Restricted Changes in the Adenovirus DNA-Binding Protein That Lead to Extended Host Range or Temperature-Sensitive Phenotypes

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Human adenovirus fails to multiply efficiently in monkey cells owing to a block to late viral gene expression. Ad2hr400 through Ad2hr403 are a set of host range (*hr*) mutants which were selected for their ability to readily grow in these cells at 37°C. The mutations responsible for this extended host range have previously been mapped to the 5' portion of the gene encoding the 72-kilodalton DNA-binding protein (DBP). DNA sequence analyses indicate that all four *hr* mutants contain the same alteration at coding triplet 130, which changes a histidine codon to a tyrosine codon. These results extend those of Anderson et al. (J. Virol. 48:31-39, 1983), which suggested that only this change in the DBP amino acid sequence can expand adenovirus host range to monkey cells. The *hr* phenotype does not appear to require phosphorylation of this tyrosine residue, since no phosphotyrosine was detected in DBP isolated from Ad2hr400-infected monkey cells. The *hr* mutants Ad2hr400 through Ad2hr403, however, are cold sensitive for growth in monkey cells. The mutant Ad2ts400, which was derived from Ad2hr400, represents a second class of *hr* mutants which can grow efficiently in monkey cells at 32.5°C. The cold-resistant *hr* mutation of Ad2ts400 has previously been mapped to the 5' region of the DBP gene (map units 63.6 through 66). DNA sequence analysis of this region shows that this mutant contains the original *hr* alteration at coding triplet 130 as well as a second alteration at coding triplet 148, which changes an alanine codon to a valine codon. We suspect that the alterations at amino acids 130 and 148 change the structure of the amino-terminal domain of the DBP, allowing it to better interact with monkey cell components required for late viral gene expression. Ad2ts400 also contains a temperature-sensitive mutation which has previously been mapped to the 3' portion of the DBP gene (map units 61.3 through 63.6). Sequence analysis of this region indicates that the DBP coding triplet 413 has been altered. This change from a serine codon to a proline codon is the same alteration reported in the previously sequenced DBP mutants Ad5ts125 (W. Kruijer et al., Nucleic Acids Res. 9:4439-4457, 1981) and Ad5ts107 (W. Kruijer et al., Virology 124:425-433, 1983). Thus it appears that only a very limited number of changes in either the 5' or the 3' portion of the DBP gene can give rise to the *hr* or temperature-sensitive phenotypes, respectively.

The nonstructural adenovirus 72-kilodalton (kd) DNA-binding protein (DBP) is a multifunctional phosphoprotein encoded in early region 2A of the adenoviral genome (coordinates 61.6 through 66.5) (10, 13, 30, 41, 55). DBP is required for viral DNA replication and appears to play a central role in regulating viral gene expression. Two classes of DBP mutants, each affecting very different activities and mapping in distinct regions of the early region 2A gene, have been isolated.

One mutant class consists of temperature-sensitive (*ts*) mutants and is typified by the adenovirus type 5 (Ad5) mutants Ad5ts125 (17) and Ad5ts107 (50). Studies with these mutants suggest a role for the DBP in viral DNA replication, early gene expression, virus assembly, and cellular transformation. At the nonpermissive temperature these *ts* mutants are unable to replicate viral DNA (17, 29), presumably owing to the thermolabile nature of the single-stranded DNA binding activity of the *ts* DBP (56). Furthermore, in Ad5ts125-infected cells the normal turnoff of early gene mRNA expression does not occur at the nonpermissive temperature (11, 12). The negative regulation of early gene expression mediated by wild-type (wt) DBP appears to act at the level of transcription for early region 4 (26, 45) and at the level of mRNA turnover for early regions 1A and 1B (8). Studies on a revertant of Ad5ts107, R(ts107)202, suggest an additional role for DBP in virus assembly (46). R(ts107)202 has re-

gained a wt phenotype in human HeLa cells but remains *ts* for growth in human 293 cells. This mutant appears to replicate DNA and express late genes normally in 293 cells at the nonpermissive temperature but fails to assemble virion particles. Finally, DBP appears to play a role in modulating the efficiency of transformation of rat cells, since Ad5ts125 transforms rat cells with three- to fivefold greater efficiency than does wt virus (24, 57).

The second class of early region 2A mutants are host range (*hr*) mutants, which have been selected for the ability to grow efficiently in monkey kidney cells (1, 32, 35). The wt adenovirus does not grow well in monkey cells, even though virus adsorption (20), early gene expression (3, 20, 23), and DNA replication (23, 27, 48) all occur normally. The defect appears to involve a block to late gene expression, since the levels of late viral proteins are diminished (9, 23, 33). This reduction can, in most cases, be accounted for by the decreased levels of late mRNA's (19, 33) which result from reduced transcription (J. Johnston, K. P. Anderson, and D. F. Klessig, manuscript in preparation). An exception is the capsid fiber protein, in which altered mRNA splicing or poor utilization of the message, or both, also contributes to the block in protein synthesis (4, 5, 34). Mutations in the early region 2A gene allow adenovirus to overcome the block to late gene expression and grow productively in monkey cells.

