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# Restoration of Interferon Responses of Adenovirus E1A-Expressing HT1080 Cell Lines by Overexpression of p48 Protein

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**We have previously shown that both alpha interferon (IFN-α) and IFN-γ signaling pathways are blocked in HeLa cells expressing the adenovirus E1A proteins (G. T. Leonard and G. C. Sen, Virology 224:25–33, 1996). Here, we report that in two other E1A-expressing cell lines derived from the HT1080 cells, neither IFN-α nor IFN-γ could induce the transcription of genes containing the IFN-stimulated response element (ISRE). In contrast, IFN-γ-mediated signaling to the gamma-activated sequence was unimpaired in these cells. This dichotomy was due to a lowered level of functional p48 protein but not of STAT1 protein in the E1A-expressing HT1080 cells. When p48 was overexpressed in those cells by stably transfecting a p48 expression vector, both types of IFN could effectively induce the transcription of ISRE-driven genes. Consequently, IFN-α was highly effective in inhibiting the replication of encephelomyocarditis virus in the E1A-expressing cells, which also overexpressed p48. These results reinforce the general conclusion that adenovirus E1A proteins block IFN signaling pathways by lowering the functional levels of one or more components of the *trans*-acting complexes that activate the transcription of IFN-stimulated genes.**

Interferons (IFNs) are cytokines with potent antiviral properties (23, 27). IFN-α, IFN-β, and IFN-γ all induce the transcription of IFN-stimulated genes (ISG). The products of these cellular genes carry out the pleiotropic cellular effects of IFNs including their antiviral effects. To counteract these effects, some viruses have developed mechanisms to block IFN actions. Adenoviruses are examples of such IFN-resistant viruses. They not only escape the actions of IFN but also allow other viruses to multiply in IFN-treated adenovirus-infected cells (2). At the cellular level, there are two main mechanisms by which adenoviruses block IFN actions. The virally encoded VAI RNA can bind to the IFN-induced double-stranded RNA-dependent protein kinase, PKR, and inhibit its action, thereby allowing viral protein synthesis to continue (15, 17, 19). However, a more global block of IFN action is mediated by the adenovirus E1A proteins. They interfere with the transcriptional signaling process of IFN, so that induced transcription of the ISGs does not occur (1, 11, 13, 14). The E1A proteins are expressed early during the adenovirus infection process (21, 24). Through alternative splicing, two major E1A proteins are produced: a 243-residue protein that is a product of the 12S E1A mRNA, and a 289-residue protein that is a product of the 13S E1A mRNA. The larger protein contains all of the smaller protein plus a 46-residue domain. The E1A proteins are potent regulators of gene expression and cell function. They can activate or repress transcription of specific cellular or viral genes and can modulate cell growth, differentiation, and oncogenic transformation (3, 7, 9, 12, 21, 24).

Both types of IFNs use JAK-STAT pathways to signal from the cell surface receptors to the ISGs (8). The common *cis* element present in the IFN-α-stimulated genes is called the IFN-stimulated response element (ISRE). The corresponding activated transcription factor that binds to ISRE is ISG factor 3 (ISGF-3). ISGF-3 is composed of three proteins: STAT1 and STAT2 form ISGF-3α, and the p48 protein forms ISGF-3γ (16). The trimeric complex binds to ISRE much more tightly than does p48, which specifically recognizes the ISRE sequence. Binding of IFN-α to the cell surface receptors activates the receptor-associated tyrosine kinases, Tyk2 and Jak1. The activated kinases Tyr-phosphorylate STAT-1 and STAT-2, which then combine with p48 and migrate to the nucleus to function as active ISGF-3 (27). The pathways of gene induction by IFN-γ are more diverse. Some genes, such as IRF-1, are induced through the gamma-activated sequence (GAS) element; others, such as 9-27, are induced through the ISRE; and the major histocompatibility complex class II genes are induced through other distinct elements. Signaling to all classes of genes requires binding of IFN-γ to the same cell surface receptor, activation of the Tyr kinases Jak1 and Jak2, and Tyr-phosphorylation of STAT1. The transcription factor that binds to GAS is called gamma-activated factor (GAF). It is a dimer of Tyr-phosphorylated STAT1. The factor that is activated by IFN-γ and stimulates transcription through ISRE is less well characterized. Genetic evidence indicates that both STAT1 and p48 are needed for this signaling pathway but STAT2 is not. Biochemical evidence also indicates that the IFN-γ-activated factor that binds to the ISRE of the 9-27 gene contains STAT1 and p48 (6, 22). It remains possible, however, that additional proteins are needed to form this factor.

