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Mutational Analysis of Human Papillomavirus Type 16 E7 Functions

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The human papillomavirus type 16 E7 gene encodes a nuclear oncoprotein (98 amino acids [AAs] long) consisting of three regions: regions 1 (AAs 1 to 20) and 2 (AAs 21 to 40), which show high homology to the sequences of conserved domains 1 and 2, respectively, of adenovirus E1A; and region 3 (AAs 41 to 98) containing two metal-binding motifs Cys-X-X-Cys (AAs 58 to 61 and 91 to 94). We constructed AA deletion (substitution) mutants and single-AA substitution mutants of E7 placed under the control of the simian virus 40 promoter and examined their biological functions. Stable expression of E7 protein in monkey COS-1 cells required almost the entire length of E7 and was markedly lowered by the mutations in region 3. Transactivation of the adenovirus E2 promoter in monkey CV-1 cells was lowered by the mutations. It was abolished by changing Cys-24 to Gly and markedly decreased by a mutation at His-2 or at the metal-binding motifs in region 3. Focal transformation of rat 3Y1 cells by E7 was eliminated by changing His-2 to Asp or Cys-24 to Gly and was greatly impaired by changing Cys-61 or Cys-94 to Gly. The transforming function survived mutations at Leu-13 and Cys-68 and deletion of Asp-Ser-Ser (AAs 30 to 32). The data suggest that regions 1 to 3 are required for its functions and that the metal-binding motifs in region 3 are required to maintain a stable or functional structure of the E7 protein.

Human papillomavirus type 16 (HPV 16) (5) is believed to cause cervical cancers (for review, see reference 47), and its open reading frame, E7, encodes a small nuclear (33) oncoprotein composed of 98 amino acid (AA) residues (35). Expression of the E7 gene can induce cell DNA synthesis in serum-starved, contact-inhibited rat 3Y1 cells (32). The E7 gene is immortalizing and transforming for primary rat cells (17, 39) and established lines of mouse and rat cells (15, 28) and is capable of transforming primary human fibroblasts in cooperation with the E6 gene (42). The E7 gene can transactivate the adenovirus E2 promoter (28). The E7 protein is nuclear when the E7-expressing monkey COS-1 cells are examined by the immunofluorescence method (33), although it is found in the cytoplasmic fraction upon subcellular fractionation (33, 37). The functions and localization of E7 resemble those of adenovirus E1A and simian virus 40 (SV40) large-T, well-studied viral nuclear oncoproteins (26, 41). It was recently reported that, like E1A and SV40 T antigens, HPV 16 E7 protein is able to bind to the retinoblastoma gene product (6).

The nucleotide sequences of HPV DNAs enable us to deduce the AA sequences of E7 and other HPV proteins (4, 34, 35). From the structural features, the HPV 16 E7 protein can be tentatively divided into three regions (Fig. 1). Region 1 (the N-terminal 20 AAs) and region 2 (AAs 21 to 40) show high homology to parts of adenovirus E1A (26, 28). AAs 6 to 20 and AAs 21 to 40 of HPV 16 E7 resemble AAs 41 to 56 (in conserved domain 1) and AAs 121 to 139 (in conserved domain 2), respectively, of adenovirus 5 E1A, both of which constitute the essential regions for the E1A transforming function (21, 26, 27, 45). Region 3, the C-terminal half (AAs 41 to 98), contains a possible zinc finger structure (1, 8). Two metal-binding motifs, Cys-X-X-Cys, are separated by a stretch of 29 AAs (Fig. 1). Like HPV 16, HPV 18 (3, 4) and HPV 6 (34) show similar E7 structural features (Fig. 1).

In this study, we prepared various HPV 16 E7 mutants by introducing deletions into the E7 gene and by site-directed mutagenesis and examined their capabilities to exert transactivating and transforming functions. Recently, Edmond and Vousden (7) reported a point-mutational analysis of HPV 16 E7 protein.

