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An Adenovirus Type 5 E1A Protein with a Single Amino Acid Substitution Blocks Wild-Type E1A Transactivation

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The E1A gene of adenovirus type 5 encodes a 289-amino-acid (289R) protein that transactivates early adenovirus promoters. We showed that the 289R protein of the E1A missense mutant gene *hr5* is novel in that it inhibits the wild-type (wt) E1A protein from stimulating transcription from each of the early viral promoters E2, E3, and E4. Since both the *hr5* and wt genes produced similar levels of E1A proteins, the ability of *hr5* E1A to block transactivation was attributed to the replacement of serine by asparagine as position 185. We confirmed that this single amino acid substitution was responsible for blocking transactivation by showing equal inhibition with an *hr5*-wt hybrid E1A gene containing this missense mutation as the only alteration. The smaller 243R E1A protein of *hr5* was not necessary for inhibition. Transcriptional activity from each early promoter was inhibited by at least 50% when the *hr5* and wt E1A genes were present in equimolar amounts; complete inhibition occurred with a fivefold molar excess of the *hr5* gene. Two other E1A missense mutant genes (*hr3* and *hr4*) with amino acid substitutions in close proximity to that of *hr5* failed to block wt E1A-induced transcription when similarly tested. Also, the *hr5* E1A gene failed to impede the pseudorabies immediate early gene from transactivating the adenovirus E3 promoter, demonstrating that *hr5* E1A inhibits wt E1A activation at the transcriptional, rather than the posttranscriptional, level. Although several possibilities were considered to account for this inhibition, the most likely is that the nonfunctional *hr5* E1A protein competes with the wt 289R protein for a cellular transcription factor required for transactivation.

Central to knowing how gene expression is controlled in eucaryotic cells is understanding the mechanism by which regulatory proteins activate transcription of other genes. E1A of adenovirus represents one of the few examples of a regulatory gene that encodes proteins which transactivate both viral and cellular genes. In adenovirus-infected cells, E1A gene products facilitate transcription from the promoters of early viral genes (2, 28, 39, 42), as well as certain cellular genes, such as heat shock and β-tubulin (29, 50). Transcription of some cloned cellular genes, including β-globin and preproinsulin, are also stimulated after introduction into cells that express E1A proteins (14, 20, 51). Furthermore, E1A can activate transcription from RNA polymerase III promoters (13, 23). In contrast to its activating functions, E1A proteins can also repress enhancer-stimulated transcription. For example, in the presence of E1A, transcription is repressed from promoters under control of the viral simian virus 40, polyoma-virus, or E1A enhancers (5, 47, 55) or the immunoglobulin heavy chain enhancer (22). The E1A proteins also play an essential role in adenovirus transformation (53). Recently, the E1A proteins of a tumorigenic serotype of adenovirus (adenovirus type 12 [Ad12]) were shown to block expression of class 1 major histocompatibility complex (MHC) genes in both rodent and human cells (4, 9, 54).

Of the several functions inherent to E1A gene products, the best understood is transactivation of early adenovirus promoters. Upon adenovirus type 5 (Ad5) infection of HeLa cells, two overlapping E1A transcripts of 12S and 13S are the first viral mRNAs synthesized (2, 28). These transcripts are 5′ and 3′ co-terminal and share the same acceptor splice site but utilize different donor splice sites (3, 41, 49). In Ad5, the 12S and 13S E1A mRNAs encode 243- and 289-amino-acid (243R and 289R, respectively) phosphoproteins. These E1A proteins are identical except that the larger protein contains an internal stretch of 46 unique amino acids (41). Analysis of E1A frameshift and splice mutants has demonstrated that only the 289R E1A protein facilitates transcription from each of the Ad5 early promoters (E1B, E2, E3, and E4) (39, 42). This suggests that the 46R region that is unique to the larger of the E1A proteins contains information that is essential for transactivation of early promoters. This concept was reinforced by the discovery that three E1A mutants (*hr3*, *hr4*, and *hr5*) defective for transactivation (2) contain distinct, single amino acid substitutions located in the unique region of the 289R E1A protein (16).

Although the mechanism by which the 289R E1A protein stimulates transcription of early adenovirus promoters is unknown, several lines of evidence suggest that the E1A protein interacts with a cellular factor rather than binding directly to DNA. (i) Binding of a putative cellular transcription factor (CTF) to sequences upstream of the adenovirus E2 promoter is greatly facilitated in the presence of E1A both in vivo and in vitro (32, 33). (ii) A specific set of sequence homologies is not observed within the DNA region 5′ proximal to the start site of transcription of E1A-inducible promoters (1). It is important to note that extensive deletion analysis of the Ad5 E2 and E3 promoter regions has failed to reveal an E1A-responsive element that can be separated from promoter elements required for transcription per se (10, 27, 31, 35, 40). (iii) The immediate early (IE) gene of pseudorabies virus and the X gene of human T-cell leukemia virus type II are both able to transactivate adenovirus early promoters (7, 11); it seems unlikely that gene products of such divergent viruses would recognize a common DNA sequence. Collectively, these studies support the concept that one or more cellular factors are intimately involved in E1A-induced promoter activation.

