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Transcription of Interferon-Stimulated Genes Is Induced by Adenovirus Particles but Is Suppressed by E1A Gene Products

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Interferon treatment of cell cultures results in the rapid transcriptional induction of a specific set of genes. In this paper we explore the effect of cellular infection by several adenoviruses, both wild type and mutant, on the expression of these genes. Infection with adenovirus induces the transcription of the interferon-stimulated genes in the absence of any protein synthesis. In fact, the inhibition of protein synthesis during a wild-type infection produces enhanced stimulation of transcription of these genes. Experiments with viral mutants indicate the ability to specifically suppress this transcription maps to the E1A gene. In addition, the E1A gene products are capable of suppressing the specific transcriptional induction of interferon-stimulated promoters during cotransfection experiments and therefore presumably during viral infection. The dual effect of adenovirus on the expression of interferon-stimulated genes may represent an example of action and evolutionary reaction between virus and host.

Exposure of cells to interferon induces an antiviral state in which the replication of a wide variety of both DNA and RNA viruses is inhibited (21). Interferon is not directly antiviral but depends on active cellular RNA and protein synthesis for its effect. After interferon binds to its specific cell surface receptor, the antiviral state takes several hours to develop. The antiviral state then disappears within a day or two. During the establishment of the antiviral state a set of cellular genes, termed interferon-stimulated genes (ISGs), are transcriptionally induced. The actions of the protein products of these genes are believed directly or secondarily to decrease the translation of viral mRNAs and possibly the synthesis of viral mRNAs (2, 6, 18). Adenovirus replication, however, is not affected by interferon treatment of cells. This is thought to be at least partially due to the action of the virus-associated (VA) RNAs. The VA RNAs suppress a kinase activity that phosphorylates and thereby inhibits a translation initiation factor, eIF2-a (17, 32; R. Schneider, personal communication).

In this paper we present evidence that adenovirus particles themselves induce the transcription of genes normally stimulated by interferon (ISGs). This induction does not appear to result from interferon synthesis and does not require the synthesis of new adenoviral proteins. In addition, the immunity of adenovirus to interferon may not be due only to the above-mentioned action of VA RNA. The E1A proteins synthesized during wild-type infection appear to substantially decrease the viral transcriptional induction of these cellular genes. This suppressive effect of E1A was also demonstrated in a transient transfection assay.

MATERIALS AND METHODS

Cells and viruses. HeLa cell cultures CCL2 and CCL2.2 were obtained from the American Type Culture Collection and were maintained in suspension or as monolayers (5 or 10% fetal bovine serum, respectively). Adenovirus type 5 mutants *dl*312 and *dl*331 were obtained from T. Shenk, and *dl*334 was obtained from R. Schneider. The adenovirus type 2 E1A mutants *pm*975 and *dl*1500 were provided by A. Berk (28). Viruses were grown and purified as described previously (27). Recombinant human alpha interferon was generously supplied by S. Pestka (Hoffman-LaRoche Inc.) and used at a concentration of 1,000 U/ml.

Infection and mRNA analysis. Adenovirus infections were performed with 1,500 to 2,000 virion particles per cell (30 to 40 PFU per cell). Cytoplasmic RNA was isolated from the cells after lysis in buffer containing 0.5% Nonidet P-40 (27). Radiolabeled RNA probes used in the hybridization analyses were synthesized with either bacteriophage T7 or Sp6 RNA polymerase from pGem plasmids (Promega Biotec Co.) in the presence of 100 μCi of [^{32}P]UTP (3,000 Ci/mmol; New England Nuclear Corp.) (25). The ISG56 gemini plasmid corresponded to an *Eco*RI-*Bgl*II subclone of the cDNA isolated from diploid fibroblasts (20) and protected a 400-nucleotide RNA fragment. The ISG54 clone corresponded to an *Eco*RI restriction fragment of the genomic clone (22) and protected a 600-nucleotide RNA product. Hybridization was performed with 20 μg of RNA in a 20-μl reaction with 80% formamide at 63°C for 12 to 16 h (24). Hybrids were digested with 50 U of T2 RNase per ml at 30°C for 1 h and were analyzed by electrophoresis in 8% polyacrylamide–8 M urea gels.

