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# Adenovirus 5 Early Region 1A Host Range Mutants *hr*3, *hr*4, and *hr*5 Contain Point Mutations Which Generate Single Amino Acid Substitutions

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**The early region 1A (E1A) gene of adenovirus 5 encodes two proteins, 289AA and 243AA, which are translated from mRNAs of 13S and 12S, respectively. These two E1A proteins are identical except for an internal stretch of 46 amino acids unique to the larger protein. The 289AA protein activates transcription from promoters of other early adenoviral genes. The adenovirus type 5 host range mutants *hr*3, *hr*4, and *hr*5 are unable to activate transcription of these early viral genes. We show here that *hr*3, *hr*4, and *hr*5 each contain a distinct missense mutation in the E1A gene. We first localized the mutations in a series of constructed wild-type–*hr* hybrid E1A genes by using a biological assay which can discriminate between functional and nonfunctional E1A proteins. We then identified the mutations by DNA sequencing. In *hr*3 lysine replaced methionine at position 176, and in *hr*4 phenylalanine replaced leucine at position 173; both substitutions occurred in the region unique to the 289AA protein. In *hr*5, due to the splicing patterns of the two mRNAs, asparagine replaced serine as the last amino acid in the unique region of the 289AA protein at position 185, while aspartic acid replaced glycine at position 139 in the 243AA protein, which is the last amino acid common to both proteins before the unique region. These results substantiate the role of the 289AA protein in transcriptional activation and underscore the importance of the unique region as the basis of the functional difference between the two E1A proteins. Implications as to how these mutations affect the structure and function of the E1A proteins in transcriptional activation and transformation are discussed.**

The early region 1A (E1A) gene of adenovirus is dynamic in its ability to regulate expression of both viral and cellular genes. The adenovirus genome consists of linear double-stranded DNA that is 35 kilobase pairs in length. The E1A gene is located between 1.5 and 4.5 map units (m.u.) on the adenovirus genome. Upon infection, expression of the E1A gene facilitates transcription from early viral promoters E1B, E2, E3, and E4 (2, 11, 26, 35, 41). In addition, E1A gene products are able to activate transcription of the mammalian heat shock gene (37) as well as certain cellular genes introduced into cells as recombinant DNA molecules by transfection or infection (12, 18, 47, 49). The E1A gene also plays a critical role in the ability of adenovirus to morphologically transform cells in vitro. Although transformation by adenovirus requires the expression of both E1A and E1B genes (8, 10, 17), recent studies have shown further that the E1A gene per se can immortalize primary cells (25) as well as cooperate with the polyoma middle-T and T24 H-*ras*-1 oncogenes to transform cells in culture (29, 43).

The E1A gene encodes overlapping RNAs of 9S, 12S, and 13S which share the same 5′ and 3′ termini but which differ in length due to the amount of intervening sequences removed by splicing (3, 6, 28, 38, 45). Each of the E1A RNAs shares the same acceptor splice site but uses a different donor splice site (3, 38, 45). The 12S and 13S RNAs of E1A are the first viral genes to be transcribed upon infection; the 9S RNA is made at late times of infection (2, 26, 45). In adenovirus 5 (Ad5), the 12S and 13S RNAs encode protein products of 243 and 289 amino acids (designated proteins 243AA and 289AA), respectively. These E1A proteins are identical except for an internal stretch of 46 amino acids unique to the large protein (38).

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Analysis of E1A mutants has helped to discriminate between the functions of the 289AA and 243AA proteins during infection. Mutants *hr*1 and *pm*975 have shown that the 289AA protein is responsible for facilitating transcription of early viral genes (35, 41). In *hr*1, the 289AA protein is truncated due to a frameshift mutation in the unique coding region, whereas the 243AA protein is of normal length (41). E1A is the only viral gene transcribed upon infection by *hr*1 (2, 41). In *pm*975, a point mutation disrupts the donor splice site of the 12S RNA and thereby permits only the 289AA protein product of the 13S RNA to be synthesized (35). The *pm*975 mutant, unlike *hr*1, undergoes a normal productive infection in HeLa cells (35).

Analysis of missense mutants may help to delineate domains of the E1A proteins essential for function. Although E1A missense mutants have not been described, three mutants, *hr*3, *hr*4, and *hr*5, were predicted to contain missense mutations in E1A (41). The mutations were generated by treatment of Ad5 virions with UV irradiation in the case of *hr*3 or with nitrous acid in the cases of *hr*4 and *hr*5 (21). The mutant viruses were isolated on 293 cells (15), which constitutively express both E1A and E1B genes (1). For each of these mutants, the defect was mapped to the E1A gene by marker rescue (9). These mutants were defective for both growth on HeLa cells (21) and their ability to transform primary rat embryo cells (14). As in the case of *hr*1, these mutants expressed only E1A transcripts upon infection (30).

*hr*3, *hr*4, and *hr*5 were suggested to be missense because they each synthesized both the 289AA and the 243AA proteins indistinguishable from wild-type (WT) virus as judged by two-dimensional gel electrophoresis (41, 42). This was true whether the E1A proteins were translated in vitro from hybridization-selected RNA (41) or synthesized in vivo and immunoprecipitated with E1A-specific antisera (42).

In this study we have determined the specific mutations in *hr*3, *hr*4, and *hr*5. For each mutant, we first identified a segment of the coding region of the E1A gene which was defective and then characterized the specific mutation by DNA sequencing. Our analysis demonstrates that *hr*3, *hr*4, and *hr*5 are indeed missense mutants. The predicted amino acid substitutions and their effects on the structure of the E1A proteins are presented.