MATERIALS AND METHODS

Plasmids. Plasmids used in this study were pSV2-E7P (15), pSV2-0 (40), pE2CAT (14), pEJ6.6 (36), pSV2*neo* (38), and pSVneo-E6 (15). Plasmid pSV2-E7P expresses the HPV 16 E7 gene controlled by the SV40 promoter. It contains an HPV 16 DNA fragment, nucleotides (nt) 554 to 874 (spanning the entire coding region for E7), inserted at the *Hin*dIII site into vector pSV2-0 containing SV40 transcriptional regulatory elements (promoter-enhancer, intron, and polyadenylation site). Mutants of HPV 16 E7 were constructed from pSV2-E7P. In pE2CAT, which was used as a reporter of transactivation by E7, the bacterial chloramphenicol acetyltransferase gene is placed under the control of the adenovirus 5 E2 promoter. Plasmid pEJ6.6, which was used in cotransfection for determination of the immortalizing function of E7, contains the activated *ras* oncogene. Plasmid pSV2*neo* expresses the neomycin (G418) resistance gene under the control of the SV40 promoter. For determination of the transforming function of E7 for WI38 human fibroblasts, pSVneo-E6 was used for cotransfection with mutated pSV2-E7 plasmids. Plasmid pSVneo-E6 contains an HPV 16 E6 (nt 25 to 657) transcription unit (15) inserted into pSV2*neo* at the *Bam*HI site. *Escherichia coli* HB101 was used for propagation of all plasmids except pEJ6.6, for which *E. coli* DH-1 was used. Preparation and purification of plasmids were done by standard methods (22).

Cells. Monkey COS-1 (9) and CV-1 cell lines, the rat 3Y1 cell line (18), primary baby rat kidney (BRK) cells, and primary human fibroblasts, WI38 (13), were used in this study. The COS-1 cell line, which is an African green monkey kidney-derived CV-1 cell line transformed by origin-minus SV40 DNA, is constitutively expressing SV40 T antigen and can support replication of the plasmids with the SV40 DNA replication origin. COS-1 cells, grown in Dulbecco modified Eagle medium with 10% fetal bovine serum,

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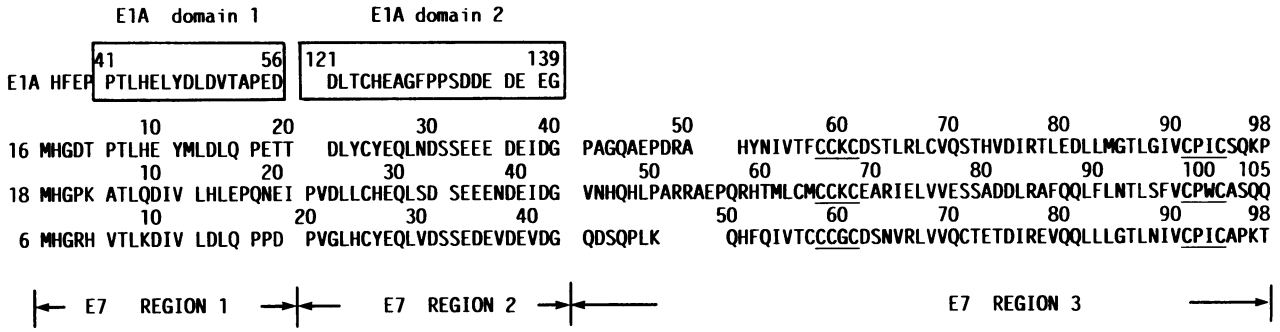

FIG. 1. AA sequences of HPV 16, 18, and 6 E7 proteins. The sequences of adenovirus 5 E1A and HPVs are based on the published DNA sequences (4, 34, 35, 41). Like HPV 16, HPV 18 DNA has been cloned from a cervical carcinoma (3) and its E7 is transforming (44). The E7 proteins are divided into three regions. Regions 1 and 2 show homology to the AA sequences in conserved domains 1 and 2, respectively, of adenovirus E1A (28). Region 3 contains two metal-binding motifs Cys-X-X-Cys (underlined).

were used to detect production of E7 protein by immunofluorescence staining (33) and immunoprecipitation (33). CV-1 cells, grown in Dulbecco modified Eagle medium with 10% fetal bovine serum, were used to determine the transactivating function of E7. Rat cell line 3Y1, grown in minimum essential medium with 10% fetal bovine serum, was used to measure the E7 function to induce focal transformation (16). Primary BRK cells were prepared from 5-day-old Fischer rats. The cells were grown in Dulbecco modified Eagle medium with 10% fetal bovine serum and were used at passage 3 for determination of E7 immortalizing function (23). WI38 primary human fibroblasts, the gift of Y. Doi (Japan Poliomyelitis Institute), were used at 25 to 30 population doublings for determination of E7 transforming function, as described previously (42).