Nuclear run-on assays. Nuclear run-on assays were performed as previously described (20) with 5×10^7 nuclei in a 200-μl reaction volume containing 200 to 250 μCi of [^{32}P]UTP. After incubation at 35°C for 10 min, nuclei were treated with DNase, and ^{32}P-labeled RNA was extracted by the hot phenol technique (38). Labeled RNA ($\sim 10^7$ cpm) was hybridized for 24 h at 65°C to various DNA samples affixed to nitrocellulose filters. The following DNA plasmids were used: pBR322 or pGem, ISG56 cDNA clone, ISG54 *Eco*RI 600-base-pair genomic clone, ISG15 3′ Pst genomic clone (34a), a chicken *b*-actin cDNA clone (obtained from D. Cleveland), and a human *b*-tubulin genomic clone (obtained from N. Cowan).

Transfections. Calcium phosphate coprecipitates containing plasmid DNAs were added to monolayer cells at ~40% confluence (43). At 72 h after the addition of the precipitate, one transfection culture was treated with interferon for 5 h. Cells were harvested lysed in 0.5% Nonidet P-40 and cytoplasmic polyadenylated RNA was isolated by oligo(dT) chromatography (24).

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RESULTS

Effect of adenovirus infection on ISG mRNA levels. A number of genes which are transcriptionally induced upon treatment with interferon have been isolated and characterized (4, 7, 9, 16, 20, 26, 41). Two of these genes, ISG54 and ISG56, are stimulated by alpha and beta interferon and encode polypeptides of 54 and 56 kilodaltons, respectively (20, 22). We examined the effect of adenovirus infection upon the expression of the ISG54 and ISG56 genes, first by analyzing cytoplasmic mRNA levels (Fig. 1). Total cytoplasmic RNA was isolated from cells and hybridized with labeled antisense RNA probes synthesized with SP6 polymerase and complementary to ISG54 or ISG56. The hybrids that were resistant to RNase T2 treatment were analyzed on polyacrylamide gels.

Mock-infected HeLa cells contained little or no ISG54 or ISG56 mRNA but after 2.5 h of interferon treatment produced amounts of mRNA which could be easily detected (Fig. 1, lanes 1 and 2). Cells which were not treated with interferon but were infected with adenovirus mutant *dl*312 for 7 h contained nearly as much ISG mRNA as the interferon-treated cells (lane 5). The *dl*312 mutant does not encode the E1A viral proteins because of a large deletion removing most of the gene (15). Interferon treatment of *dl*312-infected cells (lane 6) resulted in an increased level of ISG mRNAs compared with that seen with virus alone and slightly more than seen with interferon treatment alone.

The stimulatory effect of adenovirus on the expression of the ISG mRNAs was also seen upon infection with wild-type virus (lane 3). However, the induction of ISG mRNAs was not as great as that caused by the *dl*312 virus. In addition, interferon treatment of wild-type infected cells (lane 4) failed to yield as much mRNA as interferon treatment alone. The presence of the viral E1A product(s) therefore seems to inhibit the accumulation of the ISG mRNAs. (Hybridization of mRNA with an RNA probe complementary to the 5′ end of the ISG54 demonstrated usage of the correct transcriptional start site for the mRNAs seen in Fig. 1 [data not shown].)