Genetic mapping and DNA sequence determination of the two mutant classes and their revertants have suggested that

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the DBP contains distinct functional domains (2, 36–39, 49), since the *ts* mutations and their second site revertants map in the 3' segment of the DBP gene, whereas the *hr* mutations map in the 5' segment. Furthermore, analysis of the mutant Ad2*ts*400, which contains mutations of both types, has shown that the *hr* DBP can overcome the block to late gene expression in monkey cells even when the viral DNA replication activity of the protein is nonfunctional (49). The two functional domains of DBP may correspond to the two physical domains revealed by the sensitivity of DBP to several proteolytic enzymes. The C-terminal 44-kd fragment retains DNA binding activity (31) and is functional in an *in vitro* DNA replication system (6). The smaller, heavily phosphorylated N-terminal 26-kd fragment (31) presumably carries the activity required for late gene expression in monkey cells (49) and probably in human cells as well.

Here we report the DNA sequence analyses of Ad2*hr*400 through Ad2*hr*403, four independently derived *hr* mutants of Ad2 that grow efficiently in monkey kidney cells. Our results extend those of Anderson et al. (2), who suggested that only a specific change in the DBP amino acid sequence can expand adenovirus host range to monkey cells. However, the sequence analysis of Ad2*ts*400 shows that an additional change in the N-terminal segment of DBP can allow the virus to grow even more efficiently in monkey cells than do Ad2*hr*400 through Ad2*hr*403. Characterization of the alteration responsible for the *ts* phenotype of Ad2*ts*400, in conjunction with similar analyses of other DBP *ts* mutants (37–39), suggests that only a very limited number of changes can impart the *ts* phenotype.

MATERIALS AND METHODS

Cells, virus, and infections. Propagation of cells and isolation of virus and viral DNA were performed as previously described (49). Viral infections were performed monolayers as previously described (49).

Cloning of adenovirus DNA. The *Eco*RIB restriction fragments of Ad2*hr*400 through Ad2*hr*403 and Ad2*ts*400 were cloned in the *Eco*RI restriction site of pBR322. Plasmid DNA was prepared by the method of Tanaka and Weisblum (53). The *Kpn*I-*Sst*I fragment (map units [mu] 61.3 through 69.9) was isolated from the plasmid containing the Ad2*ts*400 *Eco*RIB fragment. A 1- μ g portion of this DNA was digested with 0.05 U of Bal 31 nuclease (Bethesda Research Laboratories) for 1 min at 16°C in 100 μ l (total volume) of the buffer suggested by Maniatis et al. (42). This DNA was then treated with the Klenow fragment of DNA polymerase (42) and cloned into the *Xba*I site of M13mp19 (42) in both orientations by using *Xba*I linkers (P-L Biochemicals, Inc.). Deletions of the parent M13 clones were generated with exonuclease III (New England BioLabs) by the method of Henikoff (28). JM105 was transformed with the various M13mp19 plasmids by the method described in the 1982 P-L Biochemicals handbook (*Manual for the M13 Cloning/Sequencing Kit*, p. 9). β -Galactosidase-negative clones were screened on LB plates supplemented with 0.03 mM isopropyl- β -D-thiogalactopyranoside (Sigma Chemical Co.) and 0.03% 5-bromo-4-chloro-3-indolyl-D-galactoside (Boehringer Mannheim Biochemicals) as described previously (44). M13 clones were grown in LB medium. The protocol of Messing (44) was used to isolate M13 replicative form DNA. M13mp19 was kindly provided by J. Messing.

DNA sequence analysis. The DNA sequence for the DBP gene of Ad2*hr*400 was determined by the method of Maxam

and Gilbert (43). Restriction enzyme digests (New England BioLabs) of plasmid DNA were performed under the conditions recommended by Maniatis et al. (42). The resultant DNA fragments were separated on agarose gels and recovered by electroelution (42). The isolated fragments were dephosphorylated with bacterial alkaline phosphatase and then 5' end labeled with polynucleotide kinase and [γ -³²P]ATP (Amersham Corp.) (43). After cleavage with appropriate restriction enzymes to yield asymmetrically labeled fragments, the fragments were fractionated by acrylamide gel electrophoresis and eluted (43). The isolated fragments were subjected to the chemical degradation reactions of Maxam and Gilbert, except that 4% formic acid was used in place of 4% pyridine formate for the adenine-plus-guanine reaction. Each treated sample was analyzed by electrophoresis through denaturing 0.4 mM gels (40 cm by 20 cm) of three acrylamide concentrations, 20%, 10 or 8%, and 6%. After electrophoresis, each gel was subjected to autoradiography at -70°C with Kodak XAR film. In general, about 150 nucleotides of contiguous sequence could be determined for each labeled fragment. The general strategy is outlined in Fig. 1A. The *Bgl*II-*Eco*RI fragment (mu 63.6 through 70.7) from plasmid DNA was first digested with *Hpa*II or *Xho*I, and after 5' end labeling, the second restriction enzyme digestion was done with *Bst*EII. The *Bgl*II fragment (mu 60.2 through 63.6) was first digested with *Sst*II or *Bst*EII and then with *Bst*EII and *Kpn*I or *Bgl*II, respectively. The *Kpn*I-*Xho*I fragment (mu 61.3 through 66) was first digested with *Bst*EII and then with *Hpa*II.