## MATERIALS AND METHODS

**Cells and viruses.** 293 cells (15) were propagated in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. Monolayer cultures of HeLa cells were grown in MEM containing 8% fetal calf serum. Seed stocks of the host range mutant viruses, Ad5 *hr*3, Ad5 *hr*4, and Ad5 *hr*5, were obtained from Jim Williams (Carnegie Mellon University, Pittsburgh, Pa.). 293 cells were infected with the mutant viruses at a multiplicity of 1 PFU. After 60 h, virions were purified by CsCl gradient centrifugation (39). Viral DNA was purified as described previously (39).

**Plasmids.** Plasmid pLA1, containing the left end 9.4% of the Ad5 genome, was obtained from F. Tamanoi (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) (48). Plasmids p3CAT(pKCAT23) and p2CAT, which consist of the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the E3 or E2 promoter of adenovirus, respectively, were generous gifts from N. C. Jones (Purdue University, West Lafayette, Ind.) (52). Plasmid constructions are described in the text. Restriction endonucleases were obtained from Bethesda Research Laboratories (BRL) and New England Biolabs, T4 DNA ligase was from Boehringer Mannheim Biochemicals, and Klenow fragment and *Eco*RI linkers were from BRL. DNA fragments were purified from agarose gels by the method of Vogelstein and Gillespie (51). Ligation and nick translation of DNA with [$\alpha^{32}$P-labeled nucleoside triphosphates (Amersham Corp.) were as described previously (32). After transformation of *Escherichia coli* (C600) to ampicillin resistance, plasmids were screened by colony hybridization (19) or restriction enzyme analysis of plasmid DNA isolated from minilysates (23). Plasmids were purified by using standard procedures (27, 40).

**Transfections and CAT assays.** Plates of HeLa cells were transfected by the calcium phosphate precipitation technique (16) followed by a glycerol shock (9). Plasmid DNA (10 μg of p3CAT alone or together with 2.5 μg of an E1A-containing plasmid) was first suspended in 438 μl of 10 mM Tris–1 mM EDTA (pH 7.9) and then 62 μl of 2 M $CaCl_2$ was added. The $CaCl_2$-DNA mixture was added to an equal volume of 2× HEPES-buffered saline (280 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 1.5 mM $Na_2HPO_4$, pH 7.1) while air bubbles were simultaneously introduced into the solution. Precipitates were allowed to form at room temperature for 30 to 45 min. The precipitate was added to a 100-mm plate of cells containing 9 ml of MEM and cultures were incubated at 37°C for 4 to 6 h. The medium was removed, and the cells were shocked for 1 min with MEM containing 20% glycerol, washed with medium, and refed with 20 ml of MEM containing 8% fetal calf serum.

After 48 h, cells were harvested and the cell extracts were assayed for CAT activity as described by Weeks and Jones (52). For standardization, CAT assays were performed with extracts containing equal amounts of protein (31). In all instances, the CAT assays were highly reproducible.

**M13 cloning and sequencing.** M13 vectors mp18 and mp19 and *E. coli* strain JM107 were used for cloning and sequencing. The relevant fragment from each host range mutant was cloned into M13, and after positive recombinants were identified, single-stranded phage DNA was prepared (33). DNA was sequenced with chain-terminating dideoxynucleotides by the method of Sanger et al. (44) with [$^{35}$S]dATP (650 Ci/mmol; Amersham) as the labeled nucleotide (4). Deoxy-dideoxynucleotide mixes were obtained from P-L Biochemicals. Primer and Klenow fragment were from BRL. Sequences were fractionated on 6 and 8% polyacrylamide gels (40% polyacrylamide–2% bisacrylamide, ultrapure reagents; BRL) containing 8 M ultrapure urea (BRL). Gels were dried and exposed for 24 to 48 h, using Kodak XAR-5 X-ray film.

**Computer analysis.** The predicted secondary structures were generated by computer analysis, using the programs of Chou and Fasman (5) and Hopp and Woods (24).

## RESULTS

**Cloning of WT and *hr*3, *hr*4, and *hr*5 E1A genes.** The left end of adenovirus encodes two overlapping E1A transcripts of 13S and 12S, as depicted by a generalized restriction map of this region in Fig. 1A. The WT E1A gene was subcloned from plasmid pLA1, which contains the 0- to 9.4-m.u. portion of the Ad5 genome. pLA1 was digested with *Eco*RI and *Kpn*I to generate the 0- to 6.1-m.u. fragment (Fig. 1B). The *Kpn*I end of the 0- to 6.1-m.u. fragment was modified by *Eco*RI linker attachment and cloned into plasmid pML to obtain pE1A (Fig. 1B).

The E1A genes of host range mutants *hr*3, *hr*4, and *hr*5 were obtained directly from their respective viral DNAs. The left end *Xho*I C fragment (0 to 15.5 m.u.) was isolated and further restricted to obtain the *Sac*II-*Sac*I fragment that extends from 354 to 1,770 base pairs (bp) (Fig. 1C). The corresponding *Sac*II-*Sac*I fragment of the WT E1A gene was removed from pE1A and replaced with the *Sac*II-*Sac*I fragment of each host range mutant (Fig. 1C). In the three resulting plasmids, pHR3, pHR4, and pHR5, the promoter, coding region, and poly(A) addition sites of E1A are derived from host range sequences, whereas flanking sequences, including upstream enhancers, are derived from the WT gene (Fig. 1A and C).

