3γ (11, 18). Antibodies specific for p91 were raised against a peptide corresponding to the C-terminal 39-amino-acid region that is not present in the splicing variant p84. Antibodies directed against the amino acids 607 to 647 of p91 and p84 recognized both proteins. A peptide identical to the amino acid sequence 125 to 174 of p113 was used to generate antibodies that specifically react with this protein. ISGF3γ antibodies were produced against a synthetic peptide corresponding to the amino acid sequence 117 to 155.

Electrophoretic mobility shift assays. Gel shift assays were performed as previously described (14, 24, 32) by using ^{32}P-end-labeled double-stranded oligonucleotides corresponding to (i) the ISGF3-binding IFN-stimulated response element (ISRE) of the ISG15 gene, 5′ GATCCATGCC TCGGGAAAGGGAAACCGAAACTGAAGCC 3′; (ii) the GAF-binding IFN-γ activation sequence (GAS) in the promoter of the GBP gene, 5′ AAGTACTTTCAGTTTCATATT ACTCTAAATC 3′; and (iii) the FcRFγ-binding IFN-γ response region (GRR) of the Fc receptor gene, 5′ AGCATGT TTCAAGGATTTGAGATGTATTTCCCAGAAAAG 3′. For

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supershift experiments, nuclear extract was incubated with the selected antibodies for 1 h at 4°C prior to gel shift analysis.

Measurement of transcriptional activity. Nuclear run-on transcription assays were performed by using isolated human fibroblast nuclei in the presence of [α-^{32}P]UTP as previously described (21): DNA probes were immobilized to Nytran membranes (Schleicher & Schuell) and hybridized to [^{32}P]RNA by using conditions previously described (21). cDNA probes included pGEM2 (to determine background), actin as an internal standard, ISG54 800-bp *Eco*RI fragment from exon 2 (19), human GBP (2), ISG15 (25), and FcRI (32). The resulting blots were quantitated on an AMBIS scintillation counter.

Immunoprecipitations and Western blotting (immunoblotting). Nuclear extracts were immunoprecipitated by using an antibody against p91. Immunoprecipitates were boiled in sodium dodecyl sulfate (SDS) sample buffer and resolved on an SDS–8% polyacrylamide gel. After transfer to Immobilon, blots were probed with PY20 anti-phosphotyrosine antibodies (ICN) and reactive proteins were visualized by enhanced chemiluminescence (Amersham). Blots were reprobed with anti-p91 or anti-p84 and -p91 antibodies by using alkaline phosphatase-conjugated secondary antibodies.

RESULTS

Vanadate maintains the assembly of IFN-α-activated transcription factors. The activation and subsequent deactivation of several IFN-α or IFN-γ-induced transcription complexes such as ISGF3, GAF, and FcRFγ parallel the induction and downregulation of transcription of the corresponding genes (3, 7, 18, 32). Because assembly of ISGF3, GAF, and FcRFγ requires tyrosine phosphorylation (5, 10, 14, 15, 29), a nuclear phosphotyrosine phosphatase (PTP) might be responsible for the observed decrease in the concentration of these transcription complexes with prolonged IFN treatment. Therefore, we investigated whether the tyrosine phosphatase inhibitor orthovanadate could prevent the inactivation of these transcription complexes. Incubation of fibroblasts with IFN-α (500 U/ml) for 1 h activated formation of GAF (Fig. 1A, lane 1 versus 2), FcRFγ (lane 6 versus 7), and ISGF3 (lane 11 versus 12). Incubation for 8 h in the continuous presence of IFN-α resulted in a disappearance of these protein-DNA complexes (lanes 3, 8, and 13). The kinetics of IFN-γ-induced formation and disappearance of GAF and FcRFγ were essentially the same as those seen with IFN-α (data not shown). When 1 mM orthovanadate was added after 1 h of IFN-α and was subsequently present for 7 more h, the integrity of all three transcription complexes (lanes 5, 10, and 15) was preserved. Vanadate was added 1 h after IFN-α because a membrane-associated PTP is also required for the activation of the complex (5, 14). The serine/threonine phosphatase inhibitors okadaic acid and calyculin A were ineffective in preserving the integrity of the transcription factors (data not shown). Although the protein synthesis inhibitor cycloheximide (30 μg/ml) is known to prevent downregulation of IFN-induced transcription (7, 16), it had only a small effect on maintaining the assembly of GAF, FcRFγ, and ISGF3 (lanes 4, 9, and 14) during incubation with IFN. To exclude the possibility that the short half-life of ISGF3γ (the DNA-binding component of ISGF3) was responsible for the disappearance of ISGF3 in these experiments, we used a reconstitution assay with in vitro-translated ISGF3γ (30) (lanes 17 to 21). ISGF3γ, which is NEM sensitive, has been demonstrated to reconstitute ISGF3 in extracts containing NEM-resistent, activated ISGF3α (those proteins which are tyrosine phosphorylated by IFN treatment of cells) (5, 12, 28). Extracts in lanes 16 to 21 were first treated with NEM, and the reaction was quenched with DTT. Samples were then incubated with in vitro-translated ISGF3γ prior to analysis by electrophoretic mobility shift assay. Increased concentrations of ISGF3γ could reconstitute ISGF3 only in extracts that contained ISGF3 already before NEM treatment (lanes 17 to 21). These results confirmed the fact that the disassembly of ISGF3 is not due to a deficiency in ISGF3γ as a result of extended exposure to IFN or treatment with cycloheximide.