The sequence around the coding triplet 130 was determined in the mutants Ad2*hr*401 through Ad2*hr*403. The *Bgl*II-*Eco*RI fragment (mu 63.6 through 70.7) from each plasmid was first digested with *Hpa*II, 5' end labeled, and

FIG. 1. Strategy used to sequence the DBP gene. The region of the adenovirus genome (mu 61.6 through 66.5) that encodes the DBP is depicted as a large open bar. Arrows indicate the direction of sequencing. (A) DNA sequence of the Ad2*hr*400 DBP was determined by the chemical degradation method of Maxam and Gilbert (43). Purified restriction enzyme fragments of DNA from pBR322 clones containing the *Eco*RIB fragment (mu 58.5 through 70.7) of Ad2*hr*400 were digested with various restriction enzymes, labeled at both ends with polynucleotide kinase, cut with other restriction enzymes to generate asymmetrically labeled fragments, purified, and sequenced. Relevant restriction sites are shown. (B) DNA sequencing of Sanger et al. (51) was used to determine the Ad2*ts*400 mutant DBP DNA sequence (mu 61.6 through 66). Thick arrows represent regions sequenced by using a 15-nucleotide universal primer and templates from the parent M13mp19 clones carrying the *Kpn*I-*Sst*I (mu 61.3 through 69.9) fragment or deletions of it generated by exonuclease III treatment (28). Thin arrows represent regions sequenced by the parent template and synthetic 20-nucleotide primers.

then digested with *Bst*EII to yield asymmetrically labeled fragments for sequence determination.

The sequence of Ad2*ts*400 DBP was determined by the dideoxynucleotide-primer extension method of Sanger et al. (51), with modifications described in the October 1983 New England Nuclear handbook (*Step-by-Step Protocol for ³⁵S-Sanger Sequencing*) for using [α -³⁵S]dATP as radioactive label. The M13 single-stranded DNA used as template was isolated by the protocol described in the 1980 Bethesda Research Laboratories handbook (*M13 Cloning/Dideoxy Sequencing Instruction Manual*, p. 29). A 15-nucleotide universal primer (New England BioLabs) was used for sequencing the full-length insert and the M13 deletion variants obtained from exonuclease III treatment. Gaps left in the DBP sequence because of lack of appropriate M13 deletions were filled by using 20-nucleotide primers synthesized and purified by preparative gel electrophoresis (Applied Biosystems, Inc.). Regions of DNA sequence containing putative mutations or sequence ambiguities were subjected to sequence analysis of the opposite strand with appropriate 20-nucleotide primers. The general sequencing strategy is illustrated in Fig. 1B.

Phosphoamino acid analysis. Ad2- or Ad2*hr*400-infected CV-1 cells were labeled from 22 to 24 h postinfection with 100 μ Ci of H₃³²PO₄ in 0.2 ml of Dulbecco modified Eagle medium minus phosphate supplemented with 2% dialyzed calf serum. Proteins were extracted at 0°C by a high-salt Nonidet P-40 extraction (15) in the presence of 30 μ g of phenylmethylsulfonyl fluoride and 100 μ M ammonium metavanadate to inhibit phosphatase activity (22). DBP was immunoprecipitated with a polyclonal rabbit antiserum made against native DBP and electrophoresed on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The proteins were electroeluted from the gel bands, passed over small Sephadex G-25 columns to desalt, and then subjected to partial acid hydrolysis (14). The hydrolyzed samples were lyophilized, solubilized in water, and dried a second time. The samples were then solubilized in 10 μ l of water containing phosphoserine, phosphothreonine, and phosphotyrosine as markers and were spotted onto chromatography paper (Whatman 3MM). Two-dimensional, high-voltage paper electrophoresis was performed as described by Clinton and Huang (14), except that electrophoresis was at pH 1.9 for 2 h in the first dimension and at pH 3.5 for 40 min in the second dimension. As a control, epidermal growth factor-activated protein kinase from human epidermoid carcinoma A-431 cell membranes was used to label an endogenous membrane-associated protein at tyrosine (54). This membrane preparation was kindly provided by S. Wiley.