**Biological assay that discriminates between WT and host range E1A genes.** Since random mutagenesis was used to produce the host range mutants, silent mutations could be present in addition to the mutation which accounts for the defective phenotype. It was thus important to have a convenient assay which could discriminate between a functional and nonfunctional E1A gene to localize the defect in each mutant. A relatively straightforward assay, recently described by Weeks and Jones (52), utilizes a plasmid, p3CAT, which contains the bacterial CAT gene driven by the early region 3 promoter of adenovirus. When p3CAT was transfected into HeLa cells, minimal levels of CAT activity were detected after 48 h (Fig. 2, lane 2). However, when p3CAT was cotransfected with pE1A, high levels of CAT activity were observed (Fig. 2, lane 3). In marked contrast, when p3CAT was cotransfected with pHR3, pHR4, or pHR5, very low levels of CAT activity were seen (Fig. 2, lanes 4 to 6). The radiolabeled spots from the silica plate were scraped and counted. CAT activity induced by the host range mutants was reduced 15- to 30-fold relative to the levels induced by WT E1A. Identical results were obtained with p2CAT, a plasmid containing the CAT gene driven by the early region 2 promoter of adenovirus (52) (data not

shown). These results were consistently reproducible and thereby enabled one to distinguish functional from nonfunctional E1A genes.

**Localization of the mutation to a specific segment of the E1A coding region in *hr3*, *hr4*, and *hr5*. (i) Construction of hybrid E1A genes.** To localize the mutation leading to the functional defect in each host range mutant, a series of hybrid E1A genes were constructed which contained sequences derived from portions of the coding regions of both WT and host range E1A genes. Plasmids containing hybrid E1A genes were constructed by reciprocally exchanging corresponding restriction fragments between the WT E1A gene and one of the host range mutant genes (Fig. 3). The three restriction fragments chosen for this exchange and the portion of the 289AA protein they contain are detailed below. The *Sac*II-*Sma*I fragment (354 to 1,007 bp of Ad5) consists of the promoter and 5'-flanking sequences as well as the coding region of the amino-terminal 150 amino acids of the 289AA protein. The second fragment, *Sma*I-*Xba*I (1,007 to 1,339 bp), encodes the middle portion of the 289AA E1A protein consisting of amino acids 151 to 222 and also contains the intron of the 13S mRNA. The third fragment, *Xba*I-*Sac*I (1,339 to 1,770 bp), encodes the carboxyl terminus of the 289AA E1A protein consisting of amino acids 223 to 289, and also contains the poly(A) additional signal and 3'-flanking sequences. In Fig. 3, all of the plasmids contain E1A-coding regions derived from the WT DNA sequence, denoted by a W, or from host range DNA sequences, denoted by an H. For example, plasmid pWHW encodes hybrid 289AA and 243AA E1A proteins of which the amino-terminal and carboxyl-terminal portions are WT and the middle portions are host range. Thus, for each host range mutant, *hr3*, *hr4*, and *hr5*, this series of six hybrid E1A genes was available for testing in the CAT assay system.

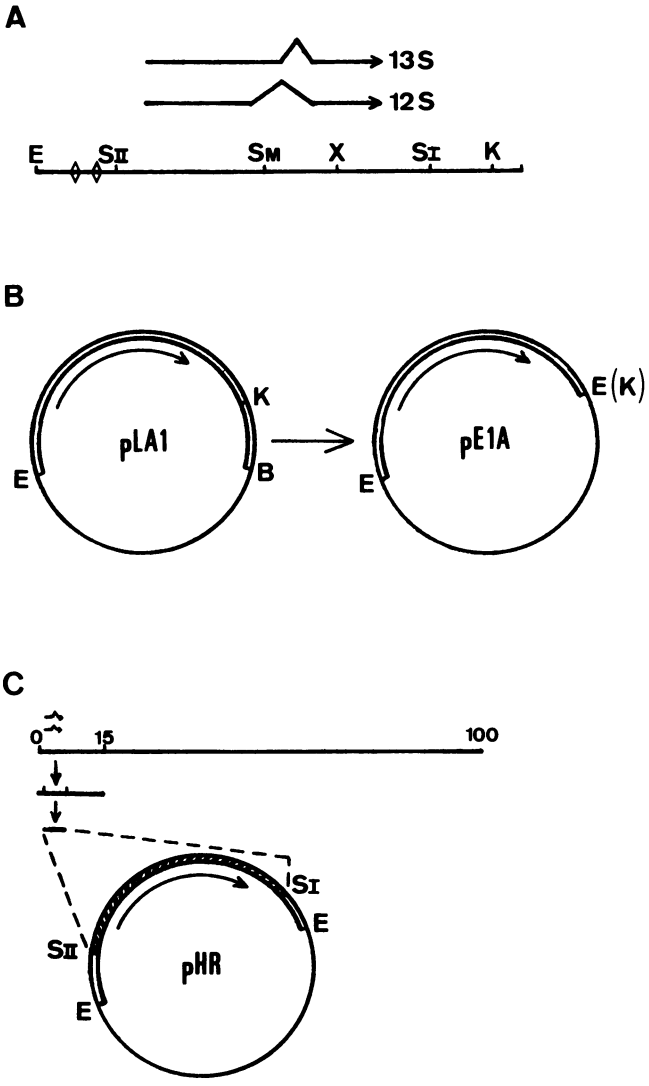


FIG. 1. Cloning of the host range E1A genes. (A) Representation of the left end of the Ad5 genome from 0 to 6.1 m.u. Shown are the positions of the 12S and 13S E1A transcripts, the E1A enhancers (open diamonds), and relevant restriction enzyme sites (E, *Eco*RI; SII, *Sac*II; Sm, *Sma*I; X, *Xba*I; SI, *Sac*I; K, *Kpn*I). (B) Construction of the WT E1A plasmid. Ad5 WT sequences are shown by open areas; pML sequences are shown by single lines. The arrow indicates the direction of transcription and extent of the E1A mRNAs. Shown are the *Bgl*I site at 9.4 m.u., B, and the *Kpn*I site modified with an *Eco*RI linker, E(K). (C) Cloning of the host range E1A genes. The pHR plasmids were constructed by ligating the *Sac*II (SII) to *Sac*I (SI) portion of the *Xho*I C fragment, 0 to 15 m.u. (derived from the host range genomic DNA, 0 to 100 m.u.), into pE1A after removal of the corresponding WT E1A fragment (SII-SI). In pHR, the host range sequences are represented by the striped area; WT E1A DNA, by the open area; and pML sequences, by the thin line.