Transcriptional induction of the ISGs by viral infection. To determine whether the effect of adenovirus infection upon the ISG mRNA levels was due to an increase in the transcriptional activity of the genes, we analyzed the synthesis of nascent RNA in an in vitro run-on transcription assay. HeLa cells were infected with *dl*312 virus for 1.25, 3, or 7 h. A sample was also infected for 7 h with interferon treatment during the last 2 h. Nuclei were isolated from each cell culture and allowed to elongate nascent RNA chains in the presence of [α-^{32}P]UTP. The radiolabeled RNA was then hybridized to various DNA samples affixed to nitrocellulose filters (Fig. 2). Quantitation of transcription assays was carried out by the method of Clayton et al. (8). Three ISGs were assayed, ISG54, ISG56, and ISG15 (4, 34a), and actin and tubulin genes served as controls for transcription of genes not affected by interferon. After 1.25 h of infection no stimulation was seen, but by 3 h of infection a strong transcriptional signal was found for all three ISGs. The transcription rate at 7 h was even higher than that at 3 h (approximately fourfold). The sample which was infected for a total of 7 h and received interferon treatment during the last 2 h of infection showed a further stimulated signal (about 1.5-fold, determined by densitometric scanning of the autoradiograms).

From these results we concluded that adenovirus infection specifically induced the transcription of at least three ISGs. However, the virus did not appear to act directly upon the specific interferon receptor. The kinetics of transcriptional induction by viral infection was slower than that produced by interferon treatment (Fig. 2B). Within 15 min after interferon addition, transcriptional induction by the ISGs was nearly maximal. After 6 h of interferon treatment, the transcription rate began to decline. This contrasted with the time course by induction of virus infection, where little stimulation of transcription was observed 1.25 h after infection.

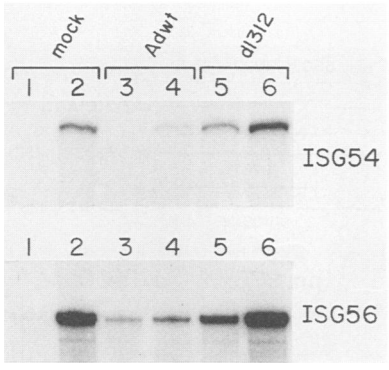

FIG. 1. ISG mRNA levels after interferon treatment or adenovirus infection. HeLa cells (CCL2.2) were either mock infected (lanes 1 and 2) or infected with wild-type adenovirus type 5 (lanes 3 and 4), or *dl*312 adenovirus (lanes 5 and 6) for 7 h. Interferon was added to one set of the cultures during the last 2.5 h of infection (lanes 2, 4, and 6). Cytoplasmic RNA was hybridized to a radiolabeled RNA probe complimentary to the ISG54 or the ISG56 as described in Materials and Methods. RNase-resistant hybrids were electrophoresed in polyacrylamide gels.

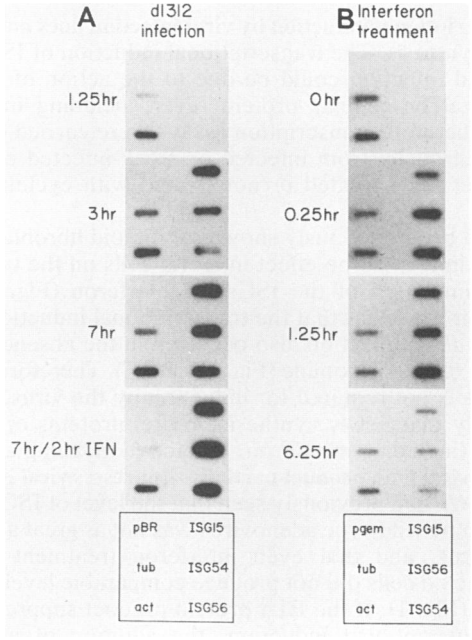

FIG. 2. Transcriptional induction of the ISGs during infection with *dl*312 adenovirus. (A) Nuclear run-on transcription assays were performed at various times after *dl*312 infection as indicated (1.25, 3, and 7 h and 7 h with 2 h of interferon treatment). (B) Similar transcription analysis at various times after treatment of cells with interferon (0, 0.25, 1.25, and 6.25 h). The patterns of the DNA samples on the filters are shown below the respective autoradiograms.

Transcriptional induction by viral infection does not require protein synthesis. The transcriptional induction of ISGs seen after viral infection could be due to the action of a newly made viral or cellular protein (even including interferon itself). Therefore transcription assays were carried out with HeLa cell nuclei from infected or mock-infected cells that had either been treated or not treated with cycloheximide (Fig. 3).