RESULTS

Nucleotide sequence analysis of Ad2*hr*400 to Ad2*hr*403. The mutants Ad2*hr*400 through Ad2*hr*403 were isolated after nitrous acid mutagenesis of Ad2 and subsequent selection for variants which could grow productively in the established monkey kidney cell line CV-1 (35). Previously, genetic mapping of the mutants by marker rescue placed the mutations in the 5' portion of the DBP gene located between mu 63.6 and 66 (36). To determine the precise location and nature of the mutation in each isolate, DNA sequence analysis of the DBP gene was performed. The strategy used for determining the sequence of the entire mutant DBP gene (mu 61.6 through 66.5) from Ad2*hr*400 is given in Fig. 1A. Only one nucleotide difference was observed in the mutant DBP gene compared with the Ad2 DBP DNA sequence published by Kruijer et al. (39). A thymine residue replaces

FIG. 2. Maxam and Gilbert (43) sequence analysis of the Ad2*hr*400 DBP gene. A portion of the sequencing gel autoradiogram that contains the single nucleotide change (*), as compared with wt Ad2, at position 1 of coding triplet 130 is shown. The change of a cytosine to a thymine in coding triplet 130 changes the codon from histidine to tyrosine. The same change was also observed in the *hr* mutants Ad2*hr*401 through Ad2*hr*403.

a cytosine residue at the first position of coding triplet 130 in the DBP gene (Fig. 2), changing a histidine codon to a tyrosine codon.

The nucleotide sequence in the region found to be altered in Ad2*hr*400 was determined for the three other *hr* mutants, Ad2*hr*401 through Ad2*hr*403. All three mutants were found to have precisely the same nucleotide change as Ad2*hr*400. No other change was observed in the region examined, which encompassed 100 to 150 nucleotides surrounding the mutation. Thus all four mutants examined here as well as the phenotypically similar mutants Ad5*hr*404 (38) and Ad2⁺ND*hr*600 through Ad2⁺ND*hr*603 (2) have precisely the same mutation. Therefore, all nine independently isolated adenovirus mutants selected for the ability to grow in monkey cells have acquired the same amino acid change in the DBP, a substitution of tyrosine for histidine at amino acid 130.

Phosphoamino acid analysis of Ad2*hr*400 DBP. To determine whether the *hr* mutant DBP might become phosphorylated on tyrosine in monkey cells, we examined the phosphorylation patterns of the DBP isolated from Ad2- and Ad2*hr*400-infected CV-1 cells. Infected cells were labeled from 22 to 24 h postinfection with [³²P]orthophosphate, and the DBP was isolated by immunoprecipitation of the cell extracts with polyclonal antiserum directed against DBP. The immunoprecipitated proteins were eluted from a sodium dodecyl sulfate-polyacrylamide gel, acid hydrolyzed, and subjected to high-voltage paper electrophoresis (Fig. 3A and B). Phosphoserine and phosphothreonine were found in both wt and *hr* DBPs in agreement with previous analyses of wt DBP synthesized in infected HeLa cells (7). However, phosphotyrosine was not detected in either the mutant or wt proteins. Phosphotyrosine was detected in the positive con-

FIG. 3. Two-dimensional separation of phosphoamino acids. The positions of the origin, phosphotyrosine (P-Tyr), phosphothreonine (P-Thr), and phosphoserine (P-Ser) markers, and [32 P]orthophosphate (P_i) are shown. $H_3^{32}PO_4$ -labeled DBP from Ad2-infected CV-1 cells (A) or Ad2hr400-infected CV-1 cells (B) was purified and subjected to acid hydrolysis. As a positive control, A-431 cell membrane fractions labeled in vitro (C) were acid hydrolyzed in parallel. The resulting products were separated by two-dimensional high-voltage electrophoresis (see text for details).

trol consisting of in vitro phosphate-labeled A-431 cell membranes which contain epidermal growth factor receptors and one or more proteins which are phosphorylated at a tyrosine residue (Fig. 3C) (54).

Nucleotide sequence analysis of Ad2ts400. Although the *hr* mutants Ad2hr400 through Ad2hr403 grow very efficiently in monkey cells at 37°C, they form relatively small plaques on CV-1 cell monolayers at 33°C, and the kinetics of plaque formation is dramatically delayed compared with that in similar infections of CV-1 cells by the Ad2-simian virus 40 (SV40) hybrid virus, Ad2⁺ND1. This cold-sensitive phenotype is seen only in monkey cells, since wt and *hr* viruses form plaques with similar kinetics on human cell monolayers at 33°C. Ad2ts400, a nitrous acid-induced mutant of Ad2hr400, was selected for its ability to grow readily in CV-1 cells at 33°C (49). The cold-resistant *hr* mutation responsible for this phenotype was mapped by marker rescue analysis to the 5' portion of the DBP gene (mu 63.6 through 66). However, Ad2ts400 also contains a *ts* mutation in the 3' segment of the DBP gene (mu 61.6 through 63.6) (49). Its *ts* phenotype is similar to that of Ad5ts125 in that viral DNA replication and the turndown of early gene expression at late times are blocked at the nonpermissive temperature.

