Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7913–7917, September 1991
Cell Biology

Repression of the interferon signal transduction pathway by the adenovirus *E1A* oncogene

(gene expression/DNA-binding factors/transcriptional regulation)

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Communicated by James E. Darnell, Jr., May 16, 1991

ABSTRACT The signal transduction pathway initiated by type I interferon (α and β interferons) is inhibited by expression of the adenovirus type 5 *E1A* oncogene. Cotransfection analyses with the *E1A* oncogene and an interferon-stimulated reporter gene show that mutations within an amino-terminal domain of the E1A oncoprotein are defective in transcriptional repression. Cotransfection experiments also revealed that the transcriptional repression is mediated through the interferon-stimulated response element (ISRE) found within the promoter of interferon-stimulated genes. Since interferon treatment activates a latent cytoplasmic DNA-binding factor that can recognize the ISRE and subsequently stimulate transcription, the appearance of this factor was analyzed in a cell line that constitutively expresses the *E1A* oncogene. The DNA binding activity of this transcriptional activator was found to be inhibited in the *E1A*-expressing cell line. *In vitro* cytoplasmic mixing experiments with extracts from control and *E1A*-expressing cells identified a specific component of this multimeric transcription factor to be defective.

Specific gene expression is activated within minutes after type I interferon (IFN), comprising α and β IFNs (IFN-α/β), binds to its cell-surface receptor (1, 2). The resultant expression of IFN-induced gene products elicits cellular responses that are both antiviral and antiproliferative (reviewed in ref. 3). Consequently, IFNs play a primary physiological role in the host defense against viral infection. Yet some viruses, such as adenovirus, have evolved mechanisms to evade this protective response system. Adenovirus utilizes at least two mechanisms to avert the action of IFN. Synthesis of the viral-associated RNAs inhibits the function of the IFN-stimulated p68 protein kinase, thereby allowing viral translation to continue unabated (4, 5). In addition, expression of the *E1A* oncogene inhibits IFN action at an even more fundamental level. Adenoviral E1A oncoproteins suppress the transcriptional induction of the IFN-stimulated genes (ISGs) (6).

After IFN-α/β binds to its receptor, a signal is generated that leads to the activation of a latent cytoplasmic DNA-binding factor, the ISG factor 3 (ISGF3) (7–9). The molecular mechanism of activation of the preexisting ISGF3 by the ligand–receptor complex remains to be determined. However, evidence has suggested the involvement of a protein kinase C isozyme or other kinase in the activation pathway (10, 11). The activation of ISGF3 appears to result from the stable interaction of two components, ISGF3-α and ISGF3-γ, to form a multimeric protein complex (9). ISGF3 translocates to the nucleus and elicits a transcriptional response by binding to a specific IFN-stimulated response element (ISRE) located in the promoter region of inducible genes (7, 12, 13).

Chimeric expression plasmids were utilized in this study to demonstrate that E1A repression of ISG expression is mediated through the ISRE. In addition, analyses of ISGF3 activation in a cell line that expresses the adenoviral *E1A* oncogene suggest that E1A interferes with the signal transduction pathway of IFN by inhibiting the DNA-binding activity of ISGF3. A DNA-binding component of the multimeric ISGF3 (ISGF3γ) (9) is nonfunctional in these cells.

Specific domains of the E1A oncoprotein are required for its functions in cellular transformation gene regulation and association with cellular proteins (reviewed in refs. 14 and 15). For this reason, various *E1A* mutations (16, 17) were used to determine their effect on transcriptional repression of the ISGs. Repression was found to require a major portion of the conserved region 1 domain of the E1A protein, a domain required for cellular transformation and stimulation of DNA synthesis (14, 15, 17).

MATERIALS AND METHODS

Cell Culture. Human Hep G2 (ATCC), HT-1080, and HT2a cells (gift of Steven Frisch; ref. 18) were maintained in Dulbecco's modified Eagle's medium with 8% (vol/vol) fetal bovine serum. Cultures were treated with 1000 units of recombinant human IFN-α2a or 100 units of recombinant human IFN-γ per ml as indicated (Hoffmann–LaRoche). Transfections were performed by $Ca_3(PO_4)_2$–DNA coprecipitations as described (12, 19). Plasmids encoding the various E1A proteins contained the native *E1A* promoter (gift of Ed Harlow; refs. 16 and 17).

mRNA Analysis. Cytoplasmic mRNA was isolated, hybridized to specific RNA antisense probes, and analyzed on polyacrylamide sequencing gels (10, 12). The antisense probe complementary to E1A mRNA was derived from linearized phage SP65 DNA containing 310–1774 adenovirus type 5 nucleotides (gift of Ronald P. Hart, Rutger's University, Newark, NJ).