As had been previously shown for diploid fibroblasts (19), cycloheximide had no effect in HeLa cells on the transcriptional stimulation of the ISGs by interferon (Fig. 3C). In addition it was clear that the transcriptional induction of the ISGs by *dl*312 infection also occurred in the absence or the presence of cycloheximide (Fig. 3D and E). Therefore protein synthesis is not required for induction by the virus, making it unlikely that newly synthesized viral proteins or cellular proteins (including interferon) are active inducing agents.

Adenoviral E1A product partially suppresses viral induction of ISGs. We had previously seen that the level of ISG mRNA induction by wild-type adenovirus was not as great as that by *dl*312 virus, and that even interferon treatment of wild type-infected cells did not produce comparable levels of ISG mRNAs (Fig. 1). If the E1A protein product suppressed the viral transcriptional induction, the addition of a protein synthesis inhibitor such as cycloheximide should dramatically increase the transcriptional response.

Nuclear run-on transcription assays were performed with nuclei from mock-infected cells and adenovirus wild type-infected cells in the presence or absence of cycloheximide. Inhibition of protein synthesis in the wild type-infected cells caused at least a 10-fold stimulation of the ISG transcription (Fig. 4). This is in contrast to a negligible effect of cycloheximide on the transcriptional induction by *dl*312 infection (Fig. 3). A protein which is synthesized during a wild-type adenovirus infection therefore appears to have a suppressive effect on the transcriptional induction of the ISGs. Since the *dl*312 virus lacks the E1A gene and does not restrain the transcriptional induction of the ISGs, it seems likely that the E1A protein products act directly or indirectly (stimulation or repression of other genes) to inhibit the maximal transcriptional induction of the ISGs.

The adenovirus E1A primary transcript is processed by alternative RNA splicing into several mRNA species. Two of these species, the 12S and 13S mRNAs, encode closely

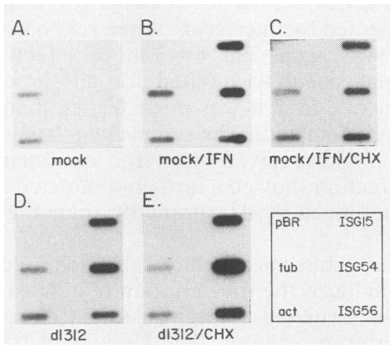

FIG. 3. Transcriptional induction of the ISGs by interferon or *dl*312 virus in the presence of cycloheximide. Nuclear run-on transcription assays were performed on HeLa cells (CCL2.2) which were mock infected (A, B, C) or infected with *dl*312 virus for 6 h (D, E). Interferon was added for the final 2 h (B, C), and 50 μg of cycloheximide per ml was included for the entire 6 h (C, E). The patterns of DNA samples on the filters are shown by the autoradiograms.

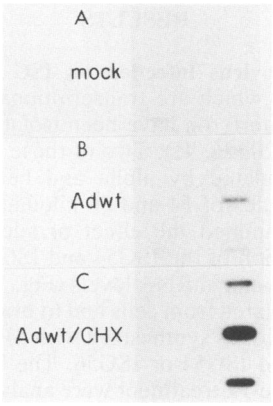

FIG. 4. Inhibition of protein synthesis during wild-type adenovirus infection dramatically increases the ISG transcription rate. Nuclear run-on transcription assays were performed on mock-infected HeLa cells (CCL2.2) (A) or cells infected with wild-type adenovirus type 5 for 6 h (B). Cycloheximide (50 μg/ml) was included at the beginning of the wild-type adenovirus infection of one set of cultures (C). The patterns of DNA samples on the filters are as in Fig. 3.