Studies from several laboratories, including ours, have shown that IFN-α fails to activate ISGF-3 in E1A-expressing cell lines (1, 11, 13). In the HeLa E1A cell line, both ISGF-3α and ISGF-3γ are nonfunctional (13), whereas in HT12s cells, only ISGF-3γ is defective (1). We have recently shown that the defects in the HeLa E1A cells are due to a lower cellular abundance of the STAT1 and p48 proteins (18). As a result, both GAS- and ISRE-mediated IFN-γ signaling pathways are also defective in the HeLa E1A cells. In this study, we have done similar analyses with two other E1A-expressing cell lines, HT12s and PCe2, derived from the HT1080 cells. The purpose of our study was to demonstrate the generality of the observed signaling defects in other E1A-expressing cells and to determine the molecular mechanism of these defects. We observed

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that the E1A-expressing HT1080 cell lines contained a lower level of p48 but the STAT1 level was unchanged. Consequently, IFN-γ signaling through the GAS element was unaffected but that through the ISRE was blocked. Similarly, IFN-α could not activate ISGF-3 in these cells. Overexpression of exogenous p48 in the PCe2 cells restored the defective IFN-α and IFN-γ signaling pathways as well as the antiviral effects of IFN-α. Thus, a lower level of functional p48 was established as the only defect in PCe2 cells that is responsible for defective IFN signaling.

## MATERIALS AND METHODS

**Cells and antibodies.** HT1080 human fibrosarcoma cells and HT1080-derived E1A-expressing cell lines, PCe2 and HT12s were the gifts of Ian Kerr (Imperial Cancer Research Fund, London, England). The HT12s cells express the 243R E1A protein, whereas the PCe2 cells express both the 243R and 289R E1A proteins (1). All cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum. PCe2 and HT12s were grown in the presence of 400 μg of G418 (Gibco BRL) per ml. Cell lines overexpressing p48 cDNA were selected and grown in the presence of 250 μg of hygromycin B (Calbiochem) per ml. The anti-p48 monoclonal antibody and the anti-STAT1 polyclonal antibodies were purchased from Transduction Laboratories (Lexington, Ky.). The goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibodies were purchased from Boehringer Mannheim (Indianapolis, Ind.).

**IFNs.** Recombinant IFN-γ was the gift of Genentech (South San Francisco, Calif.). IFN-α was from Hoffman-LaRoche (Nutley, N.J.).

**EMSA.** The GAF electrophoretic mobility shift assay (EMSA) was performed as described previously (18). The probe was from the GAS element located at −129 to −106 in the IRF-1 gene (25). Cells were either left untreated or treated for 15 min with 500 U of IFN-γ per ml. Whole-cell extracts were prepared as previously described. The ISRE EMSA was performed with the ISRE element located at −125 to −93 of the 561 gene (4). Cells were either left untreated or treated with 500 U of IFN-α per ml. Extracts were also prepared and EMSA was performed as described previously (4, 18).

**Western blot.** Whole-cell extracts were prepared as previously described (18). Whole-cell extract (50 μg) was subjected to denaturing polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membrane (Immobilon-P; Millipore) with a wet-transfer electrophoretic transfer apparatus (Mini Trans-Blot Gel Transfer Cell; Bio-Rad). The blot was probed as recommended by the antibody manufacturer (Transduction Laboratories).

**RNase protection assay.** Antisense probes to 9-27, IRF-1, and γ-actin mRNAs have been described previously (20, 22). Each probe was made with SP6 RNA polymerase (Boehringer Mannheim) to protect fragments of 163, 175, and 135 bp, respectively. Antisense probe to 561 mRNA has also been described previously (4). The 561 probe was made with T7 RNA polymerase to protect a fragment of 260 bp. Antisense probe (100,000 cpm) was hybridized overnight at 45°C with 10 μg total cytoplasmic RNA prepared with RNazol (Teltest, Friendswood, Tex.). Hybridized RNA was digested for 1 h at 30°C with a mixture of RNases A (20 μg/ml) and $T_1$ (1 μl/ml) (Boehringer Mannheim). The RNases were inactivated with proteinase K and extracted with phenol-chloroform-isoamyl alcohol and then with chloroform-isoamyl alcohol. RNA was precipitated and resuspended in loading dye (80% deionized formamide, 1 mM EDTA [pH8], 0.1% bromophenol blue, 0.1% xylene cyanol) and heated to 80°C for 5 min before being loaded onto a urea-containing 5% polyacrylamide gel. The gel was run, dried, and subjected to autoradiography (BioMax; Kodak). The dried gel was also quantitated with a PhosphorImager (Molecular Dynamics). Quantitative data are presented in the figures in arbitrary units after normalization against the γ-actin mRNA values of each sample. The highest normalized level in each experiment was assigned a value of 100, and the other values were compared to that.

**Production of p48-overexpressing cell lines.** PCe2 cells were transfected with either a eukaryotic expression vector containing the human p48 cDNA (obtained from Mathias Müller and Ian Kerr) or the empty expression vector. The vectors contained the hygromycin phosphotransferase gene. Permanent transfectants were selected in the presence of 250 μg of hygromycin B (Calbiochem) per ml. Separate cell clones were isolated by being plated sparsely. The clones were grown in the presence of 250 μg of hygromycin B per ml for maintaining the p48 plasmid and 400 μg of G418 per ml for maintaining the E1A plasmid. Levels of p48 expression in several clones were examined by Western blotting, and the most highly expressing clones were stored for further use. PCe2.γ2 and PCe2.γ6 are two such p48-overexpressing clones, whereas PCe2.pDR2 is a clone containing the empty vector.