Protein Analysis. Gel mobility-shift electrophoresis was used to study IFN-induced DNA-binding proteins (7). Nuclear protein extracts were prepared from cells as described (7, 19), and cytoplasmic extracts were clarified by centrifugation at 150,000 × *g*. A DNA fragment of the ISG15 promoter (10) or the following specific ISG15 ISRE oligonucleotide,

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5'-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3'
      3'-CCCTTTCCCTTTGGCTTTGACTTCGGCTAG-5',
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was used to study protein–DNA complexes resolved in 4.5% polyacrylamide gels.

Abbreviations: IFN, interferon; IFN-α, -β, and -γ, α, β, and γ IFNs; ISRE, IFN-stimulated response element; ISG, IFN-stimulated gene; ISGF, IFN-stimulated gene factor; TK, thymidine kinase; nt, nucleotide(s).

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RESULTS

E1A Represses Transcription Mediated by the ISRE. To determine if transcriptional repression of the ISGs by E1A was mediated through a specific DNA sequence, promoter deletions of ISG15 were analyzed (12, 19). IFN-inducible plasmids were used that contain the 5′ promoter region of ISG15 linked to the 3′ region of a reporter adenoviral *E1B* gene (12, 19). The hybrid constructs of ISG15 were cotransfected into cells with or without the *E1A* oncogene. IFN-α-inducible mRNA expression was measured by hybridization with a specific antisense RNA probe spanning the correct transcriptional start site of the ISG15-*E1B* gene (12). To provide an accurate measure of both the cotransfection efficiency and the correct expression of the *E1A* oncogene, mRNA transcripts from the cotransfected *E1A* oncogene were also analyzed.

In cotransfection experiments with the ISG15-*E1B* plasmids, the *E1A* oncogene effectively repressed IFN-α induction of a minimal ISG15 promoter that still contained the ISRE at position −108 (data not shown) (12, 19). The ability of E1A to suppress the −108/ISG15 promoter suggested that the target of repression was the ISRE, a DNA sequence both necessary and sufficient for activation by IFN (19). This was tested by analyzing the ISRE within the context of a heterologous promoter, the herpes simplex virus (HSV) thymidine kinase (TK) gene at position −109 (TK-3; ref. 19). Cotransfection of the *E1A* oncogene with the ISRE-TK construct resulted in effective repression of IFN-induced transcription (Fig. 1, lanes 3 and 4). Therefore, the ISRE appears to be the DNA target of negative regulation by E1A oncoproteins.

The primary transcript of the *E1A* oncogene is differentially spliced into several mature mRNA species. Two of the major mRNA transcripts are the 12S and 13S species encoding proteins of 243 and 289 amino acids, respectively (20) (Fig. 1, lanes 5–8). cDNA plasmids expressing the 12S or 13S mRNA were