The ISGF3 proteins are antigenically similar in cells treated with IFN-α and vanadate. The ISGF3 transcription complex is composed of the three ISGF3α proteins p84, p91, and p113, which associate with ISGF3γ, the 48-kDa DNA-binding protein described above, as a result of IFN-induced tyrosine phosphorylation (5, 12, 28). To determine whether the ISGF3 observed after 8 h of IFN-α-plus-vanadate treatment contained the same proteins as in ISGF3 seen after 1 h of IFN-α treatment of cells, extracts were prepared and incubated with specific antibodies against p91, p113, and ISGF3γ. All three antibodies formed supershifted complexes with ISGF3 from the short (Fig. 1B, lanes 2 to 5) and prolonged (lanes 7 to 10) treatment. Anti-p91 antibody also supershifted the GAF and FcRFγ complexes, which do not contain p113 or ISGF3γ, in extracts prepared from cells incubated with vanadate and IFN-α for 8 h (data not shown).

Vanadate prevents transcriptional downregulation of IFN-α-induced genes. To evaluate whether a direct correlation existed between the presence of the transcription complexes maintained by vanadate and elevated transcription rates of IFN-α-induced genes, we performed nuclear run-on assays (Fig. 2). Cells were treated as described in the legend to Fig. 1, and the nuclei were isolated and incubated in the presence of [α-^{32}P]UTP. ^{32}P-labeled RNA was isolated and hybridized to cDNA probes corresponding to the IFN-α-stimulated genes ISG54, ISG15, and FcRI and the GBP. After hybridization and autoradiography, transcription rates were quantitated by direct counting. As in previously published results (6, 17, 25), IFN-α treatment of cells for 1 h induced high rates of transcription of ISG15, ISG54, and GBP, which in the continuous presence of the cytokine for 8 h declined to nearly undetectable levels. Addition of cycloheximide 30 min prior to incubation with IFN-α maintained elevated transcription rates, whereas vanadate-treated cells exceeded maximal levels of transcription observed after 1 h of IFN-α treatment (Fig. 2). Since IFN induction of the FcRI transcription is restricted to cells of myeloid lineage, no transcription of the gene was detected in spite of FcRFγ formation (32). These data confirm that a complete correlation exists between the ability of vanadate to maintain IFN-α-induced levels of ISGF3 or GAF and the rates of transcription of their corresponding genes. However, no such correlation was noted with cycloheximide, which was able to maintain elevated transcription rates but only marginally preserved the assembly of the transcription complexes (compare Fig. 1, lanes 4 and 14, with Fig. 2).