Antisera. Rabbit antisera raised against bacterial fusion protein HPV 16 *lac*-E7 were used for immunofluorescence detection (33) and immunoprecipitation (33) of E7 protein. Preparation and characterization of HPV 16 *lac*-E7 protein and anti-*lac*-E7 sera were described previously (33). For immunofluorescence staining, antisera were extensively incubated at 4°C with acetone-powdered African green monkey kidney tissue before use. For immunoprecipitation, antisera were used without such treatment.

Construction of mutants by deletion. We introduced DNA deletions into the E7 gene by utilizing convenient restriction sites. Restriction endonucleases (*Acc*I, *Hpa*II, *Hin*fI, *Mnl*I, *Nco*I, and *Ssp*I), other enzymes, and linkers (*Eco*RI and *Hpa*I) were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan, and New England BioLabs, Beverly, Mass., and were used as recommended by the suppliers. The nucleotide sequence around the mutation site (and of the E7 coding region for the mutants accidentally made) was determined by the M13-dideoxy method (25, 31) with an M13 sequencing kit (Takara Shuzo) for each mutant constructed.

For construction of mutants 729, 747, 774, 7SS, 7DSS, and 54/61, the shorter *Hin*dIII fragment (HPV 16 nt 554 to 874) was isolated from pSV2-E7P by gel electrophoresis and circularized by ligation. The E7 minicircles were digested with appropriate restriction enzymes, blunt ended by Klenow, and, with or without insertion of appropriate linkers, recircularized by T4 ligase. The mutated and recircularized DNAs were digested with *Hin*dIII and reinserted into pSV2-0 at the *Hin*dIII site in a sense orientation.

Mutants with deletions of C-terminal AAs of the E7 gene (790, 791, 795, and 796) were constructed in a different way. The shorter *Nco*I fragment was isolated from HPV 16 pSV2-E7P, which has two *Nco*I sites: one within the E7 gene near its C-terminal end and the other within the SV40 transcriptional control region. The isolated *Nco*I fragment was digested with exonuclease III, blunt ended with S1 nuclease, and added with *Hin*dIII linkers at both ends. This product was digested with *Hin*dIII. The *Hin*dIII-digested DNA retained the intact N-terminal end of the E7 gene and the exonuclease III-digested, truncated C-terminal end of the E7 gene. The final *Hin*dIII-digested fragments were reinserted into pSV2-0 at the *Hin*dIII site in a sense orientation.

Construction of mutants by site-directed mutagenesis. A single base at a specific site in the HPV 16 E7 gene was replaced with another base by the gapped duplex DNA method (19), using Mutan-G (Takara Shuzo), a site-directed mutagenesis system. The shorter *Hin*dIII fragment from pSV2-E7P (HPV 16 DNA nt 554 to 874) was cloned into M13. For annealing, the complementary 17-mers containing a single base substitution in the middle were synthesized in a model 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.). The mutants were selected on the basis of DNA sequence (31) around the site of intended mutation. For confirmation, the entire E7 coding region was sequenced for each selected mutant by the dideoxy method (31). The E7 *Hin*dIII fragments with mutations, isolated from replicative forms, were inserted into pSV2-0 vectors in a sense orientation. In mutants 7H02D and 7L13S, C at nt 565 and T at nt 599 were replaced with G and C, respectively. In mutants 7C24G, 7C61G, 7C68G and 7C94G, T's at nt 631, 742, 763, and 841 were replaced with G's, respectively. Mutant 7CC/G was a double mutant made from mutants 7C61G and 7C94G.

Detection of E7 protein by immunofluorescence staining. COS-1 cells on cover slips were transfected by the DEAE-dextran method (24) with pSV2 plasmids containing E7 or mutated E7 DNAs (1 μg of DNA per cover slip). At 48 h after transfection, the transfected cells were processed as described previously (33) with the anti-*lac*-E7 serum, and fluorescent cells were scored.