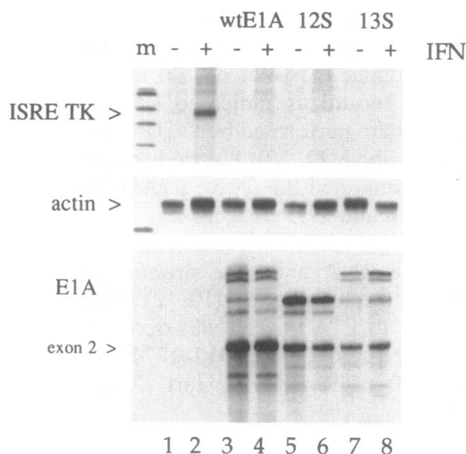

FIG. 1. E1A represses transcriptional activation by IFN-α through the ISRE. Hep G2 cells in 100-mm plates were cotransfected with the ISRE-TK reporter plasmid (10 μg) (12, 19) and either control plasmid DNA (10 μg) (lanes 1 and 2) or the *E1A* expression plasmids (10 μg) (lanes 3–8). Cultures were split 1:2 the following day, and 2 days later cytoplasmic mRNA was isolated from an untreated culture (lanes −) or a culture treated with IFN-α for 4 hr (lanes +). The antisense RNA probes protect a 202-nucleotide (nt) fragment from the 5′ end of the TK transcript, and a 135-nt fragment of the γ-actin mRNA (10, 12). The antisense RNA probe for E1A (positions 310 through 1774) protects both the first exon mRNA fragments (613 nt and 466 nt) and the second exon (405 nt) mRNA fragment. In addition to the predicted transcripts from the *E1A* gene, occasional longer RNA fragments from the first exon were noted. These transcripts may represent cryptic transcriptional start sites or splice sites. pBR322 DNA fragments generated with the *Hpa* II restriction enzyme were used as size markers (lane m). wt, Wild type.

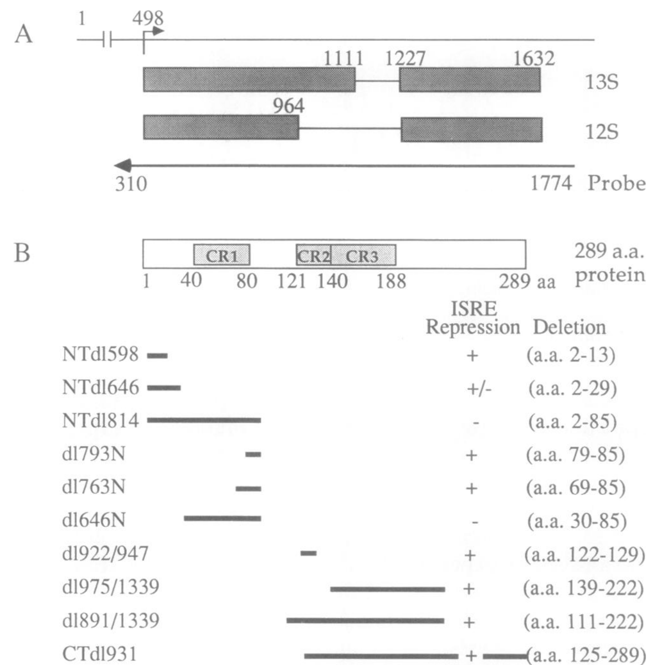

FIG. 2. Schematic representation of *E1A* products. (*A*) The transcriptional start site of the *E1A* gene is depicted with an arrow. The 13S and 12S mRNA processed transcripts are displayed with their respective exon boundaries (dark boxes) and spliced introns (thin lines) (20). The antisense RNA probe used in the *E1A* hybridizations spans the entire primary transcript. Positions within the adenovirus genome are represented by nucleotide number. (*B*) The protein product of the 13S mRNA [289 amino acids (aa)] is diagrammed to show the location of the three conserved regions CR1, CR2, and CR3 (14, 21). The translation initiation codon is at nucleotide position 560 in the mRNA. The predicted amino acid deletions of the *E1A* mutations used in this study are shown on the right and represented by a black bar (17). Mutations encoding specific amino acid substitutions are described in ref. 17. The ability of the mutations to repress (+) IFN-stimulated transcription is given in a summary column.

tested in the cotransfection analyses to determine their individual ability to repress transcription. The products of both clones were able to inhibit IFN-stimulated transcription (Fig. 2*B*). Occasionally an E1A mRNA species was detected in the 13S cDNA transfections that comigrated with the 12S 5′ exon. Since the appearance of this product was variable, it did not seem to correlate with repression. Previous experiments showed that during an adenoviral infection, the 12S product was the major negative regulator of the ISGs (6). The complexity of both positive and negative gene regulation by E1A during an adenoviral infection may be responsible for this effect.