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In this study, we showed that the 289R protein of the E1A missense mutant *hr5* inhibits the wild-type (wt) E1A protein from transactivating early viral promoters. Our experiments also demonstrated that this inhibition occurs at the transcriptional level. Although several possibilities were considered to account for this inhibition, the most likely is that the nonfunctional *hr5* E1A protein competes with the wt 289R protein for a CTF required for transactivation.

MATERIALS AND METHODS

Cells and viruses. HeLa monolayer cells were grown in minimum essential medium containing 8% fetal calf serum. We grew 293 cells (18) in Dulbecco modified Eagle medium with 5% fetal calf serum. Ad5 and host range mutant viruses (*hr3*, *hr4*, and *hr5*) were propagated as described previously (16, 21). The E1A deletion mutant virus *dl*312 (28) was propagated in 293 cells. HeLa cells in 24-well Linbro plates (4×10^5 cells per well) were infected at various multiplicities (see the legend to Fig. 2). Infected cells were labeled after 24 or 48 h with 30 mCi of [^{35}S]methionine (Amersham Corp.; >600 Ci/mmol) for 1 to 2 h. Labeled extracts (400,000 trichloroacetic acid-precipitable counts) were fractionated on a 15% polyacrylamide gel (acrylamide-bis, 30:0.8) followed by fluorography and autoradiography with Kodak XAR-5 X-ray film.

Plasmids. Constructions of the following plasmids have been previously described. p3CAT (pKCAT23) and p2CAT contain the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the E3 or E2 promoter of adenovirus (57); pE1A contains the Ad5 wt E1A gene, and pHR3, pHR4, and pHR5 contain Ad5 missense mutant E1A genes (16); pEKpm975 contains a point mutation which disrupts the E1A 12S RNA donor splice site (39); pIE (pA79) encodes the IE gene of pseudorabies virus (25). The E1A double mutant pHR5-289R was constructed by replacing the *Sac*II-to-*Sma*I fragment (base pair [bp] 354 to bp 1007) of pHR5 with the corresponding E1A fragment from pEKpm975; pHR5-289R synthesizes only the 289R protein of *hr5*. The plasmid p4CAT contains the CAT gene driven by the E4 promoter of Ad5 and was constructed as follows. A plasmid (p*Eco*RI-B) containing sequences from the right end of the Ad5 genome was digested with *Eco*RI and *Alu*I, the terminal 345-bp fragment containing the E4 promoter was isolated, and *Hin*dIII linkers were ligated to the *Alu*I end. The CAT gene was purified as an *Hin*dIII-*Bam*HI fragment from pSV2CAT (17), and the pML-tet plasmid (37) was purified after digestion with *Eco*RI and *Bam*HI. The three fragments (CAT, pML, and E4) were ligated by using standard conditions to generate p4CAT. In p4CAT, the E4 promoter is in the correct orientation to drive transcription of the CAT gene. Transformation of *Escherichia coli* (C600) and purification of plasmid DNA were carried out by using standard procedures (38). Restriction enzyme analysis of plasmid DNAs from minilysates was by the method of Holmes and Quigley (24). Isolation of DNA from agarose gels was by the glass method of Vogelstein and Gillespie (56).

Transfection and CAT assay. Transfection of plasmid DNAs into HeLa cells (60 to 80% confluent) was by the calcium phosphate precipitation method of Graham and van der Eb (19). In transfections involving two or more plasmids, pML was added to correct for quantitative differences so that each plate (100-mm diameter) in a given experiment received the same total amount of DNA. After approximately 48 h, the cells were harvested, and a soluble extract was prepared and assayed for CAT activity as previously described (17, 57). For standardization, CAT assays were performed with extracts containing equal amounts of protein (36). For quantitation of CAT activity, the acetylated forms of [^{14}C]chloramphenicol were scraped from the silica plates and counted by liquid scintillation. All experiments were repeated three to seven times.

Immunoprecipitations. HeLa cells were infected with adenoviruses in the presence of cytosine arabinoside (2 μg/ml) (14) and labeled after 36 h with 100 μCi of $^{32}P_i$ (New England Nuclear Corp.) per ml of phosphate-free medium (GIBCO Laboratories) for 5 to 7 h. HeLa cells transfected with plasmid DNA (10 μg per 100-mm plate) were labeled after 48 h under the conditions described above. Cells were washed, and extracts were prepared as previously described (46). The same number of trichloroacetic acid-precipitable counts was used in the immunoprecipitation of each extract. Extracts were incubated with a 1:20 dilution of Ad5 or Ad12 E1A monospecific antiserum produced against bacterially synthesized Ad5 or Ad12 E1A protein or normal rabbit serum overnight at 4°C and immunoprecipitated with *Staphylococcus aureus* as previously described (46). Immunoprecipitated samples were fractionated on 15% polyacrylamide gels (34), which were fluorographed, dried, and exposed to Kodak XAR-5 X-ray film.