**Antiviral assays.** Cells were plated at a subconfluent density in six-well plates. They were treated with 100 U of IFN-α per ml for 18 h and then infected with encephalomyocarditis virus (EMC virus) at 10 PFU/cell. Virus was absorbed for 1 h in medium containing 1% serum. The cells were then incubated in normal medium for 24 h. Virus was harvested by centrifugation after three freeze-thaw cycles. The viral titers from each line were determined by plaque assays on HeLa M cell monolayers. These were done in triplicate for each of the three different dilutions of the virus stock. Results are plotted as the fold decrease in virus titer upon IFN treatment. This value was obtained by dividing the mean virus titer for the untreated cells by the mean virus titer for the IFN-treated cells.

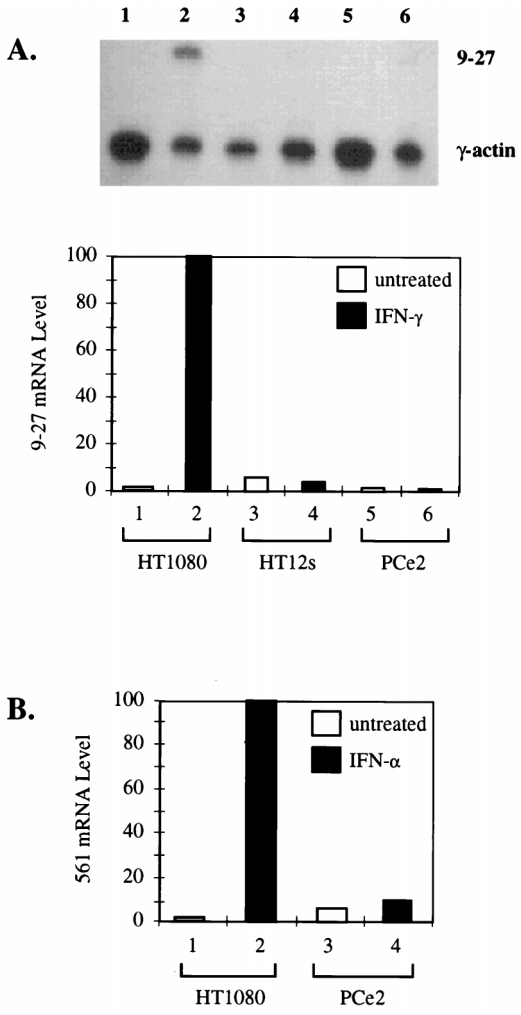


FIG. 1. Induction of 9-27 mRNA and 561 mRNA in E1A-expressing cell lines. (A) HT1080, HT12s, and PCe2 cells were treated with 100 U of IFN-γ per ml for 18 h or left untreated. Total RNA was extracted and used for RNase protection assays with the 9-27 and γ-actin antisense RNA probes. The autoradiogram is shown above, and the normalized quantitative values of 9-27 mRNA levels are shown below. The odd-numbered lanes are from untreated cells, and the even-numbered lanes are from IFN-γ treated cells. (B) HT1080 and PCe2 cells were treated with 100 U of IFN-α per ml for 6 h, and 561 mRNA levels were estimated by RNase protection assays. Lanes 1 and 3 are from untreated cells, and lanes 2 and 4 are from IFN-α-treated cells. Normalized levels of 561 mRNA are presented.

## RESULTS

**Gene induction by IFNs in E1A-expressing HT1080 cell lines.** Two E1A-expressing cell lines were used in our studies: HT12s and PCe2. Both of these lines are derived from HT1080 cells; the former line expresses the 243R E1A protein only, whereas the latter line expresses both 243R and 289R E1A proteins (1). ISRE-mediated signaling by IFN-γ and IFN-α was studied in the experiments shown in Fig. 1. We chose three probes for measuring the levels of gene induction mediated by three alternative IFN-signaling pathways. 561 mRNA is in-