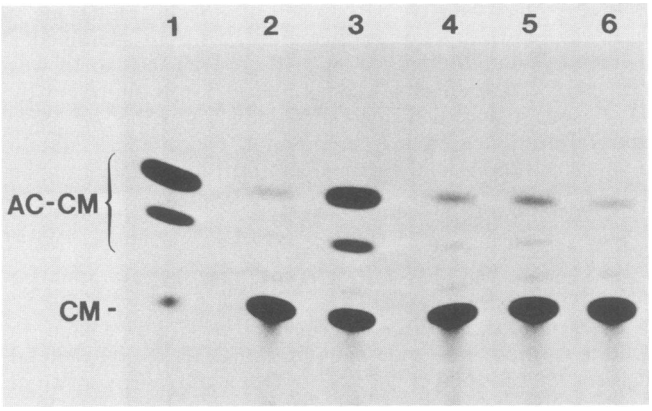


FIG. 2. Expression of CAT activity in cells transfected with p3CAT and host range E1A genes. HeLa cells were transfected with 10 μg of p3CAT plasmid alone or with 2.5 μg of an E1A-containing plasmid, using the calcium phosphate precipitation technique. After 48 h, cell extracts were assayed for CAT activity as described previously (52). For a given experiment, equal amounts of cell extract based upon protein concentration were used. [$^{14}$C]chloramphenicol (CM) and monoacetylated forms of [$^{14}$C]chloramphenicol (AC-CM) were separated by thin-layer chromatography and visualized by autoradiography. CAT assays were performed with purified CAT enzyme (P-L Biochemicals) (lane 1); extracts from HeLa cells transfected with p3CAT alone (lane 2); or p3CAT cotransfected with pE1A (lane 3), pHR3 (lane 4), pHR4 (lane 5), or pHR5 (lane 6).

**(ii) CAT assay with hybrid E1A genes.** Each of the hybrid E1A genes was tested in the CAT assay system as described above. For all of the host range mutants, high levels of CAT activity were induced when p3CAT was cotransfected with pHWW, pHWH, and pWWH (Fig. 4, lanes 6, 7, and 10, respectively). On the other hand, for each host range mutant, low levels of CAT activity were induced with pWHH, pWHW, and pHHW (lanes 5, 8, and 9, respectively). The intensity of the spots induced by the former set of hybrids

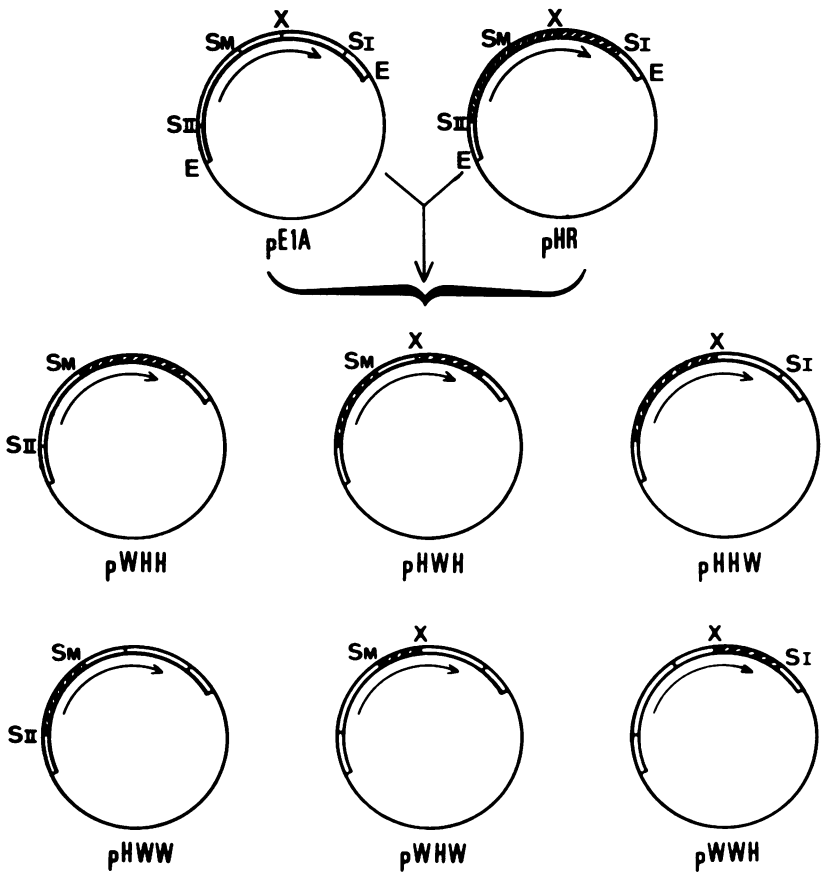


FIG. 3. Construction of hybrid E1A genes used to localize the functional defect in the coding region of *hr3*, *hr4*, and *hr5*. Ad5 WT sequences are indicated by open areas; Ad5 host range sequences from *hr3*, *hr4*, or *hr5*, by striped areas; and pML sequences, by a single line. Restriction enzyme sites are as denoted in the legend to Fig. 1. Arrow indicates the direction and extent of E1A transcripts. Construction of the plasmids and the nature of the resulting hybrid E1A proteins are described in the text. For each of the hybrid E1A plasmids (e.g., pWHH), WT sequences are designated as W and host range sequences as H.

corresponded to those induced by WT pE1A (Fig. 4, cf. lane 3 with lanes 6, 7, and 10), whereas the intensity of the spots induced by the latter set of hybrids corresponded to those induced by host range pHR3, pHR4, and pHR5 (Fig. 4, cf. lane 4 with lanes 5, 8, and 9).