RESULTS

Delayed appearance of adenovirus late proteins in cells coinfected with wt and E1A missense mutants. The E1A host range mutant viruses *hr3*, *hr4*, and *hr5* are defective for growth in HeLa cells (21) but can be propagated in 293 cells which constitutively express E1 gene products (18). In mutant-infected HeLa cells, the 12S and 13S E1A RNAs, which are the only viral transcripts synthesized (2), are translated into full-length 243R and 289R proteins, respectively (42, 43). The E1A genes of *hr3*, *hr4*, and *hr5* each code for a different amino acid substitution in the unique region of the 289R protein as a result of single point mutations (16) (Fig. 1).

In the course of coinfecting wt Ad5 with various E1A mutants, we noted that the appearance of late viral proteins seemed to be delayed in the cases of *hr5* and *hr3*. The diagnostic late viral proteins hexon (II) and penton (III) were observed after 24 h in HeLa cells infected with wt virus alone (Fig. 2A, lane 2) or coinfected with *dl*312 (lane 5), an E1A deletion mutant which fails to produce E1A products (28). In contrast, these late proteins were not detected after 24 h in HeLa cells coinfected with wt virus and either *hr5* or *hr3* (Fig. 2A, lanes 3 and 4, respectively); however, late proteins were visualized at 48 h (data not shown). In similar coinfections, neither the *hr4* E1A missense mutant nor the *hr440* E1A truncation mutant, which produces only the amino-terminal portion of the 289R E1A protein (48), altered the time cource of appearance of late viral proteins (data not shown). To verify that the E1A gene products of these missense mutants were indeed expressed, E1A phosphoproteins were immunoprecipitated from infected HeLa cells (Fig. 2B).

The E1A gene of *hr5* inhibits wt E1A from transactivating early viral promoters. From coinfection experiments, we inferred that the E1A proteins of *hr3* and *hr5* may impede the ability of the wt E1A protein (289R) to facilitate transcription from early viral promoters and thereby delay the kinetics of infection. However, these experiments were complicated by the fact that during infection many viral promoters and gene products are expressed and that examination of late proteins

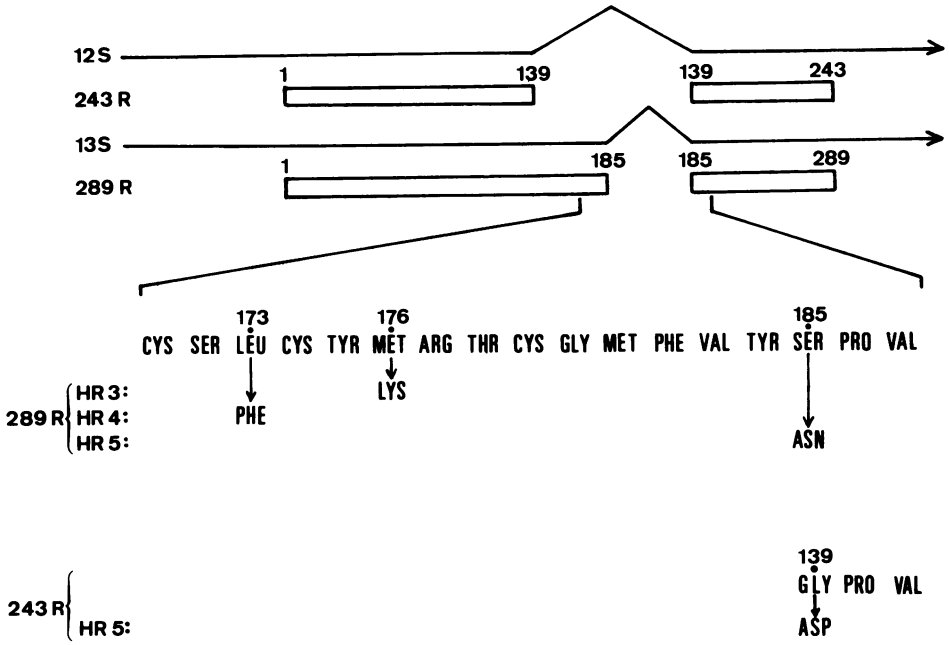

FIG. 1. Representation of the amino acid changes in the E1A proteins of *hr3*, *hr4*, and *hr5*. The E1A mRNAs (13S and 12S) are represented by the thin lines, with arrows indicating the direction of transcription and open bars designating the proteins they encode (289R and 243R). The amino acid substitutions in the E1A proteins of *hr3*, *hr4*, and *hr5* shown here were predicted from the DNA sequence (16). In *hr5*, a simultaneous missense mutation occurs in each protein because the altered nucleotide (1229) serves as the middle base of a split codon for each mRNA that is formed as a result of differential splicing.