The DNA sequence of Ad2ts400 was analyzed between mu 61.6 and 66 by the strategy outlined in Fig. 1B. This analysis indicated that Ad2ts400 carries three mutations in the DBP gene as compared with the wt DBP sequence. All three changes replaced cytosine with thymine. Ad2ts400 still maintains the *hr* alteration at coding triplet 130 which is present in Ad2hr400. An additional change was found in the 5' section of the DBP gene, which by marker rescue mapping is known to convey the cold-resistant *hr* mutation. The alteration was at position 2 in coding triplet 148 (Fig. 4A), changing an alanine codon to a valine codon. The single alteration in the 3' region, to which the *ts* mutation has been mapped, is at the first position of coding triplet 413 (Fig. 4B), changing a serine codon to a proline codon. This mutation is the same alteration seen in two other *ts* DBP mutants, Ad5ts125 (38) and Ad5ts107 (37).

DISCUSSION

The nitrous acid-induced *hr* mutants Ad2hr400 through Ad2hr403, all contain the same histidine-to-tyrosine change at position 130 in DBP, as do the spontaneously generated mutants Ad5hr404 (38) and Ad2⁺ND3hr600 through

Ad2⁺ND3hr603 (2). Thus all nine independently isolated human adenovirus mutants, selected for the ability to overcome the block to late gene expression and virus growth in monkey cells, have acquired the same mutation. By a single base substitution, the histidine codon can be changed to codons for seven different amino acids. The substitution of only a tyrosine at position 130 in the *hr* DBPs suggests that this amino acid plays an important role in the *hr* function.

The recent discovery of the tyrosine kinase activity of several oncogene products and of various membrane growth factor receptors or their associated components suggests that tyrosine phosphorylation may be involved in mediating a variety of cellular responses (18, 54). Since the *hr* phenotype depends on the substitution of tyrosine for histidine at amino acid 130 in DBP, the protein was characterized to determine whether phosphorylation of this tyrosine residue

FIG. 4. Dideoxynucleotide sequence analysis of the mutant Ad2ts400 DBP gene. The figure shows portions of the sequencing gel autoradiograms of the viral DNA noncoding strand which contain the two mutational sites in Ad2ts400 not found in parental Ad2hr400. (A) Alteration in coding triplet 148 which is responsible for the cold-resistant *hr* phenotype. The altered nucleotide (*, G-to-A; C-to-T on the coding strand) changes codon 148 from an alanine codon to a valine codon. (B) Mutation in coding triplet 413 which imparts the *ts* phenotype. The altered nucleotide (*, G-to-A; C-to-T on the coding strand) changes codon 413 from a proline codon to a serine codon.

was important for the mutant phenotype. Although both *hr* and wt DBP contained similar amounts of both phosphoserine and phosphothreonine, phosphotyrosine was not detected in either protein. This suggests that tyrosine phosphorylation is not involved in the *hr* phenotype. We cannot, however, exclude the possibility that if only a subset of DBP molecules are phosphorylated on tyrosine, the level of this phosphorylation might be below the sensitivity of our assay, which could readily detect phosphotyrosine in a control experiment.

The *hr* mutants Ad2*hr*400 through Ad2*hr*403, which were selected to grow well in monkey cells at 37°C, are relatively inefficient at late gene expression and growth in monkey cells but not in human cells at 33°C. The mutant virus Ad2*ts*400 was selected on the basis of its cold-resistant *hr* phenotype. The mutation responsible for this phenotype has been mapped to the 5' region of the DBP gene. We know that the *ts* mutation located in the 3' segment of the DBP gene is not involved in the cold-resistant *hr* phenotype, since we have obtained a virus, Ad2*hr*405, which contains a wt 3' portion of the DBP gene but retains the cold-resistant *hr* phenotype. Interestingly, Ad2*hr*405 forms larger plaques than do Ad2*hr*400 through Ad2*hr*403 at 37 and at 39.5°C (S. Rice and D. Klessig, unpublished results). DNA sequence analysis of the Ad2*ts*400 5' segment indicated that in addition to the alteration of the histidine codon to the tyrosine codon at position 130, which is found in all of the *hr* mutants, Ad2*ts*400 contained an alteration at the coding triplet 148. Since an *hr* mutant containing only a change at amino acid 148 has never been isolated, it seems likely that this change alone is insufficient to impart the *hr* phenotype. More likely, the alteration at coding triplet 148 works in concert with the change at position 130 to fine tune the structure of the DBP so that it can interact more efficiently with monkey cell factors required for expression of viral late genes.

The other alteration in the DBP gene of Ad2*ts*400 is in the 3' portion of the gene and is responsible for the *ts* phenotype. It was previously reported that Ad2*ts*400 has a phenotype similar to the mutants Ad5*ts*125 and Ad5*ts*107 at the nonpermissive temperature (49). In fact, Ad2*ts*400 has the same nucleotide change as these mutants. The alteration in coding triplet 413 of Ad2*ts*400, Ad5*ts*125, and Ad5*ts*107 changes a proline codon to a serine codon. It seems surprising that three of the four *ts* DBP mutants which have been sequenced contain the same nucleotide alteration. Since we have used the mutants Ad5*ts*125 and Ad5*ts*107 in our laboratory, we considered the unlikely possibility that Ad2*ts*400 arose by a recombination event involving Ad5*ts*125 or Ad5*ts*107. We can exclude this possibility, since Ad2*ts*400 contains Ad2 serotype-specific DNA sequences throughout the gene, including two Ad2-specific differences (relative to the wt Ad5 sequence [38]) located within 25 nucleotides of the *ts* mutation.