These analyses revealed that for each host range mutant, whenever the middle segment (*Sma*I-*Xba*I) of a hybrid E1A gene was derived from host range sequences, as in pWHH, pWHW, and pHHW, low levels of CAT activity were observed after cotransfection with p3CAT (Fig. 4, lanes 5, 8, and 9). Conversely, whenever WT sequences were present in the middle segment of the hybrid E1A, as in pHWW, pHWH, and pWWH, the resulting hybrid gene was able to induce a high level of CAT activity (Fig. 4, lanes 6, 7, and 10). This correlation holds true only for the middle segment of hybrid E1A genes. From this analysis, the amino-terminal or carboxyl-terminal coding regions of the host range E1A genes do not contain mutations that prevent transcriptional activation by E1A. Thus, these results indicated that the mutation in *hr3*, *hr4*, and *hr5* was located within the middle segment of the E1A-coding region of each mutant, between *Sma*I and *Xba*I.

**Sequence analysis of *hr3*, *hr4*, and *hr5*.** The segment of each host range mutant between *Sma*I and *Xba*I (1,007 to 1,339 bp) was cloned into M13 and sequenced, using dideoxynucleotides as chain terminators (44). The nucleotide sequence containing the mutation in each host range mutant is shown in Fig. 5. *hr3*, *hr4*, and *hr5* each contained a single nucleotide change as compared to the published sequence of Ad5 E1A (50). In addition, these three host range sequences served as controls for one another since two of them had the nucleotide of the WT E1A at the position of the mutation in the third host range sequence. There is a transversion from T to A at nucleotide 1086 in *hr3*, a transition from C to T at position 1076 in *hr4*, and a transition from G to A at nucleotide 1229 in *hr5* (Fig. 6).

The mutation in each host range mutant produces single-amino acid substitutions and thus confirms the earlier hypothesis that these are indeed missense mutants (41). The actual amino acid substitutions are depicted in Fig. 6. The mutations in *hr3* and *hr4* lie within the coding region unique to the larger E1A protein. Lysine replaces methionine in *hr3* at amino acid position 176. Phenylalanine replaces leucine in *hr4* at position 173. The single-base-pair change in *hr5* results in a different amino acid substitution in each of the E1A proteins. The mutation in *hr5* changes nucleotide 1229, the first nucleotide downstream from the acceptor splice site utilized by both 12S and 13S E1A mRNAs. This altered nucleotide serves as the middle base of a split codon in each RNA formed as a result of splicing. Thus, in *hr5*, asparagine replaces serine as the last amino acid in the unique region of the 289AA protein at position 185, while aspartic acid

replaces glycine at position 139 in the 243AA protein, which is the last amino acid common to both proteins before the unique region. It is important to note that, although the nucleotide substitution in *hr5* is adjacent to the 3′ boundary of the intron, both 12S and 13S RNAs appear to be properly spliced as determined by S1 nuclease experiments (data not shown) (30).

## DISCUSSION

We have shown here that three Ad5 mutants, *hr3*, *hr4*, and *hr5*, are missense as originally predicted by their ability to synthesize E1A proteins of normal length (41). These are the first missense mutants to be reported in E1A. These mutants are defective for both normal infection and transformation (14, 21). Since they were derived by random mutagenesis, there was the possibility that the defective E1A gene could also contain silent mutations. Therefore, prior to DNA sequencing, we localized the mutation to a specific segment of the coding region of E1A. This was done by testing hybrid E1A genes containing both WT and host range sequences in a biological assay that detects the ability of E1A to activate transcription from inducible adenoviral promoters (52).