related proteins of 243 and 289 amino acids, respectively (33). These proteins are identical except for an additional internal 46 amino acids in the larger protein. Both proteins are important for oncogenic transformation by the virus (28, 44). However, the 12S mRNA product induces a dramatically higher frequency of transformation than the 13S mRNA product (23, 28, 29, 37). To distinguish between the roles of the 12S or 13S mRNA products in transcriptional suppression of the ISGs, we performed experiments with viral mutants that could synthesize only one of the two E1A products. The point mutant *pm*975 directed the synthesis of only the 13S mRNA product, whereas the deletion mutant *dl*1500 directed the synthesis of only the 12S mRNA product (Fig. 5) (28). HeLa cells were infected with either viral mutant for 6 h and analyzed for transcription of the ISGs by nuclear run-on assays (Fig. 5). Infection of cells with *pm*975 ($13S^+$ $12S^-$) resulted in a transcriptional stimulation of the three ISGs. However, infection with *dl*1500 ($13S^-$ $12S^+$) showed only a weak transcriptional signal for the ISGs. This

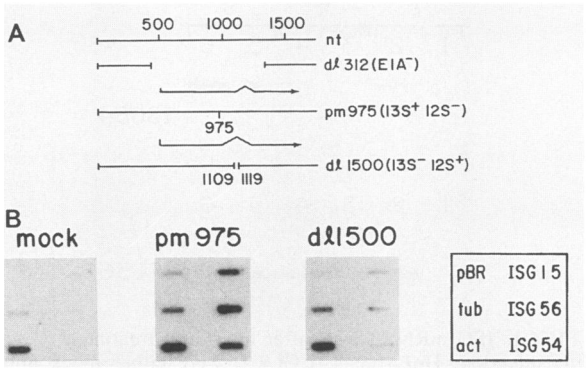

FIG. 5. Suppression of ISG transcription by the E1A 12S mRNA product. (A) Locations of the mutations in the E1A gene of the viral mutants *dl*32, *pm*975, and *dl*1500 (5, 28) relative to the nucleotide (nt) position in the adenoviral genome. The structures of the 12S and 13S mRNAs are also shown (arrowheads). (B) Results of nuclear run-on transcription assays performed with mock-infected cells, *pm*975-infected cells, or *dl*1500-infected cells.

suggests that the protein product of the 12S mRNA is responsible for transcriptional suppression of the ISGs by the E1A gene.

Interferon induction of the ISG15 mRNA is inhibited by E1A in a cotransfection. To assess more directly the inhibitory effect of the E1A gene products on interferon induction, we analyzed the induction of an ISG in a transient transfection. A recombinant plasmid was constructed by joining the upstream region of the ISG15 (34a) to the 3′ end of the adenovirus type 5 E1B gene (Fig. 6A). The 3′ portion of the E1B gene contributed the proper polyadenylation signals for mRNA processing and allowed the hybrid RNA to be distinguished from that of the endogenous gene. The ISG15 hybrid construct was transfected into monolayer cultures of HeLa cells. After 72 h one transfection culture was treated with interferon for 5 h, whereas another was left untreated. Cytoplasmic poly(A)$^+$ mRNA was isolated from the various cell cultures and hybridized to an antisense RNA probe complementary to the 5′ region of the transfected recombinant ISG15 gene. Interferon treatment clearly induced the ISG15 mRNA synthesized from the transfected gene (Fig. 6B, lanes 1 and 2).

When a plasmid containing the entire adenoviral E1A gene (nucleotides 1 through 1774) was cotransfected with the ISG15 hybrid, a significant reduction in the interferon-stimulated mRNA signal was seen (~10-fold) (Fig. 6B, lanes 3 and 4). These results together with the experiment of Fig. 5 indicate that the adenovirus 12S E1A gene product inhibits the interferon induction of the transfected ISG15. The decrease in ISG15 mRNA does not appear to be due merely to promoter competition, since cotransfection with the simian virus 40 enhancer-stimulated β-globin promoter or the adenovirus EIII promoter had no effect (data not shown). The ISG element responsive to repression by E1A must reside within the upstream region of the ISG15 which was used in the construction of the ISG15-E1B recombinant (nucleotides −366 to +40). Similar experiments with another interferon-stimulated promoter, ISG54 (22), also showed suppression by the E1A gene in a cotransfection (data not shown). Figure 6C, showing endogeneous gene function, is a control to show that interferon induction of chromosomal genes occurred in both sets of transfected cells. Because only a small minority of cells are transfected in such experiments, the E1A protein (Fig. 6C, lane 4) would not be expected to decrease the signal, since most cells in fact contain no E1A protein.

