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trans-Dominant Mutants of E1A Provide Genetic Evidence that the Zinc Finger of the *trans*-Activating Domain Binds a Transcription Factor

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The 289R E1A protein of adenovirus stimulates transcription of early viral and certain cellular genes. *trans*-Activation requires residues 140 to 188, which encompass a zinc finger. Several studies have indicated that *trans*-activation by E1A is mediated through cellular transcription factors. In particular, the ability of the *trans*-dominant E1A point mutant *hr5* (Ser-185 to Asn) to inhibit wild-type E1A *trans*-activation was proposed to result from the sequestration of a cellular factor. Using site-directed mutagenesis, we individually replaced every residue within and flanking the *trans*-activating domain with a conservative amino acid, revealing 16 critical residues. Six of the individual substitutions lying in a contiguous stretch C terminal to the zinc finger (carboxyl region$_{183-188}$) imparted a *trans*-dominant phenotype. *trans*-Dominance was even produced by deletion of the entire carboxyl region$_{183-188}$. Conversely, an intact finger region$_{147-177}$ was absolutely required for *trans*-dominance, since second-site substitution of every critical residue in this region abrogated the *trans*-dominant phenotype of the *hr5* protein. These data indicate that the finger region$_{147-177}$ binds a limiting cellular transcription factor and that the carboxyl region$_{183-188}$ provides a separate and essential function. In addition, we show that four negatively charged residues within the *trans*-activating domain do not comprise a distinct acidic activating region. We present a model in which the *trans*-activating domain of E1A binds to two different cellular protein targets through the finger and carboxyl regions.

The adenovirus 289R E1A oncoprotein is a promiscuous *trans*-activator of both viral and cellular gene transcription. Although the mechanism of *trans*-activation is not well understood, the E1A protein is thought to exert its effect primarily through interaction with cellular transcription factors. This view is based on the fact that the purified 289R protein does not bind DNA in a sequence-specific manner (5, 10). Furthermore, there is no DNA response element common to all E1A-inducible promoters (reviewed in references 3 and 24). Indeed, that a wide range of transcription factor-binding sites can mediate E1A *trans*-activation is clear from recent experiments using synthetic promoters (44, 55). For example, Pei and Berk (44) showed that the E1B TATA box, a single ATF-binding site, or two E2F sites can mediate *trans*-activation by E1A.

A number of studies have suggested that E1A activity results in the phosphorylation of sequence-specific cellular transcription factors, such as E2F, E4F, and EivF, converting them to their active forms (2, 36, 47, 48). It has also been proposed that E1A mediates its effect by interacting with, or inducing a modification of, TFIID (27, 53, 64) or other general transcription factors (55, 62). Other studies have suggested that E1A functions directly at the promoter by binding to upstream factors, such as ATF-2 (32).

The 289R E1A protein contains three regions (CR1, CR2, and CR3) representing distinct functional domains, which are highly conserved among adenovirus serotypes (reviewed in reference 41). CR3 is commonly referred to as the *trans*-activating domain because point mutations affecting only this region destroy the ability of E1A to stimulate transcription (12, 30, 42). Moreover, CR3 is absent from the otherwise identical 243R E1A protein, which is a poor *trans*-activator (40, 49, 63). The independent nature of the *trans*-activating domain was revealed by the ability of a synthetic peptide comprising the 49-amino-acid domain to stimulate transcription from an early viral promoter in microinjection experiments (31).

The salient structural feature of the *trans*-activating domain is a C4-type zinc finger sequence defined by C154, C157, C171, and C174 (7). We have shown that the 289R protein binds a single zinc ion. Since E1A does not bind DNA directly, it is different from other regulatory proteins which mediate DNA binding through their zinc fingers, e.g., TFIIIA (16, 39), Sp1 (21), and the glucocorticoid receptor (11). However, the zinc finger of E1A is clearly an essential component of the *trans*-activating domain, since individually substituting glycine or serine for each of the four cysteine residues destroyed *trans*-activation (7). Substituting serine for C157, C171, or C174 also destroyed zinc binding, whereas replacing C154 with serine surprisingly had no effect on zinc binding. X-ray absorption fine structure analysis showed that the zinc in the wild-type (wt) 289R protein is indeed coordinated to four cysteine residues; the single amino acid substitution of serine for C154 resulted in the recruitment of two histidines on the left side of the finger to bind zinc in conjunction with C171 and C174 (59). These results argue that for *trans*-activation to occur, zinc must be bound by E1A in a specific way.

Following our report of the identification of an E1A *trans*-dominant point mutant, *hr5* (185SN) (13), mutant forms of several other viral *trans*-activator proteins which also exhibit *trans*-dominance have been identified, including Tax of human T-cell lymphotropic virus type II (58), VP16 of herpes simplex virus type 1 (57), and Rev and Tat of human

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immunodeficiency virus type 1 (34, 37, 43). One mechanism by which these *trans*-dominant mutants are thought to inhibit the activity of the wt protein is by sequestering cellular transcription factors.

To better understand the basis of E1A *trans*-dominance, we have generated a series of 289R E1A mutant proteins with a conservative substitution at every position between residues 137 and 190. Mutations which produce the *trans*-dominant phenotype in E1A map to a contiguous region at the carboxyl terminus of the *trans*-activating domain (carboxyl region$_{183-188}$) encompassing the original *trans*-dominant mutation at codon 185. Furthermore, outside of this contiguous stretch, in the region from 147 to 177 which contains the zinc finger, 11 residues that were critical for *trans*-activation were also critical for *trans*-dominance; i.e., second-site substitutions at these positions abrogated the *trans*-dominant phenotype of the 185SN protein. Most importantly, a *trans*-dominant phenotype was also produced by deletion of the carboxyl region$_{183-188}$. On the basis of these results, we propose that the finger region$_{147-177}$ serves to bind a limiting cellular transcription factor. We discuss a model of *trans*-activation in which the finger region$_{147-177}$ and the carboxyl region$_{183-188}$ represent distinct factor-binding subdomains within the *trans*-activating domain of E1A.