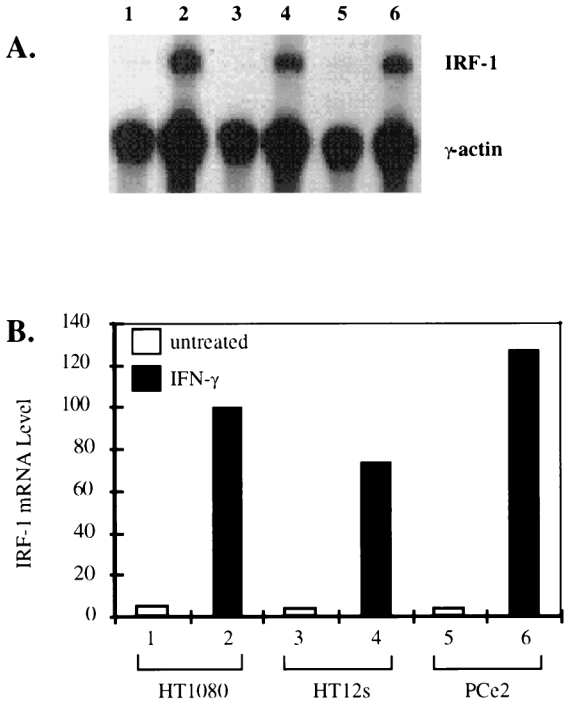


FIG. 2. Induction of IRF1 mRNA by IFN-γ. Cells were treated with 100 U of IFN-γ per ml for 18 h, and IRF-1 mRNA levels were measured by RNase-protection assays. (A) The autoradiogram is shown: odd-numbered lanes are from untreated cells, and even-numbered lanes are from IFN-γ-treated cells. (B) Quantitation and normalization of the IRF-1 mRNA values.

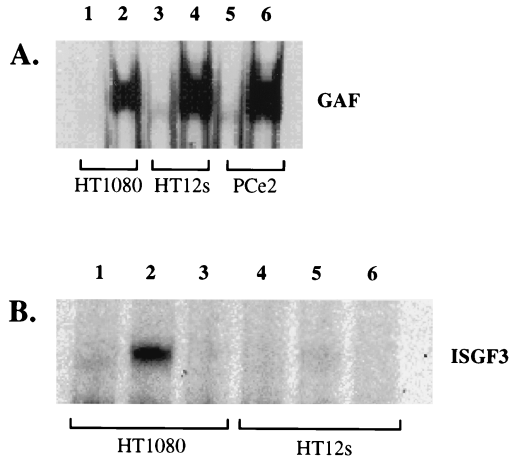


FIG. 3. Determination of transcriptional complex formation in E1A-expressing and control cell lines. (A) EMSA with a probe corresponding to the GAS element of the IRF-1 gene. Odd-numbered lanes are from untreated cells; even-numbered lanes are from cells that have been treated with 500 U of IFN-γ per ml for 15 min. (B) EMSA with a probe corresponding to the ISRE of the 561 gene. Lanes 1 and 4 are from untreated cells; lanes 2, 3, 5, and 6 are from cells that have been treated with 500 U of IFN-α per ml for 15 min. Lanes 3 and 6 contained a 50-fold excess of unlabeled probe.

duced by IFN-α using ISRE as the *cis* element and ISGF3 as the cognate transcription factor (4, 13). 9-27 mRNA is induced by IFN-γ with ISRE as the *cis* element and a poorly defined transcription factor which contains p48 and STAT-1 (1, 6, 22). IRF-1 mRNA, on the other hand, is induced by IFN-γ with GAS as the *cis* element and GAF, containing only STAT-1, as the transcription factor (8, 20, 25). Cellular levels of the IFN-γ-inducible 9-27 mRNA (22) and the IFN-α-inducible 561 mRNA (4) were measured by RNase-protection assays. The γ-actin mRNA, whose level is unaltered by IFN treatments, was used as an internal control. As reported previously (1), IFN-γ failed to induce transcription of the 9-27 mRNA in either E1A-expressing cell lines, whereas transcription was efficiently induced in the parental HT1080 cells (Fig. 1A). The same was true for induction of the 561 mRNA by IFN-α (Fig. 1B and data not shown).

In contrast to the 9-27 gene, whose induction by IFN-γ is mediated by the ISRE, the *cis*-element GAS is responsible for mediating the IFN-γ response of many other genes such as the IRF-1 gene. To test the status of the GAS-mediated IFN-γ response of the E1A-expressing cell lines, we measured the induction of the IRF-1 mRNA. Surprisingly, IRF-1 mRNA was strongly induced by IFN-γ in both the HT12s and PCe2 cell lines (Fig. 2).

**Activation of *trans*-acting factors.** The IFN-responsive DNA element-binding factors have been identified for some but not all IFN-signaling pathways. ISGF-3 is the factor used by IFN-α to signal through ISRE. ISGF-3 is a trimeric complex of activated STAT1, activated STAT2, and p48. The corresponding ISRE-binding factor used by IFN-γ remains to be completely defined. Genetic and biochemical data suggest that this putative factor contains STAT1, p48, and additional proteins. In contrast, GAF is well characterized; it is a dimer of activated STAT1. In the experiment in Fig. 3, we performed EMSAs to examine the ability of IFNs to activate GAF and ISGF-3 in the E1A-expressing cell lines. IFN-γ effectively activated GAF in both E1A-expressing cell lines (Fig. 3A). Although only one portion of the autoradiogram is shown, there was no other shifted band. That the shifted band was GAF was confirmed by its supershifting with an antibody to STAT1 (data not shown). When ISRE was used as the probe, ISGF3 was formed in IFN-α-treated HT1080 cell extract (Fig. 3B, lane 2). The mobility of this shifted band and its supershifting by antibodies to STAT1 and p48 (data not shown) confirmed its identity as ISGF3. ISGF-3 was, however, not activated by IFN-α in HT12s (Fig. 3B) or PCe2 (not shown) cells. These observations are consistent with the mRNA induction patterns shown in Fig. 1 and 2.