The restriction in the number of mutational sites seen in the N-terminal portion of the DBP is perhaps in retrospect reasonable, given the possible constraints in protein structure imposed by the strong selection for growth in monkey cells. In contrast, the conserved changes in the C-terminal portion are very surprising. Three out of five *ts* mutations in this domain have the same alteration at position 413. The exceptions are Ad2⁺ND1*ts*23, which contains an alteration at coding triplet 282 (39) and Ad2*ts*111, which is thought to be located between coding triplets 200 and 300 (16, 52). Perhaps only very limited changes in the C-terminal domain of DBP will allow this protein to function at 32.5°C but not at 39.5°C. However, this may not be the entire explanation,

FIG. 5. Schematic representation of the separate functional domains of the adenovirus DBP. The N-terminal 26-kd fragment of the protein contains the *hr* mutation which relieves the block to viral late gene expression in monkey kidney cells. All of 10 independently derived *hr* mutants contain the same alteration at codon 130, which results in the replacement of histidine by tyrosine. Ad2*ts*400 contains an additional *hr* mutation, located at amino acid 148, changing alanine to valine. This mutation further enhances the growth of the virus in monkey cells, particularly at low temperatures. The four *ts* mutants that affect early gene expression, DNA replication, DNA binding, virus assembly, and morphological transformation are located in the 44-kd C-terminal domain. Three of these four mutants have an identical alteration at codon 413, which results in the substitution of serine for proline. The fourth *ts* mutation changes amino acid 282 from leucine to phenylalanine in Ad2⁺ND1*ts*23. The two domains may be separated by a hydrophilic hinge region represented by the connecting line.

since Ad2*ts*400 was isolated after selection for cold-resistant *hr* mutants. Surprisingly, one out of the two mutants obtained, namely Ad2*ts*400, simultaneously had acquired a *ts* alteration at amino acid 413 in addition to the alteration at amino acid 148 which imparts the cold-resistant *hr* phenotype. Thus the repeated isolation of mutations at coding triplet 413 might suggest the high susceptibility of this region of the gene to mutagenesis.

The sequence analysis of Ad2*hr*400 through Ad2*hr*403 and Ad2*ts*400 further supports the model that DBP contains at least two functional domains. The *hr* mutations at amino acids 130 and 148 and the *ts* mutations at amino acids 413 and 282 are separated by an extremely hydrophilic region (centered around amino acid 160) that could act as a possible hinge between the two domains (Fig. 5). The larger domain contains the sites of the *ts* alterations, which affect early gene expression, DNA replication, viral assembly, and the efficiency of morphological transformation. The smaller domain contains the *hr* alterations, which allow normal expression of late genes in monkey cells. Coinfection of monkey cells with wt adenovirus plus SV40 also allows normal late gene expression (47), as does infection with Ad2-SV40 hybrid viruses that contain only the SV40 sequences encoding the C-terminal segment of T antigen (21, 25, 40). Thus the C-terminal segment of SV40 T antigen and the N-terminal domain of adenovirus DBP appear to share similar functions.