MATERIALS AND METHODS

Construction of mutants. To create the E1A plasmid used in these studies, pSK(−)E1A, an adenovirus type 2 (Ad2)/Ad5 E1A 13S cDNA equivalent in the context of the Ad5 E1A genomic background, was cloned from pJN20 (20) into the *Eco*RI-*Kpn*I polylinker sites of the pBluescript SK(−) vector (Stratagene). pSK(−)E1A expresses only the 289R protein. Mutagenesis of pSK(−)E1A was performed with oligonucleotides ranging from 13 to 45 nucleotides in length, using the Amersham mutagenesis kit or the method of Kunkel et al. (26). The 12 finger region/185SN double mutants were created by using pSK(−)E1A185SN as template. The E1A mutant F.S. encodes a nonfunctional 188-amino-acid protein that is shifted in reading frame after F142 by the insertion of deoxyguanylic acid between nucleotides 985 and 986 of Ad5 and contains a novel sequence from residues 143 to 188. pSK(−)E1A176MK was constructed by replacing the *Msc*I-*Kpn*I segment of pSK(−)E1A with the corresponding *Msc*I-*Kpn*I fragment of a 13S cDNA containing the *hr*3 mutation. The five mutants 173LF, 176MK, 185SN (12, 13), 180GD, and 185SG (29) were newly constructed for this study in pSK(−)E1A. All mutations were confirmed by DNA sequencing (51) using supercoiled miniprep DNA templates (8). For each mutant, the entire coding region for the *trans*-activating domain was sequenced.

Transfections. HeLa cells were maintained in Dulbecco modified Eagle medium plus 8% fetal bovine serum. E1A and reporter plasmid DNAs supplemented with pBluescript SK(−) vector to 20 μg/100-mm plate were transfected into cells by the calcium phosphate precipitation technique (14, 15). The reporter plasmid p3CAT contains the chloramphenicol acetyltransferase (CAT) gene driven by the E3 promoter of adenovirus (60). Cells were harvested 40 h posttransfection in phosphate-buffered saline and pelleted at 6,000 rpm in an Eppendorf microfuge. Cells were resuspended by gentle vortexing in 0.1 ml 0.25 M Tris, pH 8.0, and were disrupted by three consecutive freeze-thaw cycles in dry ice-ethanol 37°C baths. Extracts were assayed for CAT activity as described previously (12, 60). CAT activity was quantitated by using an Ambis Systems, Inc., radioanalytic scanner. To generate *trans*-dominant curves, the ratios of mutant/wt transfected DNAs were 1:1 (2 μg:2 μg), 2.5:1 (2.5 μg:1 μg), 5:1 (5 μg:1 μg), and 10:1 (10 μg:1 μg). To correct for inhibition in the *trans*-dominance assays due to promoter competition for cellular factors, the CAT activity for each ratio of mutant to wt DNA was divided by the CAT activity for the same ratio of the frameshift mutant (F.S.) to wt DNA. The deviation from the mean for all CAT assays was generally less than 30%.

Western immunoblot analysis. HeLa cells were transfected with 20 μg of mutant E1A plasmid plus 5 μg of the internal control plasmid pTKGH, which contains the human growth hormone gene driven by the herpes simplex virus type 1 thymidine kinase gene promoter. After 40 h, cells were harvested as described above for CAT assays. Proteins from approximately one-half of each supernatant extract, normalized by a growth hormone assay (Nichols Institute Diagnostics), were fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto an Immobilon P membrane (Millipore) for 70 min at 80 mA, using a Bio-Rad Transblot. Blocking of the membrane was performed overnight in 0.5% gelatin. E1A protein was detected with anti-E1A antisera (kind gift of Jeffrey S. Culp) and [^{125}I]protein A.

RESULTS

Single conservative substitutions define residues that are critical for E1A *trans*-activation. To identify specific amino acids critical for E1A *trans*-activation, conservative replacements were made at each position of the *trans*-activating domain and immediate flanking regions. At certain positions, nonconservative substitutions were also made (Fig. 1). In this way, a complete series of full-length 289R E1A proteins with single amino acid substitutions from positions 137 to 190 was generated and tested for *trans*-activation of an E1A-inducible promoter-driven CAT gene following cotransfection into HeLa cells.

Sixteen conservative substitutions reduced *trans*-activation to between basal and 28% of wt E1A activity (Fig. 2A). A reduction to this level of activity is considered significant since it is sufficient to severely impair productive infection by the E1A point mutant viruses *hr*3 (176MK), *hr*4 (173LF), and *hr*5 (185SN) (12). A summary of the critical residues appears in Fig. 2B. Amino terminal to the zinc finger, only two residues, V147 and P150, are sensitive to substitutions; although there is no truly conservative substitution for proline, we note that the substitution of glycine for proline at positions 167 and 190 has no effect on *trans*-activation. Conservative substitutions for each of the four cysteines, C154, C157, C171, and C174, abolish *trans*-activation. We have definitively shown these cysteines to be the Zn(II) ligands of a finger structure (7, 59). The only residue in the 13-amino-acid loop of the finger that is critical for *trans*-activation is H160. Also essential are S172, located between the carboxyl pair of cysteines of the zinc finger, and Y175, R177, V183, Y184, S185, P186, V187, and S188, which are carboxyl terminal to the zinc finger. L173, M176, and G180 represent examples of residues which can tolerate conservative substitutions but not nonconservative substitutions (Fig. 2A; 12, 30).