**Cellular levels of *trans*-acting factors.** The inability of IFN-α to activate ISGF-3 in HeLa E1A cells has been traced to lowered cellular levels of p48 and STAT1 proteins (18). We examined if the same was true for the HT12s and PCe2 cells. Western analysis showed that p48 levels were indeed lower in these two cell lines (Fig. 4). Unlike the HeLa E1A cells, however, the HT12s and PCe2 cells contained similar amounts of STAT1 proteins to the parental HT1080 cells. These observa-

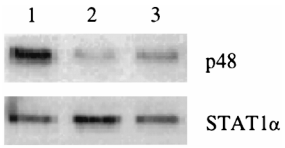


FIG. 4. Levels of signaling proteins in E1A-expressing and control cell lines. (Top) Western blot analysis of extracts from HT1080 (lane 1), HT12s (lane 2), and PCe2 (lane 3) probed with antibody to p48. (Bottom) Western blot analysis of extracts from HT1080 (lane 1), HT12s (lane 2), and PCe2 (lane 3) probed with antibody to STAT1. A 50-μg quantity of protein from each extract was used for analysis. The top and bottom panels are different blots of the same extracts. The p48 and STAT1α-specific bands did not appear when a nonimmune serum was used as the primary antibody.

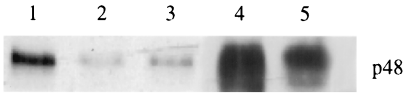


FIG. 5. Establishment of cell lines overexpressing p48. Western blot analysis of extracts from HT1080 (lane 1), PCe2 (lane 2), PCe2.pDR2 (a clone containing empty vector) (lane 3), PCe2.γ2 (lane 4), and PCe2.γ6 (lane 5) probed with antibody to p48. A 50-μg quantity of protein from each extract was analyzed. The extracts were made from IFN-untreated cells.

tions may explain why IFN-α could not activate ISGF-3 but IFN-γ could activate GAF in the PCe2 cells.

To critically examine whether the lower level of p48 protein in the PCe2 cells is responsible for the observed defect in IFN signaling, we increased the p48 level by transfecting those cells with an exogenous p48 expression vector. Stably transfected cell clones were isolated and analyzed for p48 expression. The levels of p48 protein in two such clones are shown in Fig. 5. They were much higher than those in untransfected PCe2 cells (lane 2), untransfected HT1080 cells (lane 1), or a clone of PCe2 cells transfected with the empty expression vector without the p48 coding sequence (lane 3).

**Restoration of IFN signaling by p48 overexpression.** The effects of p48 overexpression on 9-27 mRNA induction by IFN-γ were examined in the experiment shown in Fig. 6. 9-27 mRNA was poorly induced in the PCe2 · pDR2 cells but was strongly induced in the p48-overexpressing PCe2 · γ2 and PCe2 · γ6 clones. In the IFN-γ-treated γ2 clone, the 9-27 mRNA level was as high as 70% of that in the IFN-γ-treated HT1080 cells, whereas in the γ6 clone, it was more than 40% of the level in the HT1080 cells. Thus, p48 overexpression could restore, at least partially, the ability of IFN-γ to induce 9-27 mRNA in the E1A-expressing cells.

The ability of IFN-α to induce 561 mRNA in the p48-overexpressing clones was examined in the experiment shown in Fig. 7. The level of 561 mRNA in IFN-α-treated PCe2 · γ6 cells was as high as that in IFN-α-treated HT1080 cells. The same was true for the PCe2 · γ2 clone (data not shown). In contrast, 561 mRNA was not appreciably induced by IFN-α in the PCe2 cells or in the PCe2.pDR2 cells (data not shown). Note that p48 overexpression did not elevate the basal level of 561 mRNA in the PCe2 · γ6 cells but made these cells susceptible to the action of IFN-α.

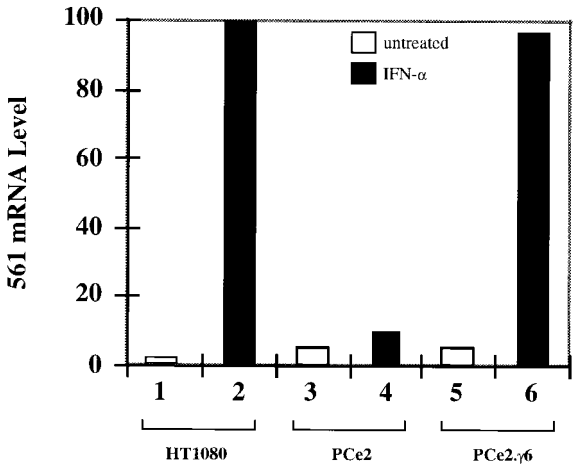


FIG. 7. Induction of 561 mRNA in p48-overexpressing cells. Odd-numbered lanes are from untreated cells; even-numbered lanes are from cells treated with 100 U of IFN-α per ml for 6 h.