Tyrosine phosphorylation of p91 parallels assembly of transcription factors and transcription rates. Because tyrosine-phosphorylated p91 is the common component found in IFN-α-activated ISGF3 as well as in IFN-α- and IFN-γ-activated GAF and FcRFγ (1, 15, 22, 23, 29), experiments

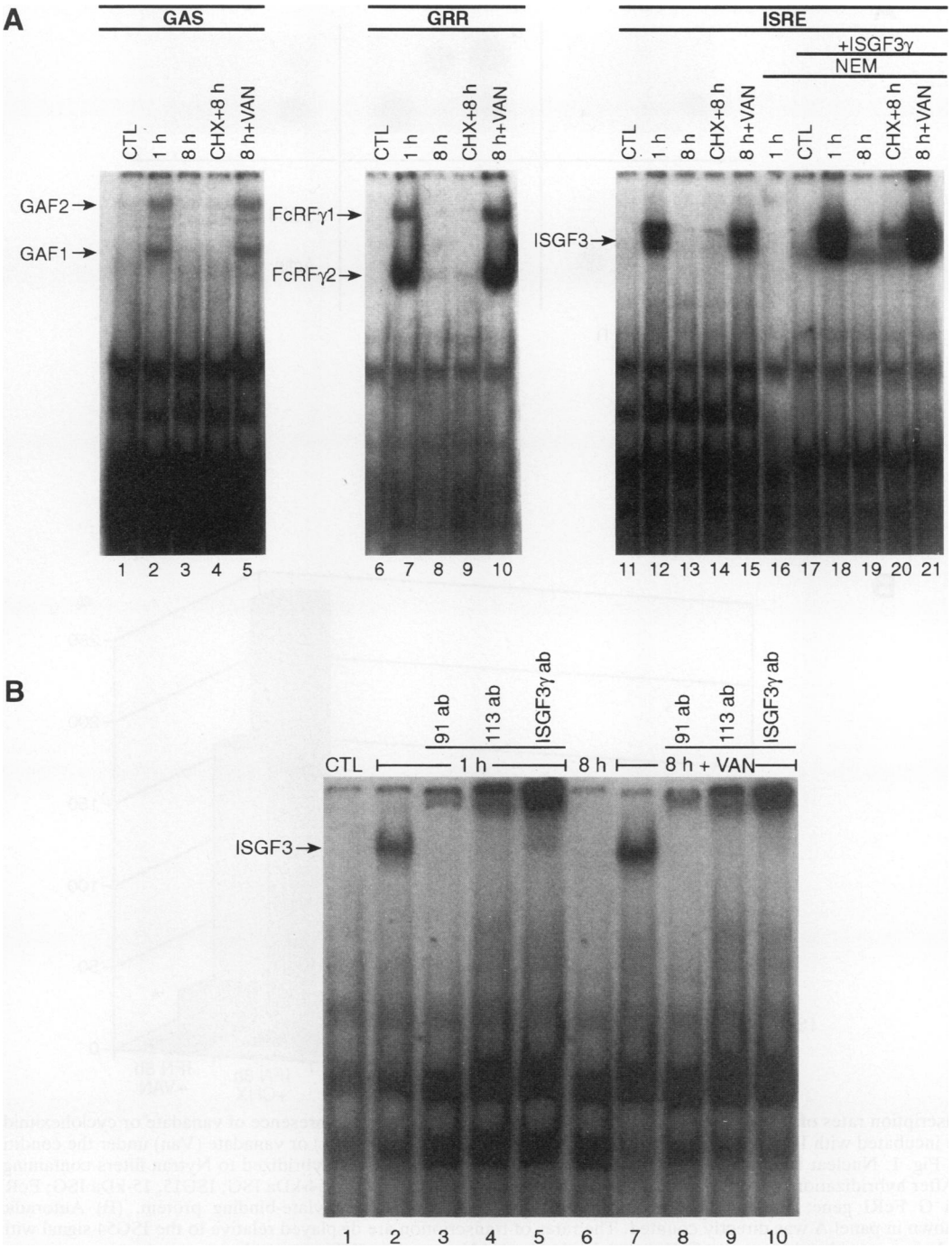

FIG. 1. Extended incubation of human fibroblasts with IFN-α and vanadate maintains the assembly of ISGF3, GAF, and FcRFγ. (A) Confluent human diploid fibroblasts (150-cm^2 dishes) were left untreated (lanes 1, 6, 11, and 17) or were incubated with IFN-α (500 U/ml) for 1 h (lanes 2, 7, 12, 16, and 18) or 8 h (lanes 3, 8, 13, and 19), with cycloheximide (CHX; 30 μg/ml) for 8.5 h and IFN-α for 8 h (lanes 4, 9, 14, and 20), or with IFN-α for 8 h and vanadate (VAN; 1 mM) for 7 h (lanes 5, 10, 15, and 21). Cells were lysed, and nuclei were isolated by sucrose density centrifugation and extracted. Electrophoretic mobility shift assays were performed as described in Materials and Methods with probes corresponding to GAS (lanes 1 to 5), the GRR (lanes 6 