Transcriptional Repression Requires a Specific Domain of the E1A Protein. The products of the *E1A* oncogene possess a multitude of biological functions (see ref. 15 for a review). They can immortalize primary cells and, in cooperation with other oncogenes, can lead to cellular transformation. The E1A oncoproteins are also transcriptional regulators. They can transactivate specific viral and cellular genes and, conversely, silence the expression of other genes. Some of the functions of E1A have been localized to specific domains of the E1A proteins. Three conserved regions of the E1A oncoproteins have been identified within the various adenovirus serotypes (Fig. 2) (21). Mutational analyses have revealed a function of CR3 (product of 13S mRNA) in transactivation and a function of CR1 and CR2 in transformation and transcriptional regulation (see ref. 14 for a review). Since E1A proteins have been found to associate with various cellular proteins including the 105-kDa retinoblastoma gene product RB and 60-kDa cyclin A, they may exert their effects through these interactions (17,

22). To investigate whether these regions of the E1A protein might mediate transcriptional repression of the ISGs, a genetic analysis was conducted (Fig. 2).

The effect of deletion mutations on the ability of E1A to suppress transcription was determined by cotransfections with the ISRE-TK plasmid. The amino-terminal boundary of the E1A protein required for activity was determined with deletions that truncate various portions of the protein from amino acids 2 through 85 (Figs. 2 and 3A) (17). The E1A protein encoded by the NTdl598 mutation was still competent in repression of the IFN signal transduction pathway. However, activity of NTdl646 was slightly impaired, and repression by NTdl814 was totally lost (Fig. 3A, lanes 5–8). To further delineate this functional domain, internal deletions were tested (Figs. 2 and 3B) (17). Mutations removing amino acids 69–85 (dl793N and dl763N) were still functional, but removal of amino acids 30–85 (dl646N) resulted in loss of activity. Therefore, a functional domain required for repression of the ISGs resides between amino acids 29 and 69 of the E1A protein, a region including a portion of CR1. Carboxyl-terminal deletions were also examined (Fig. 2) (16). These carboxyl-terminal mutations (dl975/dl1339, dl891/dl1339, and CTdl931) maintained the repressive function of E1A protein (data not shown).

To ensure that the truncated E1A proteins expressed from the transfected plasmids localized to the nucleus and associated with cellular proteins as in an adenoviral infection, immunofluorescence and immunoprecipitation studies were performed. These analyses utilized the monoclonal antibody M73 that recognizes E1A protein (23). Nuclear immunofluorescent staining was observed for E1A proteins able to repress the ISGs (wild type, NTdl598, and dl922/dl947) and for a truncated E1A protein that does not repress the ISGs (NTdl814) (data not shown). Therefore, the failure of NTdl814 to repress transcription is not a result of inappropriate cellular localization.

The *E1A* mutations used in this study were defective for binding to one or more cellular proteins. Amino acids 1–76 of the E1A protein are required for interaction with the 300-kDa protein, and amino acids 121–127 are required for interaction with the 107-kDa protein. Association with the 105-kDa retinoblastoma gene product RB or the p60 cyclin A requires two stretches of amino acids 30–60 and 121–127 in the E1A protein (17, 40). Specific protein interactions were verified by coimmunoprecipitation experiments with cells transfected with the *E1A* mutations (data not shown). Since transcriptional repression was observed with *E1A* mutations that fail to bind to the cellular proteins, these specific interactions do not appear to be involved in the ability of E1A to repress the ISGs.

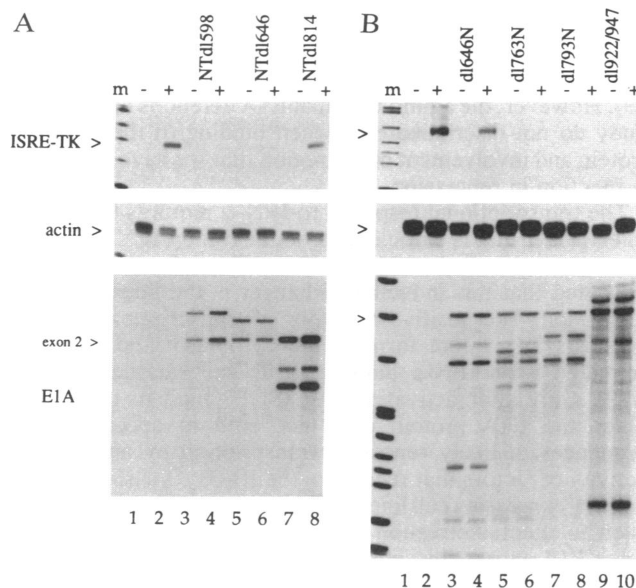

FIG. 3. Domains of the E1A protein required for transcriptional repression. Cotransfection experiments were performed with the ISRE-TK reporter construct and various *E1A* mutations as described in Figs. 1 and 2. Positions of the protected RNA fragments are noted for the ISRE-TK (202 nt), γ actin (135 nt), and the E1A second exon (405 nt).