**Antiviral action of IFN-α in p48-overexpressing cells.** It is known that as a consequence of the block in signaling, IFN-α fails to block virus replication in cells expressing E1A proteins (2). Since we observed that p48 overexpression restored the ability of gene induction by IFN-α in the PCe2 cells, we next examined the antiviral state of these cells (Fig. 8). Different cell lines were treated with IFN-α and infected with EMC virus. Virus yields from each plate were determined by plaque assays. The average of triplicate assays showed that 100 U of IFN-α per ml reduced EMC virus yield by 444-fold in HT1080 cells. The corresponding value for PCe2 cells was only 27-fold. p48 overexpression in the PCe2 · γ6 cells almost fully restored the ability of IFN-α to inhibit EMC virus replication. The virus yield from untreated PCe2 · γ6 cells was 304-fold higher than that from IFN-α-treated cells. These results indicate that overexpression of p48 could rectify all E1A-mediated defects of IFN-α action in HT1080 cells.

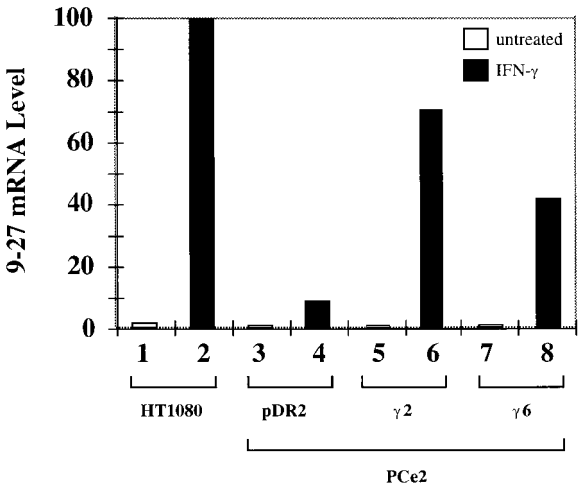


FIG. 6. Induction of 9-27 mRNA in p48-overexpressing cells. Normalized RNase protection assay results are shown. Odd-numbered lanes are from untreated cells; even-numbered lanes are from cells that have been treated with 100 U of IFN-γ per ml for 18 h.

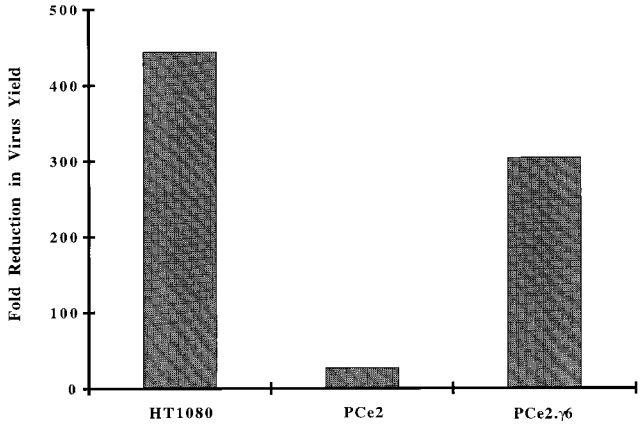


FIG. 8. EMC virus replication in p48-overexpressing cells. Data are presented as fold reduction of virus yields in each cell line upon treatment with 100 U of IFN-α per ml. The absolute virus titers in untreated cells were $1.72 \times 10^8$ PFU/ml for HT1080, $1.42 \times 10^8$ PFU/ml for PCe2, and $1.01 \times 10^8$ PFU/ml for PCe2.γ6. Results are means of triplicate plaque assays.

## DISCUSSION

Adenoviruses are resistant to the antiviral actions of IFN. Other viruses, which are susceptible to IFNs, can also multiply efficiently in IFN-treated adenovirus-infected cells (2). These observations suggested that adenoviruses can globally shut off the antiviral actions of IFN. The viral gene responsible for this effect was identified as the E1A gene, and both of its major products, the E1A 243R protein and the E1A 289R protein, were shown to be capable of interfering with the actions of IFN (1, 11, 13, 14). Studies with cell lines stably transfected with E1A expression vector established clearly that the E1A-mediated inference with the actions of IFN occurs at the level of transcriptional signaling. In E1A-expressing cells, IFN can bind to the receptor but fails to activate the transcription factors that induce the transcription of the IFN-stimulated genes. As a result, all cellular effects of IFN, including its antiviral effects, are abrogated in E1A-expressing cells.

In our previous studies, we exclusively used the HeLa E1A cells in which both IFN-α and IFN-γ signaling pathways are defective (13, 18). We have shown that in these cells, IFN-α cannot induce transcription of ISRE-driven genes, because the crucial transcription complex, ISGF-3, is not formed. Similarly, IFN-γ cannot activate GAF in the HeLa E1A cells and therefore cannot activate genes driven by the GAF-binding element GAS. Recently, we have shown that the failure to activate ISGF-3 and GAF by IFN-α and IFN-γ, respectively, can be directly attributed to a lower cellular level of STAT1 and p48 proteins in the HeLa E1A cells.