to 10), or the ISRE (lanes 11 to 21). Samples shown in lanes 16 to 21 were incubated with NEM and then with DTT prior to the addition of 2 μl of in vitro-transcribed and -translated ISGF3γ (lanes 17 to 21). Lane 16 is the same as lane 18, but without the addition of ISGF3γ. (B) ISGF3 in nuclear extracts prepared from cells treated with IFN-α for 1 h or IFN-α for 8 h and vanadate for 7 h, both contain ISGF3γ, p91, and p113. Extracts (20 μl) prepared as described above were incubated with antibodies (1:100 dilutions) for 1 h at 4°C prior to the addition of the ^{32}P-labeled ISRE probe. Lane 1, untreated control (CTL); lane 2, IFN-α, 1 h; lane 3, IFN-α, 1 h, incubated with anti-p91 antiserum; lane 4, IFN-α, 1 h, incubated with anti-p113 antiserum; lane 5, IFN-α, 1 h, incubated with anti-ISGF3γ antiserum; lane 6, IFN-α, 8 h; lane 7, IFN-α, 8 h, and vanadate; lane 8, IFN-α, 8 h, and vanadate, incubated with anti-p91 antiserum; lane 9, IFN-α, 8 h and vanadate, incubated with anti-p113 antiserum; lane 10, IFN-α, 8 h and vanadate, incubated with anti-ISGF3γ antiserum.