Western blot analysis confirmed that, in general, the *trans*-activation-defective mutant proteins and the wt protein were present at similar levels in the transfected HeLa cells (Fig. 2C). For certain mutants, especially 147VL, the

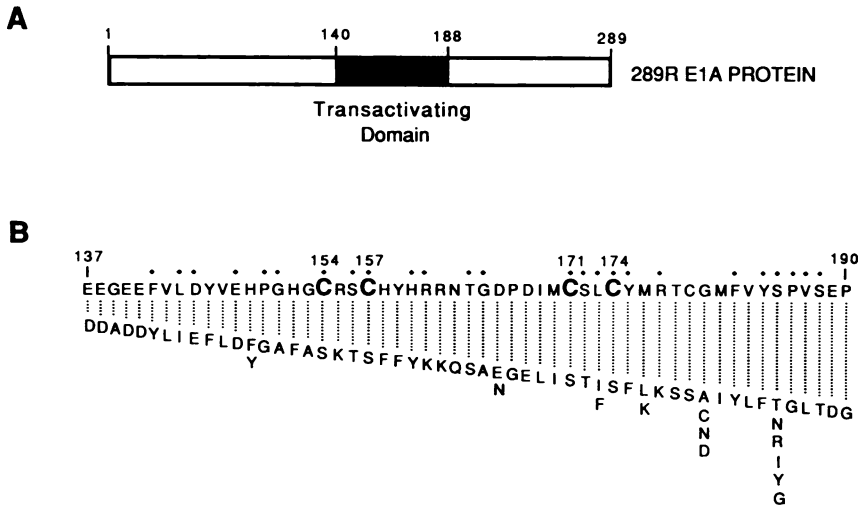

FIG. 1. Single amino acid substitutions introduced into the 289R E1A protein. (A) The 289R E1A protein. The *trans*-activating domain is composed of amino acids 140 through 188. (B) The sequence of the wt 289R protein from residues 137 to 190 encompassing the *trans*-activating domain, displayed horizontally, and the individual substitutions at each position, displayed diagonally. Except for 149HY, all substitutions below the diagonal are nonconservative. The cysteines of the zinc finger are highlighted in bold type. Residues from 137 to 190 which are highly conserved among the adenovirus serotypes (23) are indicated by diamonds. Amino acids are represented by the single-letter code: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; Y, Tyr; V, Val.

decreased protein level could be a factor in its reduced activity. However, we note that while the level of 183VL also appears to be reduced, its abundance is suggested by its ability to strongly interfere with wt activity (see below). Thus, the defect in each of the nonfunctional E1A mutants does not appear to be attributable to a decrease in protein stability.

Point mutants which generate *trans*-dominance map to a discrete region C terminal to the zinc finger. We have previously reported that a 289R protein with a single amino acid substitution at position 185 (185SN) is defective for *trans*-activation. In addition, this mutant protein interferes with the ability of the wt protein to *trans*-activate E1A-inducible promoters (12, 13). This negative *trans*-dominance is thought to be caused by the sequestration of a limiting cellular transcription factor by the 185SN protein, making the factor unavailable to the wt E1A protein. Each of the *trans*-activation-defective mutants described above, including those with nonconservative substitutions, was assayed for a negative *trans*-dominant phenotype. The wt E1A plasmid and p3CAT reporter plasmid were cotransfected with increasing amounts of each mutant E1A plasmid at mutant/wt ratios of 1:1, 2.5:1, 5:1, and 10:1. We noted that one set of mutants exhibited a strong or moderate *trans*-dominant phenotype and that these mapped to a region (residues 180 to 188) C terminal to the zinc finger (Fig. 3). The remaining mutants exhibited weak or no *trans*-dominance, and these mapped to a region (residues 147 to 177) encompassing the zinc finger. Although there is not a large difference in the strength of *trans*-dominance between a select few of the finger region$_{147-177}$ mutants and the 180–188 region mutants (compare 157CS and 184YF), the vast majority of the mutants reveal an apparent distinction between the finger region$_{147-177}$ and the carboxyl region$_{183-188}$ with respect to *trans*-dominance. We note that of the 180–188 region mutants, 180GD, 183VL, 185SN, 185SR, and 185SG were the most *trans*-dominant, whereas 184YF, 185ST, 185SI, 185SY, 186PG, 187VL, and 188ST were moderately *trans*-dominant (Fig. 3A). Since the conservative 185ST mutant was *trans*-dominant, it is not surprising that every nonconservative substitution at this position (185SI, 185SY, 185SN, 185SR, and 185SG) also produced *trans*-dominance (Fig. 3).

Is it possible that the carboxyl region$_{180-188}$ constitutes a functional subdomain, since a distinct phenotype is associated with mutations in this segment of E1A? It is generally held that a phenotypic effect produced by a conservative substitution more strongly supports a direct role for the substituted residue in the structure and function of a protein than does a nonconservative substitution. In this regard, it may be of significance that while the nonconservative substitution at position 180 (glycine to aspartate) ablates *trans*-activation and generates *trans*-dominance (Fig. 3), a conservative substitution (glycine to alanine) has only a minor effect on *trans*-activation (compare 180GD and 180GA; Fig. 2A). Therefore, the only conservative substitutions in the carboxyl region$_{180-188}$ that generate *trans*-dominance lie in a contiguous stretch from 183 to 188. Although position 180 could represent the true boundary of a functional subdomain that includes residues 183 to 188, it is perhaps more likely that the drastic nature of the glycine-to-aspartate substitution generates *trans*-dominance by simply perturbing the conformation of the 183–188 region. Therefore, we consider the 183–188 region a functional subdomain, and we refer to this contiguous stretch as the carboxyl region$_{183-188}$.