An *E1A*-Expressing Cell Line Has an Altered Response to IFN. Since transfection techniques introduce DNA into only a percentage of cells, a cell line that constitutively expresses *E1A* was analyzed to further study the mechanism of repression. The response to IFN-α was measured in a parental cell line HT-1080, and a stable *E1A* transformant HT2a (18). mRNA isolated from control or treated cells was hybridized to specific antisense probes corresponding to ISG15, actin, or E1A. Stimulation of ISG15 transcription was found to be impaired significantly in the HT2a cell line (Fig. 4A).

Transcriptional stimulation by IFN-α requires the activation of a latent ISRE binding factor, ISGF3 (7–9). To understand the effect of E1A protein on the signal transduction pathway mediated by IFN-α, activation of ISGF3 was examined in the HT-1080 and HT2a cell lines. Gel mobility shift analyses were performed with nuclear protein extracts from control and treated cells to detect the ISRE-binding factors (Fig. 4B). The ISRE is recognized by at least three distinct DNA-binding factors (7, 19). One of the factors, ISGF1, is present constitutively in control and IFN-α-treated cells. The appearance of another factor, ISGF2/IRF1 (interferon regulatory factor 1), is induced by IFN-α but requires new protein synthesis (24, 25). Since protein synthesis is not required for transcriptional stimulation by IFN-α, this factor does not play a primary role. In contrast, induction of ISGF3 by IFN-α does not require new protein synthesis and appears to be the positive regulator of transcription (7).

The constitutive ISRE binding factor, ISGF1, was detected in all of the extracts tested and served as an internal control for the quality of the protein preparation (Fig. 4B). However, induction of ISGF3 and ISGF2/IRF1 by IFN-α was prominent in the HT-1080 cell extract (lane 2) but was barely detectable in the HT2a extract (lane 4). Therefore, inhibition of ISG transcription in the HT2a cell line correlated with an inhibition of ISGF3 activation by IFN-α.

Since ISGF3 preexists in a latent form in the cytoplasm, it was possible that translocation of activated ISGF3 to the nucleus might be impaired in the HT2a cells. For this reason cytoplasmic extracts were examined for the appearance of ISGF3 after IFN treatment (Fig. 4C *Upper*). Cells were treated with IFN-α, γ IFN (IFN-γ), or both. Previous studies have shown that ISGF3 is not induced by IFN-γ alone but that pretreatment with IFN-γ leads to an increase in the appearance of ISGF3 and the transcriptional response of ISGs to IFN-α (26). The appearance of ISGF3 in the cytoplasm was seen in IFN-α-treated HT-1080 cells (lane 2), and the stimulatory effect of pretreatment with IFN-γ upon ISGF3 activation was clearly evident (lane 4). In contrast, the cytoplasmic extracts prepared from HT2a cells did not contain detectable ISGF3 even under conditions of treatment with both types of IFN (lane 8). Therefore, cytoplasmic activation of ISGF3 was impaired in HT2a cells, not merely nuclear translocation.

The stimulatory effect of IFN-γ upon activation of ISGF3 by IFN-α has been studied *in vitro* with cytoplasmic mixing experiments that mimic the synergistic response (9, 26, 27). These and other studies have revealed that ISGF3 is a multicomponent factor (27, 28). The synthesis of one constituent is induced by IFN-γ and is termed "ISGF3-γ." The other constituent is composed of at least three proteins and is termed "ISGF3-α." The amount of the ISGF3-γ compo-