Detection of E7 protein by immunoprecipitation. COS-1 cells (60-mm dish) were transfected by the DEAE-dextran method (24) with pSV2 plasmids with mutated E7 (4 μg) and were labeled 48 h later with 0.2 mCi of [^{35}S]methionine-cysteine mixture (Trans^{35}S-label; ICN Radiochemicals Inc., Irvine, Calif.) for 1 h. Then the labeled cultures were subjected to subcellular fractionation, as described by Barbosa and Wettstein (2). Samples of cytoplasmic fractions were analyzed as described previously (33). In chase experiments, the labeled cultures were maintained for 60 min in

medium without ^{35}S-labeled AAs before subcellular fractionation.

Analysis of RNA. Total RNA extracted from the pSV2 plasmid-transfected COS-1 cultures 48 h after transfection was analyzed by the Northern (RNA) blot method (20) as described previously (42). RNA on membrane filters was probed with HPV 16 E7 DNA fragment (nt 554 to 874) labeled with ^{32}P by nick translation (30).

Assay for transactivation by E7. CV-1 cells (60-mm dish) were transfected with pE2CAT (5 μg) and pSV2-E7P or pSV2 plasmids containing mutated E7 (5 μg) by the calcium coprecipitation method (11) under conditions used previously (43). Chloramphenicol acetyltransferase activities were assayed for [^{14}C]-chloramphenicol (Amersham International plc, Buckinghamshire, England) with the cell extracts prepared 48 h after transfection, as described by Gorman et al. (10).

Assay for focal transformation of rat 3Y1 cells by E7. The capacity of pSV2-E7P or pSV2 plasmids with mutated E7 to induce focal transformation was examined in rat 3Y1 cells, an immortalized normal cell line, as described previously (16). Plasmids (10 μg) were transfected to subconfluent rat 3Y1 cells (30-mm dish) by the calcium coprecipitation method (11). Foci were scored 4 to 5 weeks after transfection with cultures stained with 5% Giemsa.

Assay for immortalization of primary rat cells by E7. The immortalizing function was assayed as described by Matlashewski et al. (23). Primary BRK cells were cotransfected with pEJ6.6, pSV2 plasmids with mutated E7, and pSV2*neo* (5 μg each for a 60-mm dish culture) by the calcium coprecipitation method (11). Some 3 to 4 weeks after transfection, the cultures (resistant to G418 [Sigma Chemical Co., St. Louis, Mo.]) were fixed with methanol and stained with 5% Giemsa. Foci of the cells characteristic of those cotransformed by E7 and activated *ras* were scored.

Assay for E7 transforming function for human cells. The ability of the E7 gene to transform human cells (to extend the life span) in cooperation with the E6 gene was examined in WI38 primary human fibroblasts (42). Plasmids (with or without mutation) pSV2-E7 and pSVneo-E6 (5 μg each) were cotransfected to WI38 cells by the calcium coprecipitation method (11). The G418-resistant cells were maintained and processed essentially as described by Pirisi et al. (29). Cultures containing cells surviving beyond 65 population doublings were regarded as transformation positive (42).

RESULTS

AA deletion (substitution) mutants of HPV 16 E7. Deletion of a small segment from HPV 16 E7 DNA generated a series of frameshift mutants and AA deletion (substitution) mutants. Figure 2 is a schematic representation of the AA sequences of these mutants estimated from their DNA sequences. Mutants 729, 747, 774, 790, 791, 795, and 796 were frameshift mutants. These mutants encode truncated E7 proteins with the N-terminal 29 to 96 AAs followed by a short stretch of AA sequence originating from frame-shifted sequences of either HPV 16 or SV40 DNA. Mutants 7DSS, 7SS, and 54/61 encode mutated E7 proteins with a short AA deletion (substitution) in the midsection.

The pSV2 plasmids containing these mutated E7 genes (placed under control of the SV40 promoter) were tested for abilities to induce production of E7 protein (assayed by immunofluorescence) in monkey COS-1 cells (33), to activate expression of chloramphenicol acetyltransferase from the adenovirus E2 promoter in monkey CV-1 cells (28), to induce focal transformation of rat 3Y1 cells (16), to transform primary BRK cells in conjunction with activated *ras* (23), and to extend the life span of human WI38 cells in cooperation with the HPV 16 E6 gene (42). Table 1 summarizes the E7 functions of the HPV 16 E7 deletion mutants shown in Fig. 2.