In the present study, we examined whether the above observations can be extended to two other E1A-expressing cell lines derived from the HT1080 cells. The conclusions derived for this study are presented schematically in Fig. 9. This figure depicts the single IFN-α signaling pathway and the two IFN-γ signaling pathways. The IFN-α signaling pathway uses ISRE as the *cis* element and ISGF3, containing STAT1, STAT2, and p48, as the *trans*-acting factor. One IFN-γ signaling pathway uses GAS as the *cis* element and GAF, containing a STAT1 homodimer, as the *trans*-acting factor. The other IFN-γ signaling pathway uses ISRE as the *cis* element and a *trans*-acting factor which is composed of STAT1, p48, and possibly an unknown protein. The consequences of p48 deprivation in the E1A-expressing HT1080 cells on the above signaling pathways are shown in Fig. 9. Ackrill et al. (1) have shown that in HT12s and PCe2 cells, ISRE-mediated signaling by both IFN-α and IFN-γ is blocked, an observation consistent with our results from HeLa E1A cells. Results presented here, however, show that all IFN-γ-signaling pathways are not blocked in PCe2 and HT12s cells. GAF is activated by IFN-γ in these two cell lines, and GAS-mediated induction of the IRF-1 gene transcription occurs efficiently. In contrast, IFN-γ failed to induce the 9-27 gene, which uses ISRE, not GAS, for receiving the signal generated by IFN-γ. The exact nature of the IFN-γ-activated complex that binds to the 9-27 ISRE is obscure, although genetic studies and in vitro reconstitution studies suggest that STAT1 and p48, but not STAT2, are parts of this complex (6). As expected from the studies by Ackrill et al. (1), IFN-α failed to activate ISGF-3 in the HT12s cells and the ISRE-driven 561 gene was not induced by IFN-α in either HT12s or PCe2 cells. Thus, the results presented here demonstrate that in HT1080 cells, E1A has dichotomous effects on IFN-signaling pathways: two pathways are blocked, whereas a third pathway remains unaffected (Fig. 9).

The above dual effects of E1A could be explained by the observed changes in the cellular concentrations of the STAT1 and p48 proteins. Unlike HeLa E1A cells, PCe2 cells contained as much STAT1 protein as did the parental HT1080 cells. Consequently, activation of GAF, which is composed of STAT1α exclusively, was not impaired at all. The level of p48, on the other hand, was lower in PCe2 cells than in HT1080 cells. As a consequence, ISRE-mediated gene inductions by IFN-α or IFN-γ, which are known to require p48, were blocked in PCe2 cells. These results also explain an apparent historical discrepancy between our results (13) and those of Ackrill et al. (1). We observed that in HeLa E1A cells, IFN-α cannot activate ISGF-3, because both ISGF-3α, composed of STAT1 and STAT2, and ISGF-3γ, composed of p48, are defective (13), whereas Ackrill et al. (1) observed that in HT12s cells, ISGF-3α can be activated by IFN-α but ISGF-3γ is nonfunctional. The molecular basis of the observed discrepancy between the two cell lines is now clear; in HeLa E1A cells, both STAT1 and p48 are defective, but in HT12s cells, only p48 is defective.

Although we observed that E1A expression results in an impairment of IFN signaling and a reduction in the cellular level of p48 in both HeLa and HT1080 cells, the causal rela-