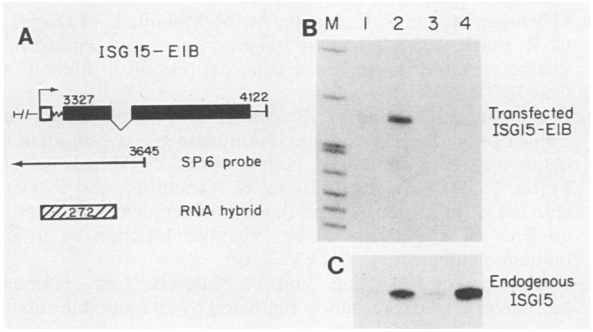

FIG. 6. Suppressive effect of adenovirus E1A upon the transcription of a cotransfected ISG15 hybrid construct. (A) Schematic representation of the ISG15-E1B hybrid construct. The 5′ portion of the ISG15 (positions −366 to +40, □) was fused with the 3′ region of the adenovirus type 5 E1B gene (■) by means of the pGem polylinker. Hybridization of mRNA with an SP6 probe complementary to the transcription start site of the fusion gene protects a fragment of 272 nucleotides. Numbers above the lines refer to nucleotide positions within the E1B gene. (B) HeLa cells (CCL2.2) (150-mm plates) were cotransfected with 25 μg of the ISG15 E1B plasmid and 25 μg of pGem (lanes 1 and 2) or cotransfected with 25 μg of the ISG15 E1B plasmid and 25 μg of the adenovirus E1A gene (lanes 3 and 4). At 72 h after transfection one set of cultures was treated with interferon for 5 h (lanes 2 and 4). Cytoplasmic poly(A)$^+$ RNA was isolated from the transfections and hybridized to the ISG15 E1B RNA probe (B). *Hpa*II restriction fragments of pBR322 served as molecular weight markers (lane M). Expression of the endogenous ISG15 gene was measured by hybridization to an RNA probe complementary to the 3′ portion of the gene (*Pst*I-*Taq*I) protecting a hybrid of 213 nucleotides (C).

DISCUSSION

Transcriptional activation by interferons is both rapid and specific. Within minutes of treatment there is a dramatic increase in the number of RNA polymerases transcribing a specific subset of genes. Little is known of the mechanism by which any receptor-mediated signals are transduced to the nucleus. Only one ISG, metallothionein II, has been reported to transcriptionally respond to other specific inducers (Zn^{2+} or dexamethasone) (10). The remainder of the known ISGs have only been shown to transcriptionally respond to interferons. In the present work, however, three different ISGs have been found to exhibit dramatic transcriptional induction during infection with an adenovirus mutant, *dl*312.

The mechanism by which adenoviral infection induces the transcription of interferon induced genes remains unclear. Direct interaction of the viral particle with the interferon receptor at the cell surface does not appear likely. The kinetics of ISG induction by adenovirus is comparatively much slower than that by interferon (3 h versus 15 min; Fig. 2), and adenovirus particles do not compete with interferon for binding to its receptor (L. Pfeffer, personal communication). These facts do not, however, rule out the possibility of an indirect effect of adenovirus on the interferon-stimulated pathway.