represents a stage far removed from activation of early viral genes. Therefore, we used recombinant plasmids to examine directly the potential of the host range mutant E1A genes to interfere with the transactivating ability of the wt E1A gene. Plasmids containing the CAT gene driven by individual early adenovirus promoters E2, E3, and E4 (referred to here as p2CAT, p3CAT, and p4CAT, respectively) are transcribed when cotransfected into HeLa cells with plasmids containing wt E1A (pE1A), but not host range mutant E1A (pHR3, pHR4, and pHR5), DNA (16, 57). High levels of CAT activity were observed when p3CAT was cotransfected with pE1A (Fig. 3A, lane 2) compared with basal levels of CAT activity obtained with p3CAT alone (lane 1). However, when increasing amounts of pHR5 were cotransfected with fixed amounts of pE1A and p3CAT, there was a concomitant decrease in CAT activity before basal levels were reached (Fig. 3A, lanes 3 to 6). In contrast, a decrease in CAT activity was not observed when pHR3 or pHR4 was substituted for pHR5 in similar cotransfections (Fig. 3B, lanes 3 to 6; data not shown). This indicated that, of the three *hr* E1A missense genes, only *hr5* can compete with the ability of the wt E1A gene to transactivate the E3 promoter. Likewise, when these experiments were performed with p2CAT or p4CAT, only *hr5* blocked wt E1A from inducing transcription.

Quantitation of these results (Fig. 4) indicated that transcription from each early promoter (E2, E3, and E4) is inhibited by at least 50% with equimolar amounts of the wt and *hr5* E1A plasmids; complete inhibition of CAT activity was obtained with a fivefold molar excess of pHR5. The fact that E1A mRNAs and proteins were readily detected in cells transfected by pHR3, pHR4, or pHR5 (data not shown) signified that the inhibition of transactivation observed only with pHR5 was a consequence of the protein product(s) synthesized by this E1A missense mutant gene and not a trivial effect of cotransfecting plasmids.

It is important to note that the single mutation at position 1229 bp is solely responsible for enabling *hr5* E1A to compete with wt E1A for transactivation since a hybrid gene containing the middle portion of the *hr5* E1A coding region (107 to 1339 bp) and wt E1A flanking sequences was equally effective in blocking transactivation (data not shown). This middle portion of the *hr5* E1A gene had been completely sequenced and was shown to contain a transition from G to A at nucleotide 1229 as the only alteration (16).

It is unclear why the *hr3* virus appeared to impede the rate of viral infection, whereas the *hr3* E1A plasmid (pHR3) failed to inhibit pE1A from transactivating the early promoter CAT plasmids. It is possible that more of the *hr3* E1A protein is required to obtain inhibition compared with the amount of *hr5* E1A protein. Perhaps more E1A protein is expressed after infection than after transfection, which would explain the discrepancy between infection and transfection with *hr3*.

The 289R protein of *hr5* blocks E1A-mediated transactivation. The single point mutation in *hr5* E1A produces a different amino acid substitution in the 289R and 243R proteins (16). These simultaneous missense mutations occur because the altered nucleotide serves as the middle base of a split codon for each mRNA that is formed as a result of differential splicing (41). In the 289R protein of *hr5*, asparagine replaces serine as the last amino acid in the unique region (residue 185), whereas in the 243R protein, aspartic acid replaces glycine as the last amino acid before the unique region (residue 139) (Fig. 1). Thus, it was not possible to discern which of the two *hr5* E1A proteins inhibits the wt 289R E1A protein from transactivating the early promoters. Therefore, a recombinant molecule, pHR5-289R, which was