***trans*-Dominance is abolished by second-site substitutions in the finger region.** Since the carboxyl region$_{183-188}$ mutants are thought to act by sequestering a transcription factor, it is important to know which region of the *trans*-activating domain might participate in this binding. Each of 11 substitutions in the finger region$_{147-177}$ which individually destroyed *trans*-activation was introduced into the *trans*-dominant mutant 185SN as a second-site substitution (Fig. 4A). Without exception, these second-site substitutions destroyed *trans*-dominance (Fig. 4B). In contrast, the 180GD substitution, which alone produced *trans*-dominance, failed

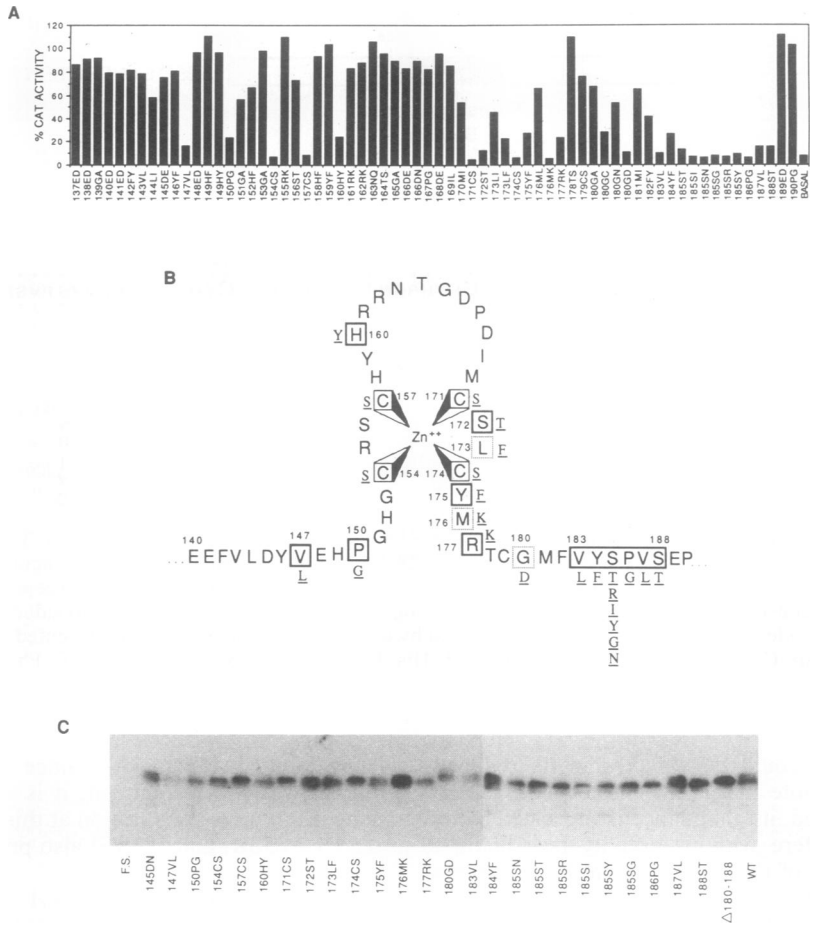

FIG. 2. *trans*-Activation by mutant 289R E1A proteins. (A) *trans*-Activation ability of each 289R E1A point mutant displayed in Fig. 1B relative to that of the wt 289R E1A protein. The mutants are designated by position number followed by the wt residue and the mutant residue. HeLa cells were cotransfected with 5 μg of mutant E1A plasmid and 5 μg of p3CAT reporter plasmid. CAT activity of cell extracts was assayed as described in Materials and Methods and quantitated by using an Ambis radioanalytic scanner. Each data point represents the average of at least three experiments. The wt 289R E1A activity is equivalent to 100%. (B) Single amino acid substitutions which most severely affect *trans*-activation. The zinc finger structure of the *trans*-activating domain is represented as a loop of peptide sequence folded around a zinc ion which is coordinated to four cysteines. Positions that cannot tolerate conservative substitutions are within solid boxes; positions that can tolerate conservative but not nonconservative substitutions are within dotted boxes (L173, M176, and G180). (C) Western blot analysis of the *trans*-activation-defective mutant E1A proteins. HeLa cells were cotransfected with 20 μg of each mutant E1A plasmid and 5 μg of pTKGH, an internal standard plasmid which expresses human growth hormone. Proteins from normalized cell extracts were fractionated by polyacrylamide gel electrophoresis, transferred to a nylon membrane, and probed with anti-E1A antisera and [^{125}I]protein A. The resolution of the 289R E1A protein into two major bands of 45 and 47 kDa is due to differential phosphorylation (50). The mutant proteins encoded by Δ180–188 and 145DN are described in Fig. 5 and 6, respectively. The frameshift mutant, F.S., which encodes a nonfunctional 188-amino-acid protein visible upon longer exposure, was used as a negative control. WT designates the wild-type 289R E1A protein. Repeated experiments showed that variation in the band intensities was random from experiment to experiment. The slight differences in migration of the mutant proteins may be attributed to altered conformation or phosphorylation. It is noteworthy that there are no apparent phosphorylation sites in the *trans*-activating domain itself (56).

to abolish *trans*-dominance as a second-site substitution. The loss of *trans*-dominance by the finger region$_{147–177}$/185SN double mutants could not be accounted for by a decrease in protein stability, as evidenced by Western blot analysis (Fig. 4C). Thus, *trans*-dominance can be abrogated by single amino acid substitutions in the finger region$_{147–177}$, implying that this region participates in the binding of a transcription factor.

Deletion of the carboxyl region produces strong *trans*-dominance. Even though the previous experiments indicated the requirement of the finger region$_{147–177}$ for binding a factor, they did not determine whether the carboxyl region$_{183–188}$ also contributes to the binding. We therefore constructed a mutant which deleted residues 180 through 188 and examined whether it generates the *trans*-dominant phenotype as do single amino acid substitutions in this region. As shown in Fig. 5, this deletion produced an E1A protein which was strongly *trans*-dominant, indicating that the finger region$_{147–177}$ can bind a factor in the complete absence of the carboxyl region. This result firmly establishes that the finger region$_{147–177}$ and the carboxyl region$_{183–188}$ each constitutes a distinct functional subdomain.