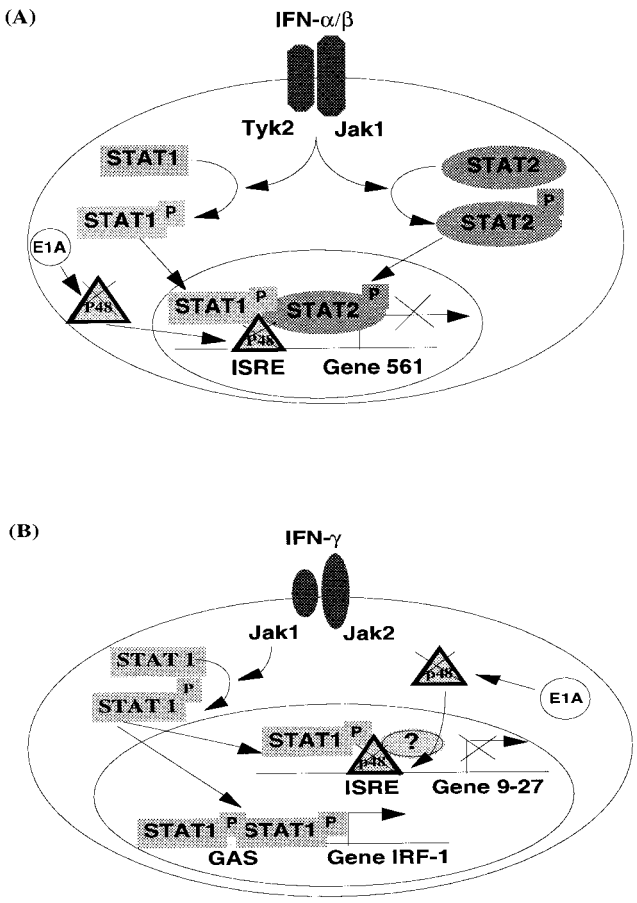


FIG. 9. Sites of blockades in IFN-signaling pathways in E1A-expressing HT1080 cells. (A) The IFN-α-signaling pathway. In E1A-expressing cells, the blockade is at the level of availability of p48 (shown by a cross-mark), so that active ISGF3 is not formed (shown by a cross-mark) and 561 mRNA is not transcribed (shown by a cross-mark). (B) Two IFN-γ-signaling pathways. The two pathways are affected differently in E1A-expressing cells. Since STAT-1 phosphorylation is not affected, STAT-1 homodimers are formed and GAS-mediated transcriptional induction of IRF-1 mRNA is unaffected. In contrast, since p48 is unavailable (shown by a cross-mark), the IFN-γ activated ISRE-binding complex is not formed (shown by a cross-mark), and transcription of 9-27 mRNA is not induced (shown by a cross-mark).

tionship between the two observations remained to be established. In this study, restoration of IFN actions as a result of overexpression of p48 clearly established that a low level of p48 is the sole reason for the defective signaling in PCe2 cells. Since only one signaling protein was affected in these cells, in contrast to the HeLa E1A cells, it was easier to overexpress the affected protein and restore IFN signaling. The next major question, which needs to be addressed in future studies, concerns what is wrong with the p48 protein in PCe2 cells. The Western blot experiments of Fig. 4 show that the cellular p48 level is reduced in the HT12s and PCe2 cells. The protein is, however, present in the PCe2 cells, and quantitation of the levels of p48 in the HT1080 and PCe2 cells showed a difference of about a factor of 2 (data not shown). It is difficult to imagine how a change of this magnitude could cause a total block of signaling. It is likely that the physical level of the p48 protein, as detected by the Western analysis, is not the crucial factor but that the level of functional p48 is the effective determinant and that functional p48 is virtually absent in the PCe2 cells. This hypothesis implies that the p48 protein can exist in both active and inactive forms and that the equilibrium is shifted toward the inactive form of p48 in the PCe2 cells. One can speculate that a posttranslational modification of the protein is essential for its activity and that E1A can directly or indirectly affect this process. From the literature, it is known that p48 is a phosphoprotein, and its phosphorylation status affects its ability to bind ISRE (26). It is also known that E1A can modulate the phosphorylation status of many regulatory proteins and thereby affect their cellular functions (5). Thus, it is tempting to speculate that the observed change in the function of the p48 protein in the PCe2 cells is brought about by changes in its phosphorylation status. The observed twofold change in the cellular level of the protein could be the consequence of a higher degradation rate of unphosphorylated p48 in the PCe2 cells, a postulate consistent with enhanced cellular instability of many unphosphorylated phosphoproteins. If this is the case, how can we explain the restoration of signaling by p48 overexpression? It is worth mentioning, in this context, that in both PCe2 clones, γ2 and γ6, p48 is highly overexpressed. At the mRNA level, where accurate quantitative estimation could be done by RNase protection assays, these clones expressed 50 to 70 times as much p48 mRNA as did untreated HT1080 cells (data not shown). It is possible that a huge excess of p48 overcomes the effects of E1A. Several alternative mechanisms can be postulated for this effect, and they need to be tested in future studies.

Not only did p48 overexpression in PCe2 cells restore IFN signaling, but also IFN-α could effectively inhibit EMC virus replication in those cells (Fig. 8). Restoration of such a complex biological phenotype indicates that the full cellular response of IFN-α has been restored in the PCe2 · γ6 cells. Thus, in this particular cellular context, all of the blocking effects of E1A on the action of IFN appear to be due to the absence of functional p48. It remains to be seen whether IFNs would be able to block adenovirus replication in p48-overexpressing cells. It is conceivable that blocking the action of E1A would not be enough and that other adenovirus-encoded gene products such as VAI RNA would block the actions of IFN at another level and thereby facilitate virus replication (27).

Finally, why are both STAT1 and p48 proteins affected in HeLa E1A cells, whereas only the latter protein is affected in HT1080 cells? Resolution of the above problem has to await elucidation of the mechanism by which E1A affects p48 functions. However, it is worth noting that HeLa cells constitutively express several human papillomavirus proteins, which are not expressed in HT1080 cells. These HPV proteins, in concert with adenovirus E1A, may bring out cellular changes not attainable by E1A alone. Such a mechanism is all the more likely since adenovirus E1A and certain HPV proteins are known to interact with the same cellular proteins and thereby modulate their functions (10). In spite of the noted difference between the E1A-expressing HeLa and HT1080 cells, our results clearly demonstrate the general mechanism by which E1A interferes with IFN signaling: it lowers the functional level of one or more proteins which constitute the activated transcription complex.

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