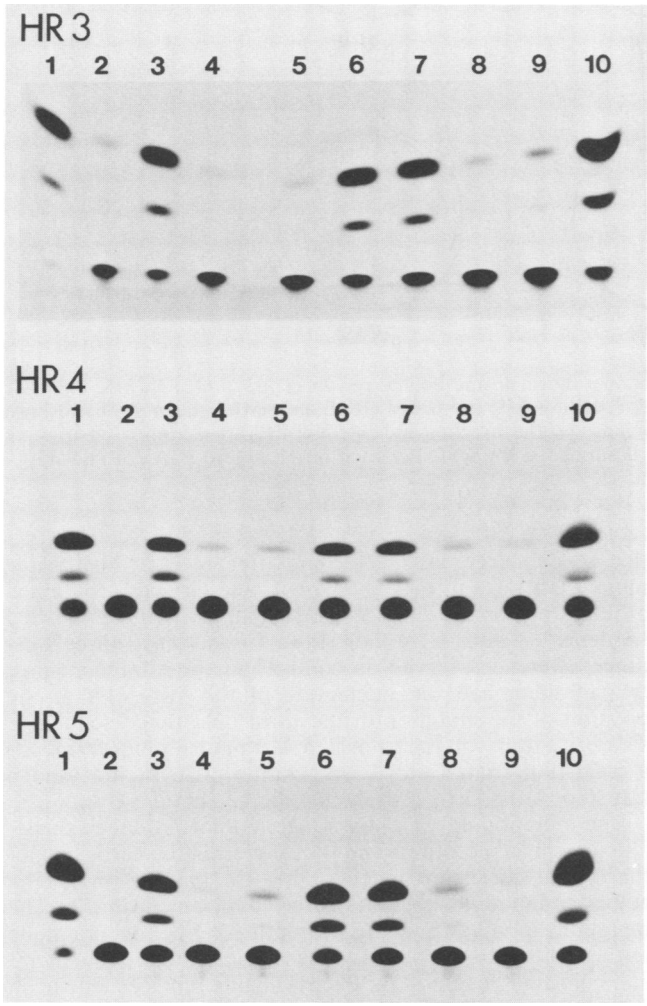


FIG. 4. Expression of CAT activity when p3CAT is induced by the hybrid E1A genes. The transfection procedure and positions of the [$^{14}$C]chloramphenicol and acetylated [$^{14}$C]chloramphenicol are as described in the text and the legend to Fig. 2. CAT assays were carried out with purified CAT enzyme (lane 1); extracts from HeLa cells transfected with p3CAT alone (lane 2); or p3CAT cotransfected with pE1A (lane 3), pHR (lane 4), pWHH (lane 5), pHWW (lane 6), pHWH (lane 7), pWHW (lane 8), pHHW (lane 9), or pWWH (lane 10) for each of the three host range mutants.

In *hr3* and *hr4*, single-base-pair changes occur within the region unique to the 13S RNA and generate amino acid substitutions which affect only the 289AA protein. In *hr5*, a single-base-pair alteration produces missense mutations which affect both the 289AA and the 243AA proteins. In the 289AA protein, the last amino acid of the unique region is changed and in the 243AA protein the last amino acid common to both proteins before the unique region is changed. These results substantiate the role of the 289AA protein in transcriptional activation (35, 41) and underscore the importance of the unique region as the basis of the functional differences between the two E1A proteins.

The novel feature of the defect in *hr5* is that this single mutation is able to simultaneously produce a missense mutation in both the 289AA and the 243AA proteins in nonidentical regions. The split codons [i.e., 5′G (974)/G(1229)-T(1230)3′ in the 12S RNA and 5′A(1112)/G(1229)-T(1230)3′ in the 13S RNA] are brought together by the joining of different donor splice sites to a common acceptor splice site and encode amino acid 185 of the 289AA protein and amino acid 139 of the 243AA protein. These are the only codons which can be simultaneously altered to affect both proteins in different regions. Any other missense mutation would affect (i) both E1A proteins in identical overlapping regions, (ii) only the 289AA protein in the unique region, or (iii) the 289AA protein at a position which disrupts the donor splice site of the 12S RNA.

It is noteworthy that, although the nucleotide substitution of G to A at position 1229 in *hr5* is adjacent to the 3′ boundary of the intron, both the 12S and the 13S RNAs are properly spliced as determined by S1 analysis (30; unpublished data). This is not unexpected since both G and A are frequently used as the first nucleotide of the downstream exon (36).

Although the precise mechanism of action of E1A remains unknown, it is important to consider how these mutations may render the E1A protein unable to facilitate transcription from early viral promoters. The substituted amino acids in *hr3*, *hr4*, and *hr5* may either prevent a productive interaction of E1A with DNA or protein or disrupt the conformation of the E1A protein, thus rendering it nonfunctional. It is interesting to note that leucine at position 173 (which is changed to phenylalanine in *hr4*) and serine at position 185 (which is changed to asparagine in *hr5*) are identical among adenovirus serotypes 2, 5, 7, and 12. The methionine at position 176, which is changed to lysine in *hr3*, is chemically conserved among these serotypes (methionine in Ad2, Ad5, and Ad7; leucine in Ad12).

The predicted secondary structures of the 289AA and 243AA E1A proteins were generated by computer analysis, using programs instructed by Chou and Fasman rules for secondary structure (5) and Hopp and Woods rules for hydrophilicity (24), and are shown in Fig. 7A. When the secondary structures of *hr3*, *hr4*, and *hr5* were compared with that of WT E1A, no major changes were observed. However, in *hr3* and *hr4*, changes in the local environment of the region containing the mutation were seen. When compared with WT E1A, the stretch of amino acids from positions 169 to 173 was more hydrophilic in *hr3* but more hydrophobic in *hr4* (Fig. 7B). In *hr5*, there was no apparent