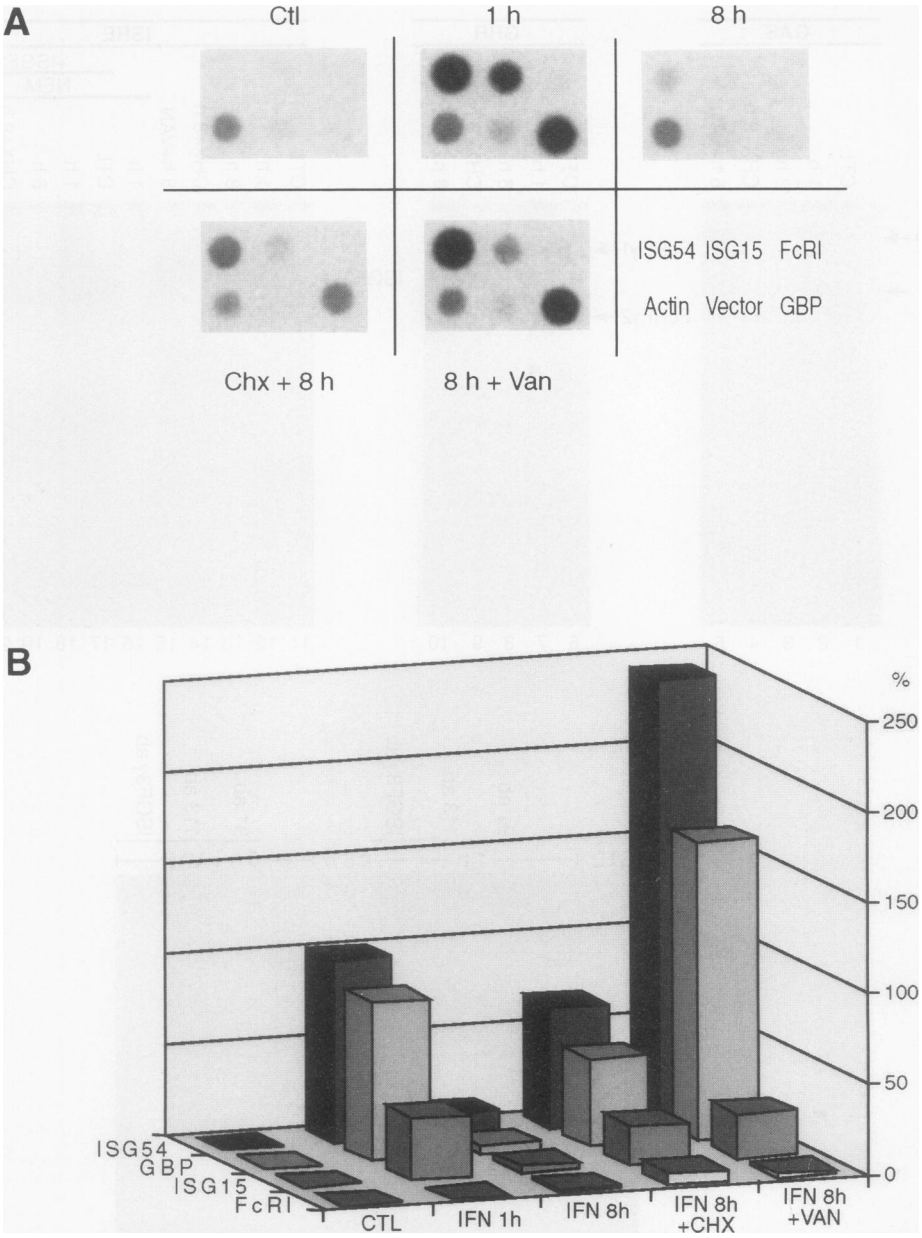

FIG. 2. Transcription rates of ISGs are enhanced after 8 h of IFN-α treatment in the presence of vanadate or cycloheximide. (A) Human fibroblasts were incubated with IFN-α in the presence or absence of cycloheximide (Chx) or vanadate (Van) under the conditions described in the legend to Fig. 1. Nuclear run-on assays were performed, and labeled RNA was hybridized to Nytran filters containing the indicated cDNA clones. After hybridization, the filters were subjected to autoradiography. ISG54, 54-kDa ISG; ISG15, 15-kDa ISG; FcRI, high-affinity immunoglobulin G FcRI gene; Actin, chicken β-actin; Vector, pGEM; GBP, guanylate-binding protein. (B) Autoradiograph of the hybridization shown in panel A was directly counted. The rates of transcription are displayed relative to the ISG54 signal with 1 h of IFN-α (= 100%), after being normalized for actin. CHX, cycloheximide; VAN, vanadate; CTL, control.

were initiated to characterize the fate of tyrosine-phosphorylated p91 as a result of extended treatment of cells with IFN-α or IFN-γ. Human fibroblasts were treated with either IFN for 1 or 8 h or for 8 h in the presence of vanadate or cycloheximide. After lysis, nuclei were isolated by sucrose density centrifugation and extracted, and the extracts were immunoprecipitated with an antibody against the p91 protein. The immunoprecipitates were resolved on SDS-polyacrylamide gels, blotted, and probed with anti-phosphotyrosine antibodies. Treatment of cells for 1 h with either IFN-α or IFN-γ resulted in tyrosine-phosphorylated p91 (Fig. 3A, lanes 1 and 6 versus 2 and 7), and in agreement with the gel shift experiments, further incubation for 7 more h led to the complete loss of tyrosine phosphorylation of p91 (lanes 3 and 8). Incubation of cells with either IFN-α or IFN-γ for 8 h in the presence of vanadate maintained p91 as a tyrosine-phosphorylated species in the nuclei (lanes 5 and 10), while cycloheximide had very little effect (lanes 4 and 9). These results not only confirmed the data from the gel shift and nuclear run-on experiments, but they also reinforced the