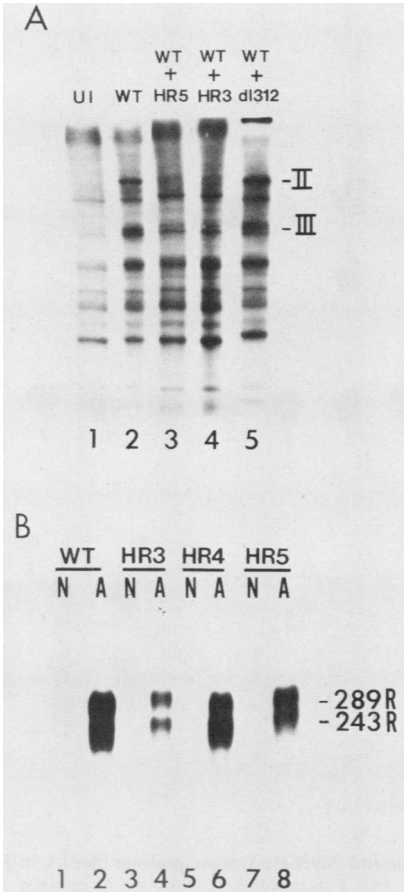

FIG. 2. Coinfections of Ad5 wt and host range mutant viruses. (A) Late viral proteins synthesized in HeLa cells coinfected with wt and host range mutant viruses. HeLa cells were infected with wt Ad5 at a multiplicity of infection of 1 and with *hr*3, *hr*5, and *dl*312 viruses at a multiplicity of infection of 2. Cells were labeled 24 h postinfection with [^{35}S]methionine, and proteins were fractionated on a 15% polyacrylamide gel. The diagnostic late viral proteins hexon (II) and penton (III) are indicated. The uninfected control is shown in lane 1, infection with wt Ad5 alone is shown in lane 2, and coinfections with wt Ad5 and *hr*5, *hr*3, or *dl*312 are shown in lanes 3, 4, and 5, respectively. (B) Immunoprecipitation of ^{32}P-labeled E1A proteins from Ad5 host range mutant-infected cells. Infected HeLa cells were labeled with $^{32}P_i$, and E1A proteins were immunoprecipitated and fractionated on a 15% sodium dodecyl sulfate-polyacrylamide gel. The positions of the 289R and 243R E1A proteins are indicated. Immunoprecipitation was with normal rabbit serum (lanes N) or monospecific Ad5 E1A antiserum (lanes A). Cells were infected with wt Ad5 (lanes 1 and 2), *hr*3 (lanes 3 and 4), *hr*4 (lanes 5 and 6), or *hr*5 (lanes 7 and 8).

capable of expressing only the *hr*5 289R protein, was constructed. This was accomplished by replacing the *Sac*II-to-*Sma*I fragment of pHR5 with the corresponding fragment from pEKpm975. In pEKpm975, a tranversion in the second base of the 12S intron at nucleotide 975 prevents splicing and synthesis of the 12S RNA which encodes the 243R protein but does not alter the 289R protein encoded by the 13S RNA (39). When pHR5-289R and p3CAT were cotransfected into HeLa cells, no CAT activity was observed (Fig. 5A, lane 3). However, CAT activity was readily detected upon cotransfection of pEKpm975 and p3CAT (lane 2). This result established that failure of pHR5 to transactivate early viral promoters is attributable to the asparigine-to-serine substitution in the 289R protein. Of greatest relevance was the fact that pHR5-289R inhibited the wt 289R E1A protein from inducing transcription from the E3 promoter (Fig. 5A, lanes 4 to 7). In fact, the inhibition curve generated with pHR5-289R was nearly identical to that obtained with pHR5, which expresses both E1A missense proteins (compare Fig. 5B with Fig. 4). Verification of E1A expression was by immunoprecipitation of both E1A proteins from HeLa cells transfected with pHR5 (Fig. 5C, lane 4) and only the 289R protein from cells transfected with pHR5-289R (lane 6).

Even though the amino acid substitution in *hr*5 E1A renders the 289R protein incapable of transactivating early viral promoters, this mutant protein apparently retains some partial function which allows it to inhibit the transactivating ability of the wt E1A protein. Although there are other possible explanations, this partial function retained by the 289R E1A protein of *hr*5 likely involves interacting with (e.g., binding to) and titrating out a CTF.