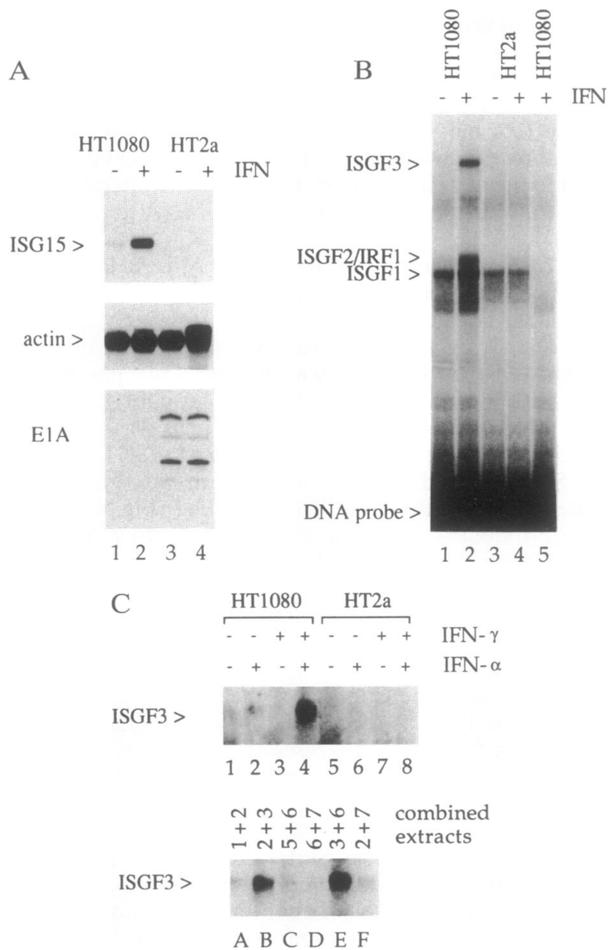

FIG. 4. Inhibition of the IFN-α signal transduction pathway in an *E1A*-expressing cell line. (*A*) Comparison of IFN-induced expression of endogenous ISG15 mRNA in the parental HT-1080 fibrosarcoma cell line and an HT2a cell line expressing the *E1A* oncogene (18). Cytoplasmic mRNA was isolated from untreated cells (lanes 1 and 3) or cells treated with IFN-α for 2 hr (lanes 2 and 4). Levels of ISG15, actin, and E1A mRNAs were measured by specific hybridization to antisense RNA probes. (*B*) IFN-α induction of nuclear ISRE-binding factors. Gel mobility-shift analyses were performed with nuclear extracts prepared from HT-1080 and HT2a cells either untreated (lanes 1 and 3) or treated with IFN-α for 2 hr (lanes 2, 4, and 5) (7, 19). The DNA binding reaction used a probe generated from the ISG15 promoter (10). Specific competitor ISG15 DNA containing the ISRE (100-fold excess) was included in a binding reaction to demonstrate ISGF specificity (lane 5). (*C Upper*) IFN activation of latent cytoplasmic ISGF3 in HT-1080 or HT2a cells. Cytoplasmic extracts were prepared from control cells (lanes 1 and 5), cells treated with IFN-α for 1 hr (lanes 2 and 6), cells treated with IFN-γ for 15 hr (lanes 3 and 7), or cells treated with both IFN-γ and IFN-α (lanes 4 and 8). Protein extracts were incubated with a labeled ISRE oligonucleotide probe and analyzed by gel mobility-shift electrophoresis. (*C Lower*) Reconstitution of ISGF3 activity by complementation of cytoplasmic extracts *in vitro*. Equal amounts of protein from cytoplasmic extracts described in *C Upper* were mixed in the binding reactions. The two numbers above each lane correspond to extracts in specific lanes of *C Upper*.

nent is a rate-limiting factor for ISGF3 activation in many cell lines. Therefore, the synergistic effect of treatment with IFN-γ and IFN-α on the appearance of ISGF3 is due to an increase in ISGF3-γ levels by IFN-γ. To further elucidate the inhibitory influence of E1A on the activation of ISGF3, cytoplasmic mixing experiments were performed with HT-1080 and HT2a extracts (Fig. 4*C Lower*). Mixing of the separately treated IFN-α and IFN-γ extracts from HT-1080 cells generated a synergistic response of ISGF3 (lane B). As expected, mixing of the separately treated IFN-α and IFN-γ extracts from HT2a cells did not lead to an increase in ISGF3 (lane D). However, adding the IFN-γ extract from HT-1080 cells to the IFN-α extract from the HT2a cells led to a reconstitution of ISGF3 (lane E). These results indicate that the ISGF3-α component in HT2a cells is functional, and that the deficiency in ISGF3 activation is due to a nonfunctional ISGF3-γ component. The fact that the ISGF3-α component is activated after IFN-α treatment of HT2a cells also ensures that the IFN-α receptor is functional in these cells.