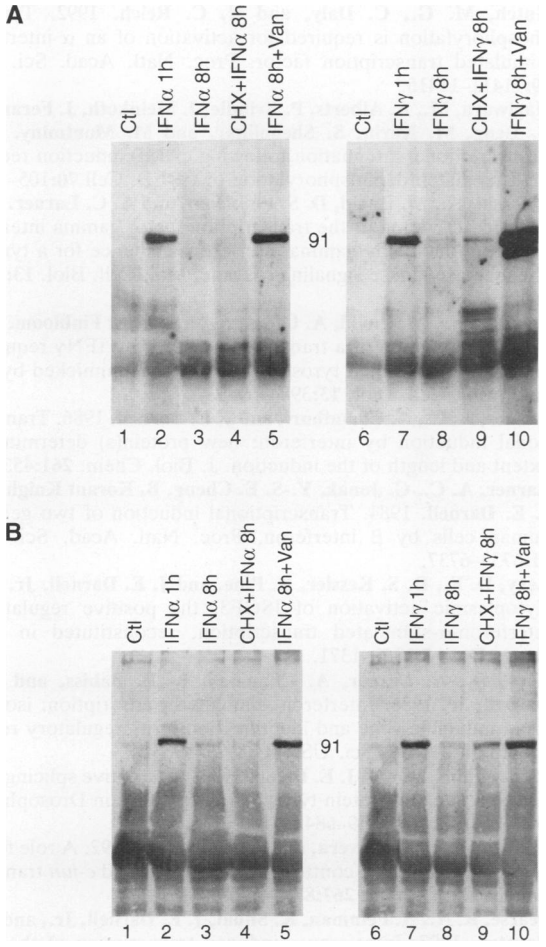

FIG. 3. Tyrosine phosphorylation and nuclear localization of p91 as a function of extended treatment with IFN-α or IFN-γ. (A) Phosphotyrosine blots of immunoprecipitated p91. Fibroblasts were exposed to either IFN-α (500 U/ml) or IFN-γ (5 ng/ml), and nuclei were isolated and extracted under the conditions described in the legend to Fig. 1. Extracts were incubated with the antiserum against p91 for 16 h at 4°C, and the resulting immunoprecipitates were resolved on 8% polyacrylamide-SDS gels. The gels were transferred to Immobilon and probed with an anti-phosphotyrosine antibody. The presence of tyrosine-phosphorylated proteins was detected by enhanced chemiluminescence. Migration of p91 is indicated. (B) The same blot shown in panel A was reprobed with anti-p91 antibody and developed with an alkaline phosphatase-conjugated secondary antibody. Ctl, control; CHX, cycloheximide; Van, vanadate.

specificity of the in vivo effects of vanadate's action as a PTP inhibitor. Interestingly, another protein besides p91 with a molecular mass of 84 kDa became tyrosine phosphorylated when cells were treated with IFN-γ and vanadate for 8 h (lane 10). An antibody which recognized both p91 and its spliced variant p84 (27) reacted with the 84-kDa protein, suggesting that under certain circumstances IFN-γ can cause tyrosine phosphorylation of both proteins.

The phosphotyrosine blots revealed that no tyrosine-phosphorylated p91 was present in the nuclei of either untreated cells or cells exposed to IFNs for 8 h. To determine whether p91 was present in the unphosphorylated form in the nucleus, the same blots initially probed with anti-phosphotyrosine antibodies were reprobed with anti-p91 antibodies. As shown in Fig. 3B, p91 was only present in the nuclei of those samples shown to be tyrosine phosphorylated in Fig. 3A (compare lanes 2, 5, 7, and 10 of Fig. 3A and B).