***hr*5 E1A does not inhibit the pseudorabies IE gene from transactivating adenovirus early promoters.** The pseudora-

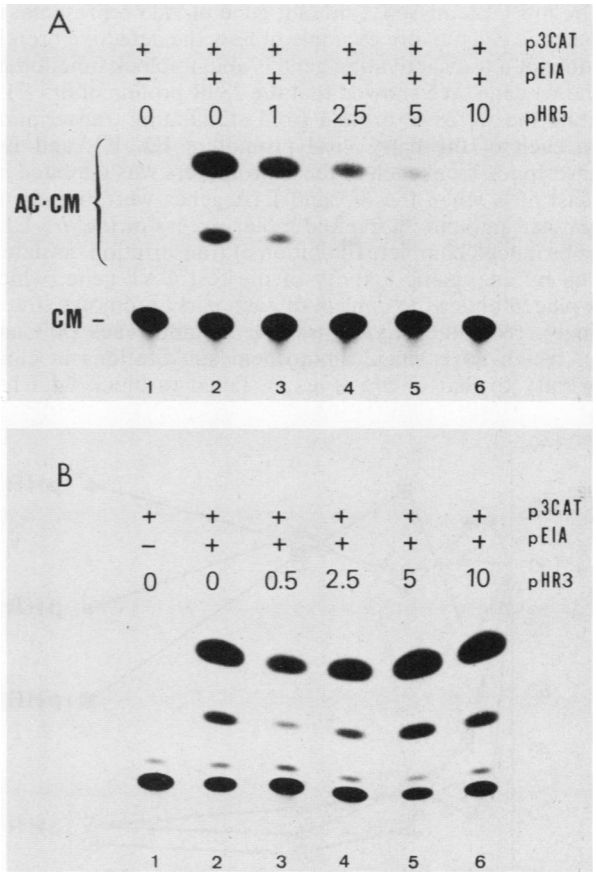

FIG. 3. *hr*5 E1A inhibits the ability of wt E1A to transactivate the E3 viral promoter. (A) Effect of *hr*5 E1A on wt E1A-induced p3CAT activity. HeLa cells were transfected with 5 μg of p3CAT alone (lane 1) or 1 μg of pE1A (lanes 2 to 6). In lanes 3 to 6, increasing amounts of pHR5 DNA (1, 2.5, 5, and 10 μg, respectively) were also added to the cotransfection. To ensure that each plate received the same amount of DNA, appropriate amounts of plasmid pML were added to the cotransfections. CAT assays were carried out 48 h after transfection. [^{14}C]chloramphenicol (CM) and its acetylated forms (AC-CM) are indicated. (B) Effect of *hr*3 on wt E1A-induced p3CAT activity. Cotransfections were carried out as described above except that pHR3 was substituted for pHR5. The amounts of DNA used were the same as in A except that lane 3 contained 0.5 μg of pHR3 in the cotransfection.

bies virus IE protein can also transactivate the early promoters of adenovirus (11). We tested whether the *hr5* E1A gene could also block IE protein-mediated transactivation. High levels of CAT activity were observed when p3CAT was cotransfected with pIE (Fig. 6, lane 2) compared with basal levels of CAT activity obtained with p3CAT alone (lane 1). When increasing amounts of pHR5 were cotransfected with fixed amounts of pIE and p3CAT, no change in the levels of CAT activity were observed (Fig. 6, lanes 3 to 6). This is in sharp contrast to the effect that *hr5* E1A has on wt E1A-induced transactivation. This result rules out the possibility that *hr5* E1A acts at the level of posttranscription, since CAT activity (i.e., CAT mRNA expression) could occur in the presence of pHR5. It will be of future interest to determine whether the separate responses of *hr5* E1A to the wt E1A and IE genes reflect differences in their mechanisms of transcriptional activation (11, 20, 26).

DISCUSSION

The *hr5* E1A missense mutant gene of Ad5 represents, in eucaryotic cells, a rare example of how the defective protein product of a transactivating gene is able to block functioning of the wt gene. We showed that the 289R protein of *hr5* E1A inhibits the wt 289R protein from stimulating transcription from each of the early viral promoters E2, E3, and E4. Transcription from each of these promoters was inhibited by at least 50% when the *hr5* and E1A genes were present in equimolar amounts; a fivefold molar excess of the *hr5* E1A gene produced complete inhibition of transcription, as determined by enzymatic activity of the test CAT gene, which was placed under the control of each early promoter. Interestingly, two other E1A missense mutant genes (*hr3* and *hr4*), which have single amino acid substitutions in close proximity to that of *hr5* (Fig. 1), failed to block wt E1A-induced transcription when similarly tested. The fact that each of the *hr* genes produced levels of E1A protein similar to that of the wt gene indicated that the inhibition of transactivation observed only with *hr5* was specifically due to replacement of serine by asparagine at amino acid position 185. It is important to note that the defective 289R protein of *hr5* was capable of inhibiting transactivation in the absence of the 243R protein, as determined by testing a plasmid construct (pHR5-289R) which was capable of expressing only the larger of the two *hr5* E1A proteins. We also discovered that the *hr5* E1A protein did not impede the ability the pseudorabies virus IE protein from transactivating the E3 promoter-driven CAT gene. From this result it follows that, since CAT activity (and hence, CAT mRNA)

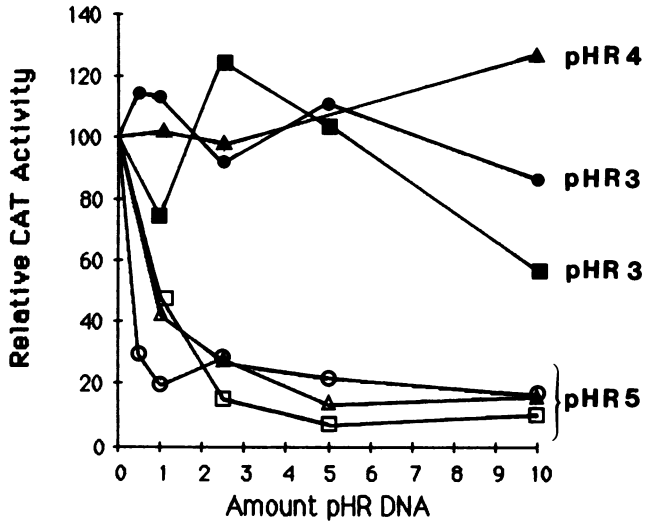

FIG. 4. *hr5* E1A blocks wt E1A-induced transcription from each early promoter. HeLa cells were cotransfected with plasmids, and CAT assays were performed as described in the legend to Fig. 3. CAT activities were quantitated from the acetylated forms of [^{14}C]chloramphenicol. For each experiment, the level of CAT activity induced by wt E1A alone was set as 100%. Represented are cotransfections with wt E1A and the following: pHR5 with p3CAT (○), pHR5, with p2CAT (△), pHR5 with p4CAT (□), pHR3 with p2CAT (●), pHR3 with p4CAT (■), and pHR4 with p3CAT (▲). The values indicating the amount of host range mutant DNA represent the microgram amounts used in the transfections.