The E1A *trans*-activating domain does not contain an acidic activating region. The *trans*-activating domain contains four negatively charged residues N terminal to the zinc finger (E140, E141, D145, and E148) which were originally pro-

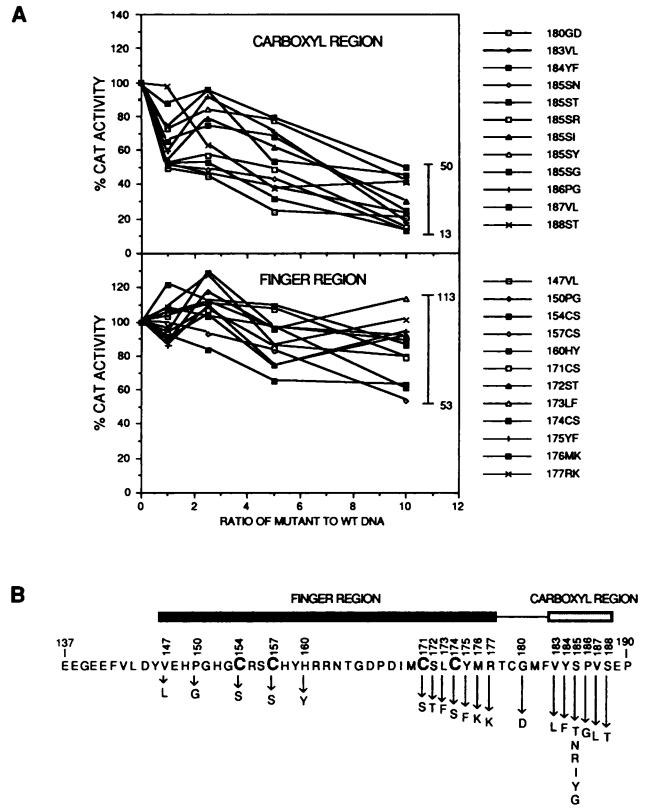

FIG. 3. Mutations that generate *trans*-dominance map to a discrete region C terminal to the zinc finger. (A) Ability of *trans*-activation-defective point mutants to inhibit *trans*-activation by the wt E1A 289R protein. HeLa cells were contransfected with the p3CAT reporter plasmid and increasing ratios of mutant E1A to wt E1A plasmid DNAs. The vertical bar in each set of plots represents the range of CAT activity at the highest ratio (10:1). Each *trans*-activation-defective mutant in Fig. 2B was individually tested. To correct for inhibition due to promoter competition for cellular factors, the CAT activity for each ratio of mutant to wt DNA was made relative to the CAT activity for a nonfunctional frameshift (F.S.) mutant to wt DNA at the same ratio. *trans*-Dominant plots for the carboxyl region$_{183-188}$ and the finger region$_{147-177}$ mutants are shown separately. Each point represents the average of three experiments. (B) Mapping of substitutions. Conservative substitutions which most effectively produced *trans*-dominance map to the contiguous carboxyl region$_{183-188}$ (stippled bar). Conservative and nonconservative substitutions that were only weakly *trans*-dominant or were not *trans*-dominant map to the finger region$_{147-177}$ (black bar). The G-to-D substitution at 180 generated strong *trans*-dominance, which suggests either that 180 represents the true boundary of the carboxyl region$_{183-188}$ subdomain or that the radically nonconservative nature of this substitution alters the conformation of the carboxyl region$_{183-188}$.

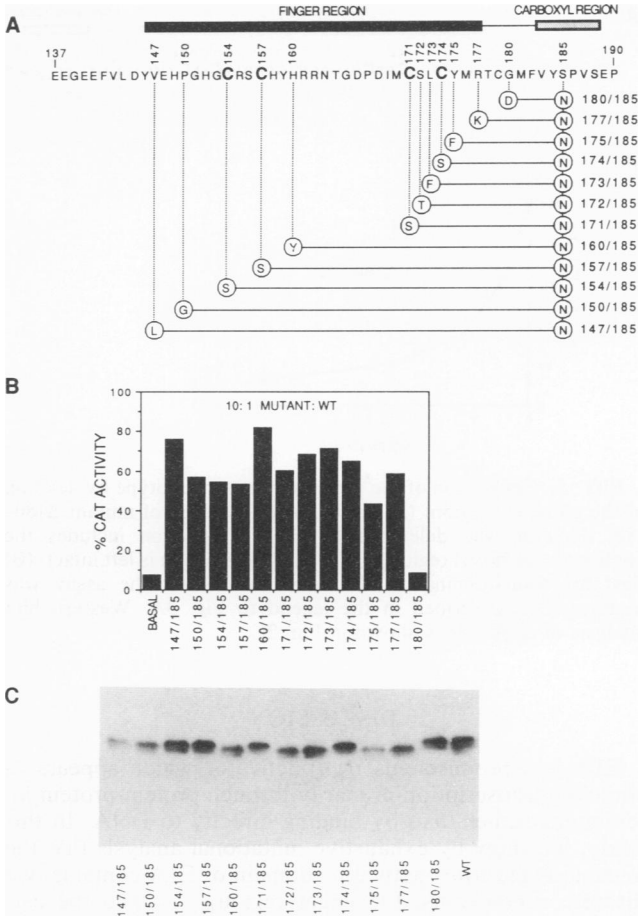

FIG. 4. Destruction of the *trans*-dominant phenotype by second-site substitutions in the finger region$_{147-177}$. (A) E1A double mutants. Eleven deleterious substitutions in the finger region as well as the *trans*-dominant 180GD substitution were individually introduced into E1A 185SN protein. (B) Test for *trans*-dominance. The ability of double mutants to inhibit wt E1A *trans*-activation was assayed as described for Fig. 3A, using a mutant/wt ratio of 10:1. Only the 180GD/185SN double mutant retains significant *trans*-dominance. (C) Western blot analysis. The expression of double mutant E1A proteins in transfected HeLa cells was assayed as described for Fig. 2C.