DISCUSSION

This study describes the ability of a viral oncogene to block the signal transduction pathway mediated by IFN-α. The adenoviral *E1A* oncogene can cooperate with other oncogenes to fully transform cells and encodes a multifunctional protein that can immortalize cells, activate or repress transcription, and induce cellular DNA synthesis and mitosis (see ref. 15 for a review). Some of the biological functions of E1A have been localized to specific domains within the protein, and some of these domains have been shown to be required for association with cellular proteins (17). Cotransfection analyses with *E1A*-deletion plasmids used in this study have defined a region of the E1A protein (amino acids 29 through 69) that is involved in transcriptional repression of the ISGs. This protein region overlaps a major portion of the CR1 domain. The CR1 domain also appears to be required for the negative regulation of the insulin enhancer, muscle-specific enhancers, and the phorbol ester response element (29–31). However, E1A can elicit either positive or negative effects on the phorbol ester response element, apparently dependent upon promoter context and cell type (18, 32–35). Analyses of enhancer repression in other systems have either suggested a role for both CR1 and CR2, or have generated results that do not precisely map an E1A region critical for activity (refs. 34 and 35 and references therein). The E1A protein binds to several characterized cellular proteins and may exert its effects through such interactions. Our studies with specific *E1A* mutations do not suggest the involvement of previously characterized *E1A*-binding proteins in repression of the IFN response. This is in contrast to negative regulation of the insulin enhancer, which has been reported to require a region of the E1A protein that binds to the 300-kDa cellular protein (29). However, the amino-terminal E1A deletions used in that study do not discriminate between binding to the 300-kDa protein and involvement of the region that we have identified to function in repression.

The transcriptional response to IFN-α requires the ISRE, an enhancer that is sufficient for induction by IFN-α (7, 19). Transfection analyses with an ISRE-TK construct have demonstrated that this inducible enhancer is the target of E1A repression. The positive regulator of transcription, ISGF3, preexists in a latent form in the cytoplasm and becomes activated upon IFN-α binding to its cell surface receptor (7–9). Only after activation can ISGF3 bind to the ISRE. Since the E1A protein does not bind to specific DNA sequences, it likely represses transcription by altering the activity of factors that recognize the ISRE. Our studies with an *E1A*-expressing cell line, HT2a, have provided evidence of such an effect. Activation of ISGF3 by IFN-α is defective in these *E1A*-expressing cells. *In vitro* complementation studies with extracts from HT-1080 and HT2a cells have identified a deficiency in a specific component of ISGF3 designated "ISGF3-γ." This deficiency could reflect an impairment in ISGF3-γ gene expression and/or protein function since the *in vitro* reconstitution assay of ISGF3 binding is at this time the only test for ISGF3-γ activity (9, 22, 27). Although the presence of E1A protein in HT2a cells correlates with a

functional inhibition of ISGF3-γ, future studies are needed to establish a specific cause and effect relationship.

The *E1A* oncogene has been shown to repress transcriptional activity of a number of enhancer elements (ref. 35 and references therein). The mechanism of transcriptional repression by E1A may be unique for specific enhancer elements, or alternatively may involve a shared consequence of E1A action. The modification of transcription factors by phosphorylation has been described in many systems including those that positively respond to E1A action (36–38). Since protein kinase activity appears to be necessary for ISGF3 activation by IFN-α, negative regulation by E1A may be mediated through such protein modifications (10). E1A can also physically interact with a number of cellular proteins and may in this manner activate or inhibit transcription factors (39, 41–44). Future studies should impart insight into the mechanism of transcriptional repression by E1A, and provide a better understanding of the signal transduction pathway utilized by interferon.

We thank our colleagues for helpful discussions, Drs. Deborah French, Patrick Hearing, and James Quigley for reading of the manuscript, and Michele Greenless for technical assistance. We are grateful to Dr. Ed Harlow for kindly supplying E1A deletion plasmids and E1A monoclonal antibodies for these studies, and Dr. Steven Frisch for generously providing the HT2a cell line. Human recombinant interferon αA was a gift of Dr. Michael J. Brunda of Hoffmann-LaRoche. This work was supported by grants from the American Cancer Society (JFRA-300) and the National Institutes of Health (R29CA50773) to N.C.R.

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