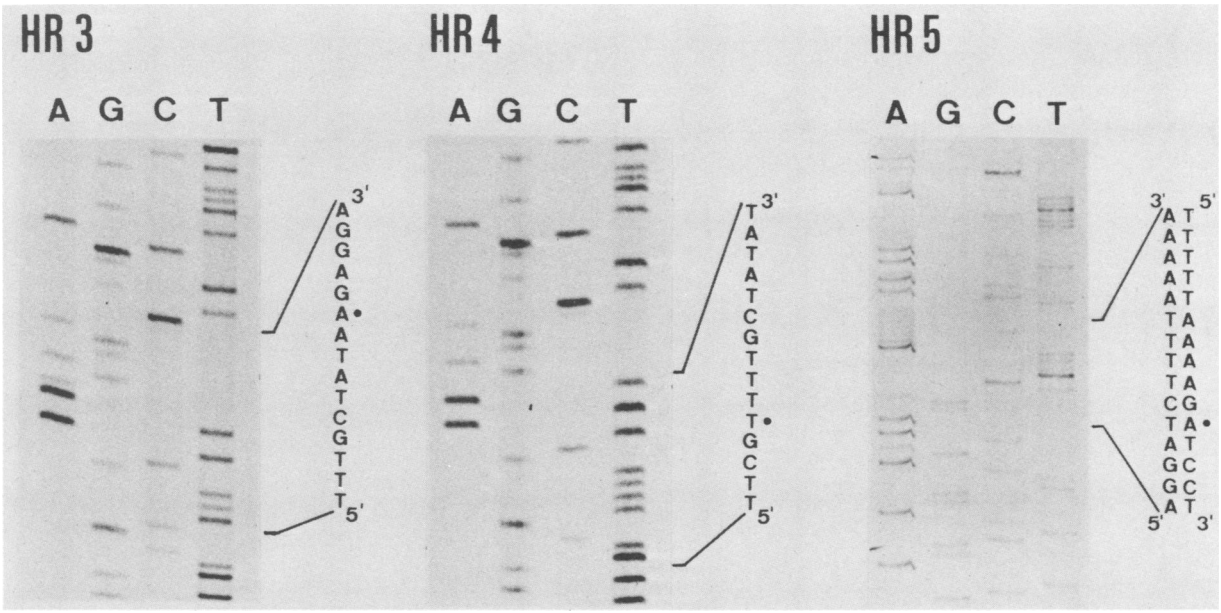


FIG. 5. DNA sequences containing the mutation in *hr3*, *hr4*, and *hr5*. The region of each host range mutant identified as containing the mutation was cloned into M13 vectors mp18 and mp19 and sequenced with dideoxynucleotides. For each mutant, only the region containing the mutation is shown. The mutation is designated by a dot alongside the affected nucleotide.

change in the hydrophilicity in the region of the mutation of the 289AA protein (Fig. 7B) or the 243AA protein (not shown). Thus, if the mutations in *hr3*, *hr4*, or *hr5* affect the secondary conformation of the 289AA protein, it is not obvious by this analysis.

Interestingly, the mutations in *hr3* and *hr4* are proximal to a cysteine residue which is one of a set of four cysteines found at positions 154, 157, 171, and 174 in Ad5. This set of cysteine residues is found in the unique region and its spacing is conserved among all serotypes that have been sequenced (7, 13, 38, 46, 50). In *hr3*, a charged amino acid (lysine) replaces a nonpolar one (methionine) two residues

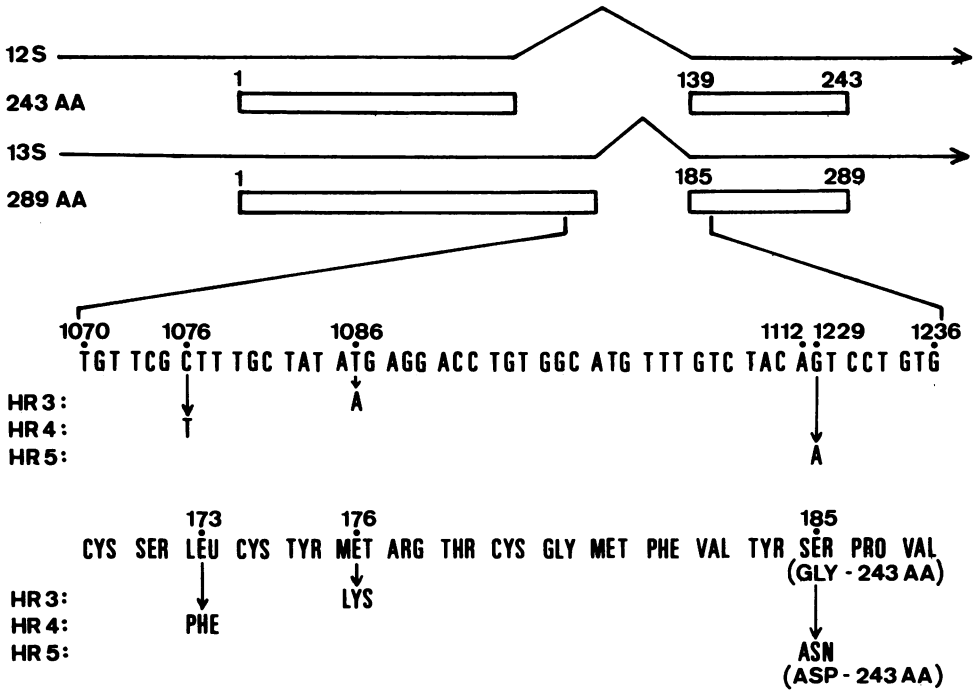


FIG. 6. Representation of the nucleotide and amino acid changes in the E1A genes of *hr3*, *hr4*, and *hr5*. The mRNAs (13S and 12S) and proteins (289AA and 243AA) they encode are indicated. Nucleotide numbers are from the left end of the Ad5 genome. Nucleotides 1112 and 1229 are joined by splicing of the 13S mRNA. Amino acid numbers are from the amino terminus of the E1A proteins. For host range mutants *hr3*, *hr4*, and *hr5*, the specific nucleotide and amino acid substitutions are indicated by the arrows.