posed by Lillie and Green (29) to comprise an acidic activating structure, since they could be functionally replaced by the acidic activating region of VP16. As shown above, each of these acidic residues can be substituted by another acidic residue (140ED, 141ED, 145DE, and 148ED) with no loss of *trans*-activation (Fig. 2A). However, the overall net negative charge contributed by these residues was found not to be important for *trans*-activation. We generated 11 point mutants in which the neutral amino acids glutamine and asparagine replaced glutamate and aspartate, respectively, either singly, in double or triple combinations, or all together (Fig. 6). Neither single, double, nor triple nonconservative substitutions of glutamine for E140, E141, and E148 had a significant effect on *trans*-activation. Only combinations that included the substitution of asparagine for aspartate 145 reduced *trans*-activation, by about threefold. Thus, while negative charge at position 145 does appear to be important, the cumulative negative charge supplied by the other three acidic residues plays no role in *trans*-activation. A similar conclusion is supported by the recent findings of Martin et al. (35).

It is formally possible that the negatively charged residues located between positions 133 and 138 functionally replace the lost acidity in our mutants or are important for activation in the wt molecule. However, the latter possibility is not supported by experiments using GAL4-E1A fusion proteins containing progressive N-terminal deletions of E1A sequences (29, 35). We conclude that E140, E141, D145, and E148 are not specifically required for *trans*-activation.

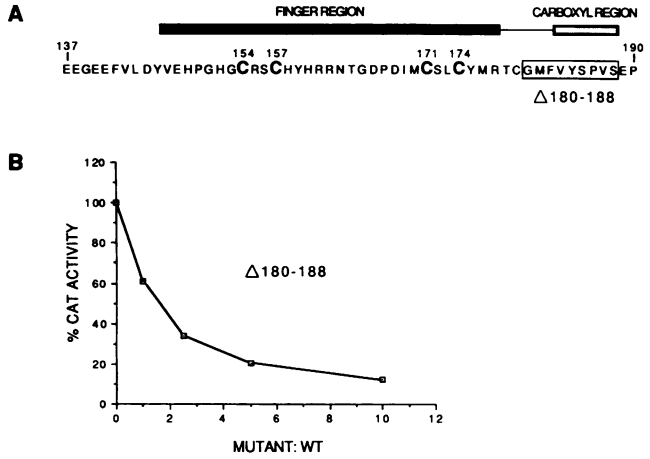

FIG. 5. Generation of the *trans*-dominant phenotype by deletion of the carboxyl region. (A) Amino acid sequence of mutant Δ180–188, depicting the deleted residues. The deletion includes the contiguous carboxyl region$_{183–188}$. The finger region is left intact. (B) Test for *trans*-dominance of mutant Δ180–188. The assay was performed as described in the legend to Fig. 3A. Western blot analysis of Δ180–188 is shown in Fig. 2C.

DISCUSSION

E1A is a promiscuous *trans*-activator which appears to stimulate transcription primarily through protein-protein interactions rather than by binding directly to DNA. In this study, we show by exhaustive mutational analysis that the 49-amino-acid *trans*-activating domain of E1A contains two discrete subdomains, the finger region$_{147–177}$ and the carboxyl region$_{183–188}$. While mutations in either subdomain can destroy *trans*-activation, only mutations in the carboxyl region$_{183–188}$ produce a strong *trans*-dominant phenotype. Our results indicate that the finger region$_{147–177}$ binds a limiting cellular transcription factor. The possibility that the carboxyl region$_{183–188}$ binds a distinct cellular factor is discussed below.

The *trans*-activating domain is comprised of two distinct subdomains. A systematic point mutational analysis was first used to delineate all critical residues in the *trans*-activating domain of E1A. Conservative substitutions were made since they most effectively reveal residues whose side chains are strictly required for structure or function (4). Of the 49 amino acids of the *trans*-activating domain, only 16 were found to be critical for *trans*-activation. They include V147 and P150 N terminal to the finger; C154, C157, C171, and C174, which function as zinc ligands; H160 in the large central loop of the finger and S172 in the C-terminal knuckle of the finger; and Y175, R177, V183, Y184, S185, P186, V187, and S188 C-terminal to the finger. Every one of these critical residues is highly conserved among adenovirus serotypes except for V147, which in all other serotypes is proline. It is noted that 11 other residues in the *trans*-activating domain which are also highly conserved were relatively unaffected by conservative substitutions.

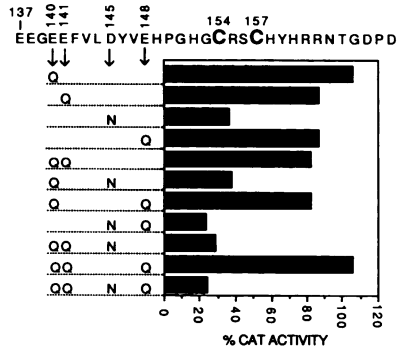

FIG. 6. Evidence that the negatively charged residues of the *trans*-activating domain do not comprise an acidic activating region. Eleven mutants in which the four acidic residues E140, E141, D145, and E148 were replaced with the neutral amino acid glutamine (Q) or asparagine (N) either singly, in double or triple combinations, or all together were assayed for *trans*-activation as described for Fig. 2A. A negative charge appears to be important only at position 145.

The E1A point mutant *hr5* (185SN) represented the first example of a negative *trans*-dominant viral *trans*-activator (13). The 185SN protein interfered with *trans*-activation of early promoters by the wt E1A protein in both infected and plasmid-transfected cells. This interference appeared to result from the ability of the 185SN protein to sequester a limiting cellular transcription factor, making it unavailable to the wt E1A protein (13). We wished to know whether serine-to-asparagine is the only substitution at position 185 capable of generating a *trans*-dominant phenotype. That each of five additional substitutions made at position 185 also manifested the *trans*-dominant phenotype suggests that it is the absence of serine at this position which is the specific determinant of this phenotype, rather than the nature of the particular amino acid substituted.