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LITERATURE CITED

- Anderson, C. W. 1981. Spontaneous mutants of the adenovirus-simian virus 40 hybrid, Ad2*ND3, that grows efficiently in monkey cells. *Virology* **111**:263-269.
- Anderson, C. W., M. M. Hardy, J. J. Dunn, and D. F. Klessig. 1983. Independent, spontaneous mutants of adenovirus type 2-simian virus 40 hybrid Ad2*ND3 that grow efficiently in monkey cells possess identical mutations in the adenovirus type 2 DNA binding protein gene. *J. Virol.* **48**:31-39.
- Anderson, K. P., and D. F. Klessig. 1982. Synthesis of human adenovirus early RNA species is similar in productive and abortive infections of monkey and human cells. *J. Virol.* **42**:748-754.
- Anderson, K. P., and D. F. Klessig. 1983. Posttranscriptional block to synthesis of a human adenovirus capsid protein in abortively infected monkey cells. *J. Mol. Appl. Genet.* **2**:31-43.
- Anderson, K. P., and D. F. Klessig. 1984. Altered mRNA splicing in monkey cells abortively infected with human adenovirus may be responsible for inefficient synthesis of the virion fiber polypeptide. *Proc. Natl. Acad. Sci. U.S.A.* **81**:4023-4027.
- Ariga, H., H. Klein, A. Levine, and M. Horwitz. 1980. A cleavage product of the adenoviral DNA-binding protein is active in DNA replication *in vitro*. *Virology* **101**:307-310.
- Axelrod, N. 1978. Phosphoproteins of adenovirus 2. *Virology* **87**:366-383.
- Babich, A., and J. R. Nevins. 1981. The stability of early adenovirus mRNA is controlled by the viral 72 kd DNA-binding protein. *Cell* **26**:371-379.
- Baum, S. G., M. S. Horwitz, and J. V. Maizel, Jr. 1972. Studies of the mechanism of enhancement of human adenovirus infection in monkey cells by simian virus 40. *J. Virol.* **10**:211-219.
- Berk, A. J., and P. A. Sharp. 1978. Structure of adenovirus 2 early mRNAs. *Cell* **14**:695-711.
- Carter, T. H., and R. A. Blanton. 1978. Autoregulation of adenovirus type 5 early gene expression. II. Effect of temperature-sensitive early mutations on virus RNA accumulation. *J. Virol.* **28**:450-456.
- Carter, T. H., and R. A. Blanton. 1978. Possible role of the 72,000-dalton DNA-binding protein in regulation of adenovirus type 5 early gene expression. *J. Virol.* **25**:664-674.
- Chow, L. T., T. R. Broker, and J. B. Lewis. 1979. Complex splicing patterns of RNAs from the early regions of adenovirus-2. *J. Mol. Biol.* **134**:265-303.
- Clinton, G. M., and A. S. Huang. 1981. Distribution of phosphoserine, phosphothreonine and phosphotyrosine in proteins of vesicular stomatitis virus. *Virology* **108**:510-514.
- Crawford, L., K. Leppard, D. Lane, and E. Harlow. 1982. Cellular proteins reactive with monoclonal antibodies directed against simian virus 40 T-antigen. *J. Virol.* **42**:612-620.
- D'Halluin, J. C., C. Cousin, C. Niel, and P. Boulanger. 1984. Characterization of an early temperature-sensitive and cytotoxic double mutant of adenovirus type 2. *J. Gen. Virol.* **65**:1305-1317.
- Ensinger, M. J., and H. S. Ginsberg. 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. *J. Virol.* **10**:328-339.
- Erikson, R. L., and A. F. Purchio. 1982. Avian sarcoma viruses, protein kinases, and cell transformation, p. 43-57. *In* G. Klein (ed.), *Advances in viral oncology*, vol. 1. Raven Press, New York.
- Farber, M. S., and S. G. Baum. 1978. Transcription of adenovirus RNA in permissive and nonpermissive infections. *J. Virol.* **27**:136-148.
- Feldman, L. A., J. S. Butel, and F. Rapp. 1966. Interaction of a simian papovavirus and adenoviruses. I. Induction of adenovirus tumor antigen during abortive infection of simian cells. *J. Bacteriol.* **91**:813-818.
- Fey, G., J. B. Lewis, T. Grodzicker, and A. Bothwell. 1979. Characterization of a fused protein specified by the adenovirus 2-simian virus 40 hybrid Ad2*ND1 dp2. *J. Virol.* **30**:201-217.
- Frackelton, A. R., Jr., A. H. Ross, and H. N. Eisen. 1983. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor-stimulated cells. *Mol. Cell. Biol.* **3**:1343-1352.
- Friedman, M. P., M. J. Lyons, and H. S. Ginsberg. 1970. Biochemical consequences of type 2 adenovirus and simian virus 40 double infections of African green monkey kidney cells. *J. Virol.* **5**:586-597.
- Ginsberg, H. S., J. J. Ensinger, R. S. Kauffman, A. J. Mayer, and U. Lundholm. 1974. Cell transformation: a study of regulation with types 5 and 12 adenovirus temperature-sensitive mutants. *Cold Spring Harbor Symp. Quant. Biol.* **39**:419-426.
- Grodzicker, T., C. Anderson, P. A. Sharp, and J. Sambrook. 1974. Conditional lethal mutants of adenovirus 2-simian virus 40 hybrids. I. Host range mutants of Ad2*ND1. *J. Virol.* **13**:1237-1244.
- Handa, H., R. E. Kingston, and P. A. Sharp. 1983. Inhibition of adenovirus early region IV transcription *in vitro* by a purified DNA binding protein. *Nature (London)* **302**:545-547.
- Hashimoto, K., K. Nakajima, K. Oda, and H. Shimojo. 1973. Complementation of translational defect for growth of human adenovirus type 2 in simian cells by a simian virus 40-induced factor. *J. Mol. Biol.* **81**:207-223.
- Henikoff, S. 1984. One-way exonucleolytic digestion to target detection breakpoint for DNA sequencing. *Gene* **28**:351-359.
- Horwitz, M. S. 1978. Temperature-sensitive replication of H5ts125 adenovirus DNA *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **75**:4291-4295.
- Kitchingman, G. R., S. P. Lai, and H. Westphal. 1977. Loop structures in hybrids of early RNA and the separated strands of adenovirus DNA. *Proc. Natl. Acad. Sci. U.S.A.* **74**:4392-4395.
- Klein, H., W. Maltzman, and A. J. Levine. 1979. Structure-function relationships of the adenovirus DNA-binding protein. *J. Biol. Chem.* **254**:11051-11060.
- Klessig, D. F. 1977. Two adenovirus mRNAs have a common 5' terminal leader sequence encoded at least 10 kb upstream from their main coding regions. *Cell* **12**:9-21.
- Klessig, D. F., and C. W. Anderson. 1975. Block to multiplication of adenovirus serotype 2 in monkey cells. *J. Virol.* **16**:1650-1688.
- Klessig, D. F., and L. T. Chow. 1980. Incomplete splicing and deficient accumulation of the fiber messenger RNA in monkey cells infected by human adenovirus type 2. *J. Mol. Biol.* **139**:221-242.
- Klessig, D. F., and T. Grodzicker. 1979. Mutations that allow human Ad2 and Ad5 to express late genes in monkey cells map in the viral gene encoding the 72K DNA binding protein. *Cell* **17**:957-966.
- Klessig, D. F., and M. P. Quinlan. 1982. Genetic evidence for separate functional domains on the human adenovirus specified, 72kd, DNA binding protein. *J. Mol. Appl. Genet.* **1**:263-272.
- Kruijer, W., J. C. Nicolas, F. M. A. van Schaik, and J. S. Sussenbach. 1983. Structure and function of DNA binding proteins from revertants of adenovirus type 5 mutants with a temperature-sensitive DNA replication. *Virology* **124**:425-433.
- Kruijer, W., F. M. A. van Schaik, and J. S. Sussenbach. 1981. Structure and organization of the gene coding for the DNA binding protein of adenovirus type 5. *Nucleic Acids Res.* **9**:4439-4457.
- Kruijer, W., F. M. A. van Schaik, and J. S. Sussenbach. 1982. Nucleotide sequence of the gene encoding adenovirus type 2 DNA binding protein. *Nucleic Acids Res.* **10**:4493-4500.
- Lewis, A. M., Jr., J. J. Levin, W. H. Weise, C. S. Crumppacker, and P. H. Henry. 1969. A nondefective (competent) adenovirus-SV40 hybrid isolated from the Ad2-SV40 hybrid population. *Proc. Natl. Acad. Sci. U.S.A.* **63**:1128-1135.
- Lewis, J. B., C. W. Anderson, and J. F. Atkins. 1977. Further mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. *Cell* **12**:37-44.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