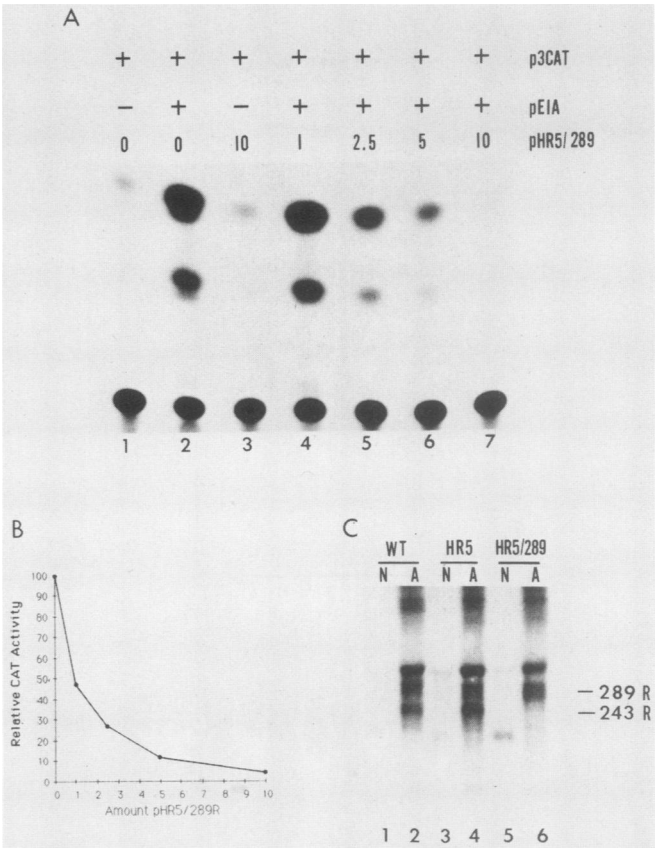

FIG. 5. The *hr5* 289R E1A protein alone blocks wt E1A-induced transcription. (A) Effect of *hr5* 289R E1A protein on wt E1A-induced p3CAT activity. CAT activity after transfection of HeLa cells with 5 μg of p3CAT alone (lane 1), 1 μg of pE1A (lane 2), or 10 μg of pHR5-289R (lane 3). In lanes 4 to 7, p3CAT (5 μg) and pE1A (1 μg) were cotransfected with increasing concentrations of pHR5-289R (1, 2.5, 5, and 10 μg, respectively). The amounts of plasmid DNA used in the cotransfections were made equivalent as described in the legend to Fig. 3. (B) Quantitation of CAT activity obtained from cotransfection of p3CAT and pE1A with increasing amounts of pHR5-289R. The acetylated forms of [^{14}C]chloramphenicol were quantitated and represent an average of three experiments. (C) Immunoprecipitation of ^{32}P-labeled E1A proteins synthesized in transfected cells. HeLa cells were labeled with $^{32}P_i$, and immunoprecipitated extracts were fractionated on a 15% polyacrylamide gel. Immunoprecipitation was with normal rabbit serum (lanes N) or monospecific Ad5 E1A antiserum (lanes A). The positions of 289R and 243R E1A proteins are indicated. HeLa cells were transfected with 10 μg of pE1A (lanes 1 and 2), pHR5 (lanes 3 and 4), or pHR5-289R (lanes 5 and 6).

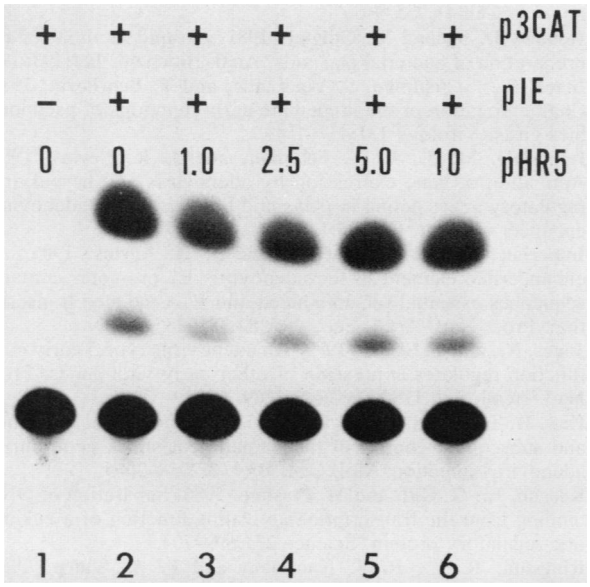

FIG. 6. *hr5* E1A does not affect the ability of the pseudorabies virus IE gene to transactivate the E3 promoter. The CAT assay was performed on extracts of HeLa cells transfected with 5 μg of p3CAT alone (lane 1) or combined with 3.4 μg of pIE alone (lane 2) or combined with 1, 2.5, 5, or 10 μg of pHR5 (lanes 3 to 6, respectively). The molar equivalents of pIE and pHR5 are 3.4 and 1.0 μg, respectively.