Significantly, the *trans*-dominant phenotype is shared by proteins with single conservative amino acid substitutions immediately adjacent to serine 185; these substituted positions form a contiguous stretch carboxyl to the finger, from V183 to S188. A nonconservative substitution at position 180 also produced *trans*-dominance, but because of the drastic nature of this substitution, it is not clear whether position 180 represents the true N-terminal boundary of a subdomain that includes residues 183 to 188. In contrast, none of the amino acid replacements in the finger region$_{147–177}$ generated a strong *trans*-dominant phenotype; moreover, these substitutions abrogated the *trans*-dominance of the 185SN protein. Most importantly, the finger region$_{147–177}$ can confer *trans*-dominance in the absence of the carboxyl region$_{183–188}$. The phenotypic disparity between the carboxyl region$_{183–188}$ and the finger region$_{147–177}$ mutants indicates that they are distinct functional subdomains within the *trans*-activating domain of E1A.

Sequestration of a cellular transcription factor is mediated by the finger region. Although there is no clear evidence that E1A multimerizes, we cannot rule out the possibility that the wt E1A and *trans*-dominant mutant proteins form nonproductive oligomers. Alternatively, the E1A *trans*-dominant mutants may mediate their effect by titrating a cellular transcription factor. We propose that the primary function of the E1A zinc finger region is to bind a limiting cellular transcription factor (Fig. 7A). Mutation of the carboxyl region, but not the finger region, generates a strong *trans*-dominant phenotype (Fig. 7B and C). This suggests that the sequestration of a limiting cellular transcription factor is mediated by the finger region. An intact finger region is required for *trans*-dominance, since each substitution in the finger region which destroys *trans*-activation also abolishes the *trans*-dominance produced by an accompanying substitution in the carboxyl region (Fig. 7D). The most likely

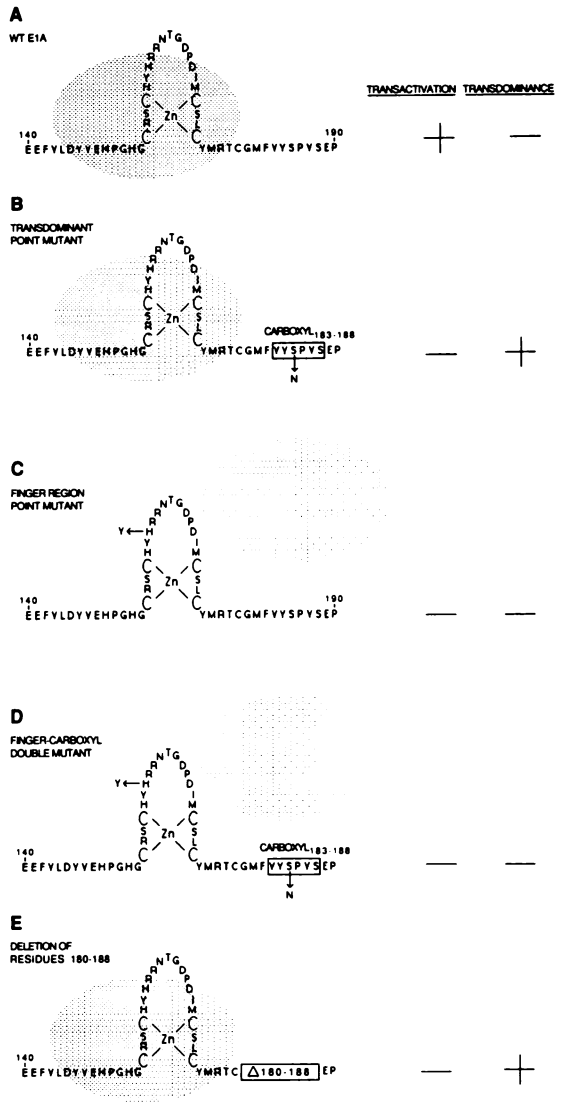

FIG. 7. Proposed mechanism of E1A *trans*-dominance. The shaded oval represents a limiting cellular transcription factor bound by E1A through the finger region (A, B, and E) or no longer bound by E1A as a result of mutation of the finger region (C and D). *trans*-Activation requires both an intact finger region and an intact carboxyl region. *trans*-Dominance occurs when the carboxyl region is mutated and the finger region is left intact.

interpretation is that the finger region performs the same function in *trans*-dominance as in *trans*-activation. That the finger region is capable of binding a transcription factor independently of the carboxyl region is confirmed by the observation that deletion of residues 180 to 188 produces a powerful *trans*-dominant phenotype (Fig. 7E).

Purified E1A does not bind DNA in a sequence-specific manner (5, 10). In this sense E1A is unique because the zinc finger regions of other regulatory proteins serve as sequence-specific DNA-binding structures (9; reviewed in reference 25). There is evidence, however, that some zinc fingers additionally interact with proteins. For example, certain mutations in the CII region zinc finger of the glucocorticoid receptor and in the zinc finger region of the yeast activator HAP1 affect *trans*-activation without altering DNA binding (22, 52). Likewise, it is conceivable that when complexed with cellular proteins, the E1A zinc finger could also bind to DNA.