43. Maxam, A. M., and W. Gilbert. 1980. Sequencing end labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
44. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20-78.
45. Nevins, J. R., and J. J. Jensen-Winkler. 1980. Regulation of early adenovirus transcription: a protein product of early region 2 specifically represses region 4 transcription. *Proc. Natl. Acad. Sci. U.S.A.* **77**:1893-1897.
46. Nicolas, J. C., P. Sarnow, M. Girard, and A. J. Levine. 1983. Host range temperature conditional mutants in the adenovirus DNA binding protein are defective in the assembly of infectious virus. *Virology* **126**:228-239.
47. Rabson, A. S., G. T. O'Connor, I. K. Berezesky, and F. J. Paul. 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. *Proc. Soc. Exp. Biol. Med.* **116**:187-190.
48. Reich, P. R., S. G. Baum, J. A. Rose, W. O. Rowe, and S. M. Weissman. 1966. Nucleic acid homology studies of adenovirus type 7 - SV40 interactions. *Proc. Natl. Acad. Sci. U.S.A.* **55**:336-341.
49. Rice, S. A., and D. F. Klessig. 1984. The function(s) provided by the adenovirus-specified, DNA-binding protein required for viral late gene expression is independent of the role of the protein in viral DNA replication. *J. Virol.* **49**:35-49.
50. Rubenstein, F. E., and H. S. Ginsberg. 1974. Transformation characteristics of temperature-sensitive mutants of type 12 adenovirus. *Intervirology* **3**:170-174.
51. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**:5463-5467.
52. Stillman, B. W., E. White, and T. Grodzicker. 1984. Independent mutations in Ad2ts111 cause degradation of cellular DNA and defective viral DNA replication. *J. Virol.* **50**:598-605.
53. Tanaka, T., and B. Weisblum. 1975. Construction of a colicin E1-R factor composite plasmid in vitro: means for amplification of deoxyribonucleic acid. *J. Bacteriol.* **121**:354-362.
54. Ushiro, H., and S. Cohen. 1980. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. *J. Biol. Chem.* **255**:8363-8365.
55. Van der Vliet, P. C., and A. J. Levine. 1973. DNA binding proteins specific for cells infected by adenovirus. *Nature (London) New Biol.* **246**:170-173.
56. Van der Vliet, P. C., A. J. Levine, M. J. Ensinger, and H. S. Ginsberg. 1975. Thermolabile DNA binding proteins from cells infected with a temperature-sensitive mutant of adenovirus defective in viral DNA synthesis. *J. Virol.* **15**:348-354.
57. Williams, J. F., C. S. H. Young, and P. E. Austin. 1974. Genetic analysis of human adenovirus type 5 in permissive and nonpermissive cells. *Cold Spring Harbor Symp. Quant. Biol.* **39**:427-437.