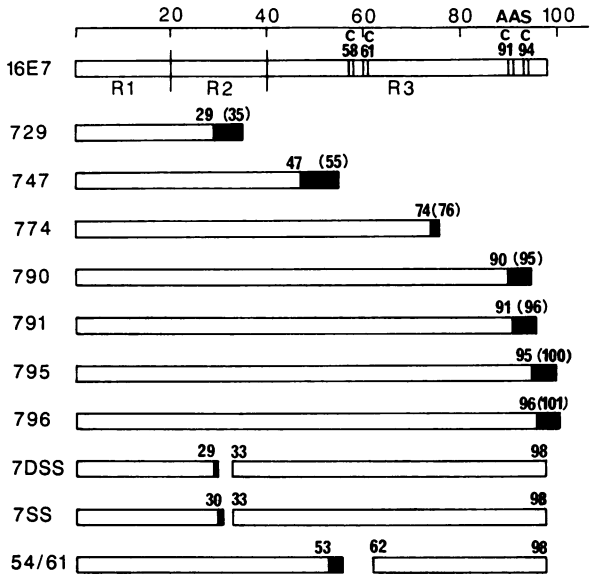

FIG. 2. Schematic representation of E7 deletion mutants of HPV 16. HPV 16 wild-type E7 (98 AAs) is indicated by 16E7. Regions 1, 2, and 3 (Fig. 1) are indicated by R1, R2, and R3, respectively. C's in R3 indicate cysteines in metal-binding motifs. Open rectangles represent AA sequences originating from E7 protein, and the number above them shows the end AA from E7. Filled rectangles represent AA sequences from fame-shifted DNA sequences and the number in parentheses shows the total number of AAs of mutant proteins. Filled rectangles for HPV 16 represent Glu-Arg-Arg-Arg-Met-Lys (in 729), Arg-Thr-Glu-Pro-Ile-Thr-Ile-Leu (747), Gly-Cys (774), Lys-Leu-Gly-Ser-Leu (790, 791, and 795), Ala-Cys-Gly-Ser-Leu (796), Glu (7DSS), Arg (7SS), and Pro-Asn-Ser (54/61).

Steady-state expression of the mutated HPV 16 E7 from the SV40 promoter was examined in COS-1 cells by immunofluorescence staining (Fig. 3 and Table 1). As described previously (33), E7-producing cells were recognized as those with fluorescent nuclei stained with rabbit anti-*lac*-E7 serum 48 h after transfection. Comparison of the frameshift mutants 729, 747, 774, 790, 791, 795, and 796 shows that, for full or stable expression, almost the entire length of E7 (95 of 98 AAs) was required. Probable mutant polypeptides of 729 (35 AAs long) and 747 (55 AAs long) were undetectable by immunofluorescence. The 774 protein (76 AAs long) was detected in a markedly reduced number of cells, and the positive nuclei with the presumably truncated 774 protein appeared differently under a UV microscope. Whereas most of the E7-positive nuclei, like SV40-T- or adenovirus E1A-positive nuclei, contained immunofluorescent dots with variable density (Fig. 3A and B), the 774 E7-positive nuclei contained condensed masses of immunofluorescence (Fig. 3C, D, and E). These abnormally stained nuclei constituted about 0 to 3% of the positive cells in the wild-type (*wt*) E7 transfection and about 30% in transfection with mutant 790 or 791. Deletion of Asp-Ser-Ser at AAs 30 to 32 or Ser-Ser at AAs 31 to 32 (in mutants 7DSS and 7SS) did not affect the level of E7-positive cells in the transfected COS-1 cultures much, but deletion of AAs 54 to 61 (in mutant 54/61)