Our results using mutant forms of the full-length 289R protein which indicate a role for the E1A zinc finger as a factor-binding structure are consistent with recent experiments involving fusion proteins (29, 32, 35). E1A residues 121 to 223 appended onto the yeast GAL4 or the bacterial LexA DNA-binding domains activate transcription from promoters bearing GAL4 or LexA binding sites, respectively. With a reporter containing LexA sites, squelching of the LexA-E1A$_{121-223}$ fusion protein by the GAL4-E1A$_{121-223}$ fusion protein was observed. This is very likely the same phenomenon as the *trans*-dominance described in our studies. Importantly, the squelching of LexA-E1A$_{121-223}$ was destroyed by point mutations in or near the E1A zinc finger of GAL4-E1A$_{121-223}$ which also destroyed the ability of the mutant itself to activate a promoter bearing GAL4 sites. As was originally proposed by Glenn and Ricciardi (13) for the *trans*-dominant 185SN E1A protein, Martin et al. (35) suggested that the squelching of activation resulted from the titration of a cellular transcription factor.

The *trans*-activating domain lacks an acidic activating region. The DNA-binding and activation functions encoded by most gene regulatory proteins map to separate domains (reviewed in reference 45). Activating regions require the presence of negatively charged residues, as in the case of the GCN4, GAL4, and VP16 proteins (17, 33, 57), or glutamine and proline, in the case of Sp1 and NF-1, respectively (6, 38). The important feature of acidic activating regions appears to be the total number of negatively charged residues rather than any one residue in particular. Activating regions are thought to stimulate transcription by making critical contacts with the basal transcription machinery, in particular TFIID (1, 18, 19). The most direct support for this view is the recent demonstration that an affinity column bearing the acidic activating region of VP16 selectively retained the TATA-binding factor, TFIID (54).

Lillie and Green (29) proposed that the negatively charged residues (E140, E141, D145, and E148) in the amino-terminal portion of the *trans*-activating domain constitute an acidic activating region. They showed that the fusion protein GAL4-E1A$_{139-223}$ consisting of the yeast GAL4 DNA-binding domain, and E1A residues 139 to 223, which encompass the *trans*-activating domain, could stimulate the adenovirus E4 promoter. The significant loss of *trans*-activation by the deletion of E1A residues 139 to 149 in GAL4-E1A$_{150-223}$ could be restored by adjoining the 78-amino-acid acidic activating region of VP16 to the carboxyl terminus of this fusion protein. It was concluded that the four acidic residues between 140 and 149 function as an acidic activating region.

In contrast, our experiments employing full-length 289R proteins do not support the existence of an acidic activating region in the *trans*-activating domain of E1A. We replaced E140, E141, D145, and E148 with neutral amino acids either singly or in combination. A significant effect on *trans*-activation was observed only with mutants that included the neutral substitution at position 145. Since the substitution of glutamate for aspartate 145 did not significantly affect *trans*-activation, a negative charge may be specifically important at position 145. However, as the hallmark of acidic activating regions is a correlation of net negative charge with activating strength, and since no such correlation was found with the mutants in this study, we conclude that E140, E141, D145, and E148 do not comprise an independent acidic activating region. We note that while it is possible that our

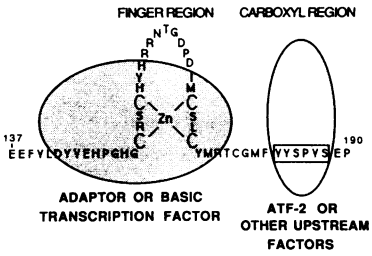

FIG. 8. Proposed model of transcription factor-binding sites in the E1A *trans*-activating domain. The finger region may make contact with the basal transcription machinery through association with an adaptor protein or a component of the basic transcription machinery, and the carboxyl region may bind to an upstream sequence-specific factor such as ATF-2 (see text).

fully functional glutamate-to-glutamine mutants may constitute a switch from an acidic activating region to a glutamine-rich activating region, substitution of the same residues with alanine in the background of a GAL4-E1A fusion protein had a similar effect (35). Indeed, Martin et al. (35) arrived at essentially the same conclusion as the one drawn from this study.

Model of factor binding to the E1A *trans*-activating subdomains. We propose a model in which the *trans*-activating domain of E1A binds to two different cellular protein targets through the finger region$_{147-177}$ and the carboxyl region$_{183-188}$ (Fig. 8). This model is based on the genetic evidence presented here and on recent findings which suggest an association between E1A and cellular transcription factors.

Carboxyl region. Using a promoter containing GAL4 sites, Liu and Green (32) showed that the GAL4 DNA-binding domain fused to either the retinoblastoma protein or the ATF-2 protein mediated *trans*-activation by the E1A 289R protein. This apparent ability of ATF-2 to direct E1A to a promoter was abolished by the E1A 180GD substitution (32). This mutation had no effect on the ability of GAL4-retinoblastoma to direct E1A to the same promoter, since retinoblastoma binds not to the *trans*-activating domain but to conserved regions 1 (residues 30 to 60) and 2 (residues 121 to 127) (61). Since the 180GD substitution abrogates the interaction of E1A with ATF-2 and since, as shown in the present study, 180GD and mutants with substitutions from 183 to 188 shared the *trans*-dominant phenotype, we suggest that one function of the carboxyl region$_{183-188}$ is to bind to transcription factors such as ATF-2 (Fig. 8). Because not all E1A-inducible promoters have ATF sites, it is intriguing to consider that the region of the ATF-2 protein with which the highly conserved carboxyl region$_{183-188}$ interacts may itself be an evolutionarily conserved structural motif common to a variety of sequence-specific transcription factors.

Finger region. We have previously demonstrated by spectrophotometric and X-ray absorption fine structure analysis that E1A has a C_4 zinc finger structure, which is required for *trans*-activation (7, 59). Our results from this study indicate that the finger region$_{147-177}$ binds a limiting cellular transcription factor and that this interaction is independent of the carboxyl region$_{183-188}$ (Fig. 8). This factor could be an adaptor protein (reviewed in references 28 and 46) through which E1A contacts the basic transcription apparatus. Alternatively, this factor may be a component of the basic transcription apparatus itself, e.g., TFIID. Such factor binding to the finger region$_{147-177}$ would result in augmented transcription only if it were accompanied by the binding of an upstream factor to the carboxyl region$_{183-188}$.

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