can be produced in the presence of *hr5* E1A, *hr5* E1A inhibits wt E1A activation at the transcriptional, rather than the posttranscriptional, level.

Several possibilities were considered to account for how the *hr5* E1A protein inhibits wt E1A transactivation. First, since it is not known whether the E1A protein functions as a monomer or as an oligomer, the possibility exists that the 289R *hr5* and wt E1A proteins could form nonfunctional hetero-oligomers. However, we observed that pHR5 generated essentially the same inhibition curves of p3CAT activity (Fig. 4). when cotransfections were performed with amounts of pE1A well above that required to stimulate maximal CAT expression (L. C. Webster, G. M. Glenn, and R. P. Ricciardi, unpublished data). This suggests that, since inhibition by *hr5* E1A occurs equally well with an excess of pE1A present, the block in transactivation is unlikely due to depletion of wt E1A protein in the form of inactive *hr5*-wt E1A oligomers. Rather, this argues that some cellular factor required for transactivation is titrated out regardless of whether E1A acts as a monomer or an oligomer. However, there is the caveat that we cannot be certain that the saturating concentrations of E1A plasmid used in these cotransfections actually resulted in a concomitant increase in E1A protein. In other experiments, *hr5* E1A inhibited the wt E1A gene of Ad12 (a divergent serotype) equally from transactivating the E3 promoter (unpublished data). Although not definitive, this result suggested that the *hr5* E1A protein equally blocks transactivation of the wt Ad5 and Ad12 E1A proteins by interacting with the same cellular factor rather than by simply forming nonfunctional hetero-oligomers.

A second possibility is that *hr5* E1A may function as a more potent autoregulator than wt E1A (47, 52), which could diminish expression of the wt E1A proteins. However, this does not appear to be the case since similar levels of E1A proteins were detected in cells transfected with either pE1A or pHR5 (Fig. 5C); if the *hr5* E1A protein were a potent autoregulator, its own synthesis would be significantly reduced compared with that of wt E1A. It is noteworthy that *hr5* E1A can block transactivation of the E3 promoter in 293 cells, in which there is an endogenous pool of cellular E1A (unpublished data).

Third, it could be argued that the *hr5* E1A protein decreased the stability of CAT mRNA synthesized from wt E1A-induced promoters. This is unlikely since p3CAT activity induced by pIE was not altered in the presence of pHR5.

A fourth and highly likely explanation is that the *hr5* E1A protein competes with the wt E1A protein for a CTF that is involved in transactivation. For example, the *hr5* E1A protein could bind to and titrate out a CTF and thereby reduce its availability to the wt E1A protein. Although factors that interact with E1A have never been isolated, a number of studies (indicated earlier) indirectly suggest their involvement. Recently, specific transcription factors have been identified by using in vitro systems. For example, in addition to RNA polymerase II, four factors (TFIIB, TFIID, TFIIE, and MLTF) were shown to be required for specific initiation of transcription from the Ad2 major late promoter in vitro (6, 44). One of these factors, TFIID, binds to DNA in the TATA region upstream of the major late promoter (45), whereas MLTF binds to sequences upstream of the TATA box (6). These factors may be involved in the formation of preinitiation complexes before interactions with RNA polymerase II (8, 12). Most relevantly, Kovesdi et al. (32, 33) have shown that binding of a cellular factor to sequences upstream from the E2 promoter of adenovirus is greatly facilitated in the presence of E1A.

The fact that the 289R E1A protein of *hr5* fails to transactivate early viral promoters but blocks the wt E1A protein in this capacity suggests that the *hr5* protein retains partial activity such as binding to a putative CTF. Should this prove true, it would imply that E1A-induced transcription involves at least two steps: CTF binding and transactivation. It would also lead to the question of whether E1A protein sites for putative CTF binding and transactivation are separate. In this regard, it is interesting that in the yeast *GAL4* protein there appears to be a separation of functions as revealed by the analysis of amino-terminal derivatives which were shown to be still capable of binding to specific DNA sequences upstream of target genes but unable to activate their transcription (30). The novel property of *hr5* E1A described here could prove useful for identifying and isolating the transcription factors which are postulated to interact with the E1A protein.

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