DISCUSSION

Homeostasis requires downregulation of many cellular responses resulting from extended exposure to external stimuli such as cytokines, growth factors, and hormones. Initial studies indicated that a protein synthesis-dependent mechanism functioned to shut off IFN-activated transcription of early response genes (9, 16). However, recent reports, which have shown that IFN-α- and IFN-γ-induced formation of several transcription complexes (ISGF3, GAF, and FcRFγ) required tyrosine phosphorylation (5, 10, 12, 14, 15, 22, 23, 28, 29) suggested that a nuclear tyrosine phosphatase might be one mechanism to downregulate IFN-induced gene expression. Although no evidence has so far been presented for the role of nuclear PTPs in regulation of gene expression, it has been reported that type 1 or 2A serine/threonine phosphatases downregulate the cyclic AMP-responsive transcription factor CREB after forskolin induction (13, 31). In this report we describe a nuclear PTP activity that is able to dephosphorylate IFN-activated transcription factors and to turn off the transcription of ISGs. Exposure of IFN-stimulated cells to the PTP inhibitor vanadate prevented the downmodulation of the transcription complexes observed during extended incubation. Furthermore, nuclear run-on experiments demonstrated that the transcription rates of several IFN-α-induced genes remained elevated for 8 h in the presence the cytokine and vanadate (Fig. 1 and 2). To characterize what role tyrosine phosphorylation contributed to maintenance of elevated transcription rates, fibroblast nuclear extracts were immunoprecipitated by using an antibody against p91, the common component of ISGF3, GAF, and FcRFγ. The results of these experiments demonstrated that incubation with either IFN-α or IFN-γ and vanadate maintained p91 in its tyrosine-phosphorylated state within the nucleus and that the presence of nuclear, tyrosine-phosphorylated p91 correlated with elevated rates of transcription. These data clearly support the role of a nuclear PTP as a transcriptional downregulator of ISGs.

Interestingly, none of the conditions used in these experiments permitted detection of unphosphorylated (tyrosine) p91 in the nuclei. This finding implied that tyrosine phosphorylation is needed not only for assembly of the transcription complexes but also for targeting the proteins to the nucleus and maintaining their nuclear localization. At the moment it is not evident whether dephosphorylated p91 is degraded in the nucleus or is recycled to the cytoplasm to be reactivated. Although we assume that p84 and p113 are processed in a manner similar to that for p91, the antibodies presently available do not immunoprecipitate these proteins efficiently enough to perform these studies.

Those few nuclear-localized PTPs which have thus far been purified are very substrate specific (8, 20, 26), and their activity does not appear to be regulated by treatment of cells with cytokines or growth factors. The nuclear PTP(s) activity which dephosphorylates the IFN-induced transcription complexes described in this work also appears to be constitutively expressed. This conclusion is suggested by the fact that preincubation with cycloheximide did not substantially maintain IFN-induced ISGF3, GAF, or FcRFγ formation or retain tyrosine-phosphorylated p91 in the nucleus. The role of protein synthesis in downregulating IFN-induced gene transcription remains unclear. From the data presented, cycloheximide may function to maintain elevated rates of

transcription of ISGs through a mechanism which functions independently of these transcription complexes binding to their enhancer elements. Alternatively, the PTP may have a short half-life, and cycloheximide prevents resynthesis of the degraded nuclear PTP. This latter possibility is attractive, since Fig. 1 and 3A indicated a slightly enhanced concentration of both the ISGF3 and FcRFγ transcription complexes and nuclear tyrosine-phosphorylated p91 in the presence of cycloheximide (compare Fig. 1, lanes 19 and 20, and Fig. 3A, lanes 8 and 9). However, it is unclear whether the small amount of ISGF3 maintained by cycloheximide can completely account for the elevated transcription of the ISGs seen in the nuclear run-on assays. Further characterization and purification of the nuclear PTP(s) which is associated with downregulation of the transcriptional response will permit both resolution of this question as well as an understanding of a new function that nuclear tyrosine phosphatases provide in regulation of gene expression.

ACKNOWLEDGMENTS

D. Levy generously provided the vector for in vitro translation of the ISGF3γ protein.

M.D. was supported by a Schroedinger fellowship from the Fonds zur Foerderung der wissenschaftlichen Forschung from Austria.

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