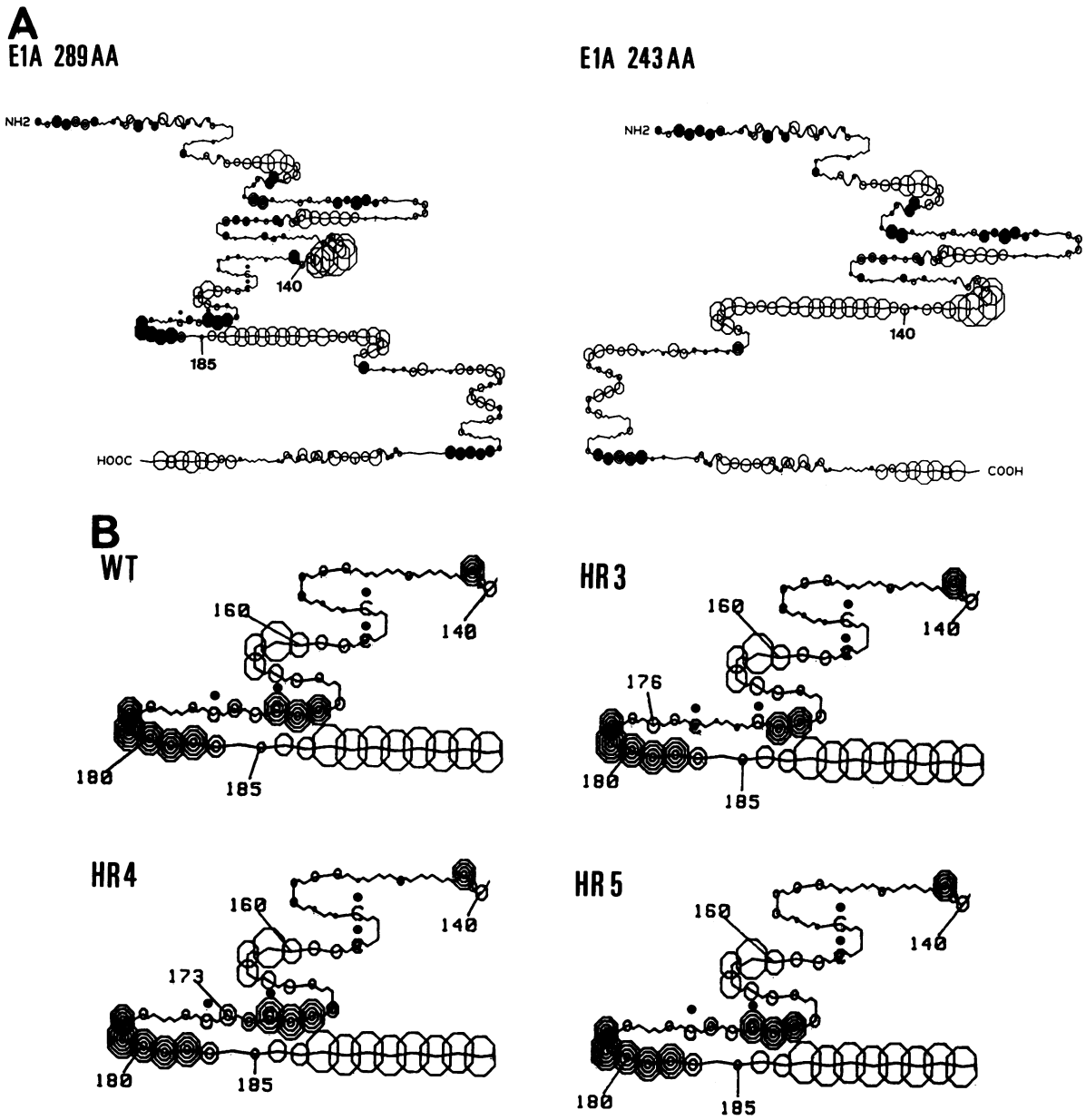


FIG. 7. Predicted secondary structure and hydrophilicity of the WT and host range E1A proteins. Open circles indicate hydrophilic regions, and filled circles indicate hydrophobic areas. The radius of a circle is proportional to the average hydrophilicity/hydrophobicity calculated for that residue and the next five residues. Numbers correspond to amino acid residues from the amino terminus. The four conserved cysteine residues in the unique region are inidcated with a dot above each. (A) Predicted structures of the WT 289AA and 243AA E1A proteins. (B) Comparison of the predicted structures of the unique region (amino acids 140 to 195) of the 289AA protein of WT and the three host range mutants.

from the cysteine at position 174. In *hr*4, an amino acid with an aromatic group (phenylalanine) replaces one which is less space filling (leucine) at a position adjacent to cysteine 174. It is possible that these mutations may interfere with potential intra- or intermolecular disulfide bridges formed by these cysteines and thereby disrupt the tertiary conformation of the 289AA protein.

The host range mutant viruses, *hr*1, *hr*2, *hr*3, *hr*4, and *hr*5, are equally defective in their abilities to infect HeLa cells (21), but exhibit differences in their abilities to transform primary rat embryo cells (22). *hr*1 and *hr*2 are cold sensitive for transformation; i.e., they form foci at 38.5°C but not at 32.5°C, whereas *hr*3, *hr*4, and *hr*5 are unable to form foci at either temperature (22). An important difference between these two classes of mutants is that *hr*1 and, predictably, *hr*2 encode a 243AA protein of normal length but truncated forms of the 289AA protein (41). In contrast, *hr*3, *hr*4, and *hr*5 are each missense mutants as shown here and encode both proteins of normal length (41, 42). The cold-sensitive phenotype observed with *hr*1 could be due to the action of the 243AA protein since viral mutants which express the 243AA protein, but not the 289AA protein, are also cold sensitive for transformation (20, 34). In light of these observations, there are two possible explanations which could

account for the reason that *hr3*, *hr4*, and *hr5* fail to transform primary rat embryo cells at the high temperature. First, there may be an overlooked mutation in another region of the E1A gene of *hr3*, *hr4*, or *hr5* which makes them defective for transformation since this study only measured the ability of E1A to activate transcription from viral promoters. However, in the case of *hr5*, the single mutation could account for the transformation-defective phenotype since it affects both the 289AA and the 243AA E1A proteins. If second mutations specifically affecting transformation do exist, they are predicted to reside in the 5′ end of the coding region in the case of *hr4* and *hr5* (nucleotides 560 to 1007) since the 3′ ends of these mutants (nucleotides 1339 to 1572) were sequenced and found to be completely wild type (data not shown). In *hr3* such a mutation could reside in either the 5′ or the 3′ end of the gene. A second explanation may account for the inability of *hr3*, *hr4*, and *hr5* to transform at the higher temperature. In each of these mutants, the full-length 289AA protein which contains a single-amino-acid substitution may interfere with the transforming activity of the 243AA protein.

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