It is clear from the experiments with cycloheximide that adenoviral induction of ISGs does not require new protein synthesis (Fig. 3). Thus the stimulatory effect upon transcription cannot be due to the action of a newly made viral or cellular protein product (including interferon). The transcriptional inducing ability of the virus may be due to a component of the virion capsid. It is also possible that the introduction of a relatively large amount of adenoviral DNA into the nucleus of a cell can in some way trigger the specific transcriptional response of the ISGs. Since transcription occurs on both DNA strands of the adenoviral genome, an accumulation of viral RNA or double-stranded RNA could mediate the ISG induction. However, very little viral transcription occurs with a 3-h infection with *dl*312 virus (15, 30). We have also seen induction of ISG transcription within 4 h of infection with mutants deleted in the VAI RNA gene (*dl*331) and in both the VAI and VAII genes (*dl*334) (data not shown). Therefore the VA RNAs, which late in virus infection appear to suppress the activity of an interferon-induced protein kinase (18, 32; R. Schneider, personal communication), do not themselves play a role in transcriptional induction of ISGs.

The most strikingly homologous system of transcriptional induction by a virus is that for the interferon genes themselves. Both alpha and beta interferons are efficiently induced by members of many virus groups (21). This induction may be due to the action of viral double-stranded RNA in most cases, since expression of the interferon genes can also

be induced by synthetic double-stranded RNA (34). The regulatory sequences of the genes which are responsive to the double-stranded RNA or viral inducer are located in the 5'-flanking region of the interferon genes (11, 12, 36). It is possible that a similar regulatory element exists in the ISGs which is responsive to virus infection as well as to interferon treatment. Future experiments with more defined promoter sequences of the ISGs should reveal any shared functional elements.

A second aspect of the transcriptional regulation of ISGs in adenovirus-infected cells was related to suppression of transcription by the adenovirus E1A gene products. The role of E1A was first suggested by adenovirus infection experiments in the absence or presence of cycloheximide. Cycloheximide had little effect on the transcription of the ISGs after infection with a mutant lacking the E1A gene. In contrast, the inhibition of protein synthesis during a wild-type adenovirus infection resulted in at least a 10-fold increase in the ISG transcription rate (Fig. 4). This implied that the E1A proteins could inhibit the transcription of interferon-inducible genes. More definitive evidence of transcriptional repression by E1A was afforded by cotransfection experiments. In these studies the presence of the E1A gene dramatically decreased the interferon-stimulated transcription of the cotransfected ISG15 hybrid (Fig. 6). This negative effect of E1A proteins on the ISG15 is mediated by sequences within the region of positions −366 to +40 of the gene. It will be interesting to determine whether the same short enhancer region responsible for induction by interferon is also the region susceptible to the negative effects of E1A (34a).

The E1A gene products are expressed early after adenovirus infection and have been found to be transcriptional regulators. They have been shown to stimulate the transcription of other adenovirus genes (E1B, EII, EIII, EIV, VA) (14, 15, 30) as well as a number of cellular genes (heat shock, *B*-tubulin) (31, 39). On the other hand, they have also been found to repress the transcription of enhancer-dependent viral genes of simian virus 40 and polyomavirus (5, 42) and some cellular genes encoding insulin and immunoglobulin (13, 40). All of these facts are most likely due to the action of the E1A proteins on preexisting cellular transcription factors. This idea fits the case of ISG induction which is brought on by interferon without the need for new protein synthesis (19). Transcriptional regulation by E1A is believed to be the cause of its complex effect on the growth properties of cells. Expression of the E1A gene can result in the immortalization of primary cells or in a fully transformed phenotype when acting in cooperation with E1B or the *ras* oncogene (3, 35). Since interferon treatment causes an arrest of cell growth as well as an inhibition of the replication of many viruses, it may not be surprising to find that E1A acts to suppress the interferon stimulated genes.

The dual effect that adenovirus exerts upon the transcription of ISGs is of potential interest in the natural history of adenovirus infections. If attachment of virions alone stimulates the establishment of an antiviral state, then adenovirus infection in nature might be innocuous. However, adenovirus encodes at least two gene products, E1A and VAI, which differentially act to suppress the development of the antiviral state. This suppressive effect of adenovirus has recently been shown to allow replication of a superinfecting virus, vesticular stomatitis virus, in interferon-treated cells (1). The stimulation and subsequent suppression of ISGs by adenovirus is possibly an example of action and evolutionary reaction between host and virus and may explain why adenoviruses are generally regarded as resistant to the action of interferon.

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