TABLE 1. Functions of HPV 16 E7 deletion mutants

Plasmid[a]	Steady-state E7 protein in COS-1 cells (no.)[b]	Transactivation (%) for adenovirus E2 promoter in CV-1 cells[c]	Transforming activity for:		
			Rat 3Y1 (no. of foci)[d]	BRK (immortalization efficiency)[e]	Human WI38 (transformation efficiency)[f]
pSV2-0	0	1.0	0	0/2	0/2
pSV2-E7P	1.00	9.2	1.00	4/4	4/4
pSV2-729	0	0.9	0	0/4	NT[g]
pSV2-747	0	1.0	0	0/4	NT
PSV2-774	0.01	1.7	0	0/4	0/4
pSV2-790	0.16	1.7	0.01	NT	0/4
pSV2-791	0.18	1.6	0	0/4	1/4
pSV2-795	0.77	4.5	0.77	4/4	NT
pSV2-796	0.98	5.5	0.68	4/4	NT
pSV2-7DSS	1.12	4.0	0.34	4/4	2/2
pSV2-7SS	1.34	4.2	0.18	4/4	2/2
pSV2-54/61	0.14	3.4	0	0/4	2/4

[a] AA sequences of the protens encoded by these plasmids are shown in Fig. 2.
[b] Transfected COS-1 cell cultures were stained with anti-*lac*-E7 48 h after transfection. Number of immunofluorescence-positive cells were normalized to that in pSV2-E7P-transfected cultures, whose positive cells constituted about 0.5%. Average of four experiments.
[c] Average of two experiments. The chloramphenicol acetyltransferase activities (percent conversion to acetylated form per protein content) of pE2CAT with each mutant were normalized to that with pSV2-0, which is a nontransactivating backbone vector.
[d] Average of three experiments. Number of foci were normalized to that of pSV2-E7P. Efficiency of focal transformation by pSV2-E7P was 66 foci per 10 μg of DNA.
[e] Primary BRK cells were cotransfected by E7 and activated *ras*, and cultures with foci of the cotransformed cells were scored as immortalization positive. Immortalization efficiency is expressed as a ratio of cultures with colonies of transformed cells to cultures initially replated.
[f] Primary human fibroblasts WI38 were cotransfected with mutated E7 and HPV 16 E6 (pSVneo-E6). Transformation efficiency is expressed as a ratio of cultures with transformed cells to cultures initially replated (42).
[g] NT, Not tested.

decreased the number of E7-positive nuclei. Deletion of AAs 92 through 98 (in mutants 790 and 791) drastically lowered the steady-state E7 protein expression.

Capacity to activate chloramphenicol acetyltransferase expression from the adenovirus E2 promoter was examined with pSV2 plasmids containing the mutated E7 (Fig. 2) in monkey CV-1 cells. Cell extracts prepared from cultures 48 h after transfection were measured for ability to convert chloramphenicol to acetylated forms; the relative activities (compared with backbone vector pSV2-0 and *wt* E7) are shown in Table 1. The transactivating function was lowered by all mutations shown in Fig. 2, but it was less affected by the deletion of AAs 96 to 98 at the C terminus or AAs 30 to 31 than by the other deletions.

Transforming functions of the mutated E7 genes were tested in rat 3Y1 cells, primary BRK cells, and WI38 primary human fibroblasts. The E7 gene controlled by the SV40 promoter is capable by itself of focal transformation of immortalized rat 3Y1 cells (15). pSV2 plasmids with the mutant E7 genes (Fig. 2) were transfected into 3Y1 cells, and foci of the transformed cells were scored 4 to 5 weeks later (Table 1). Comparison of the frameshift mutants (729, 747, 774, 790, 791, 795, and 796) indicates that, for efficient focal transformation, almost the entire length of the E7 polypeptide (95 of 98 AAs; in mutant 795) was required. Transformation by mutants with 90 to 91 AAs was drastically reduced (mutants 790 and 791). Whereas deletion of AAs 30 to 32 (in mutant 7DSS) and AAs 31 to 32 (in mutant 7SS) decreased focal transformation, deletion of AAs 54 to 61 (in mutant 54/61) totally abolished the transforming function for 3Y1 cells. Mutants whose activity for focal transformation survived showed an immortalizing function, which was assayed as transforming activity for primary BRK cells after cotransfection with plasmids with the activated *ras* (Table 1). Mutants 7DSS and 7SS extended the life span of WI38 cells, in cooperation with the E6 gene. Mutant 791 and mutant 54/61 lacking AAs 54 to 61 were weakly transforming for WI38 cells.

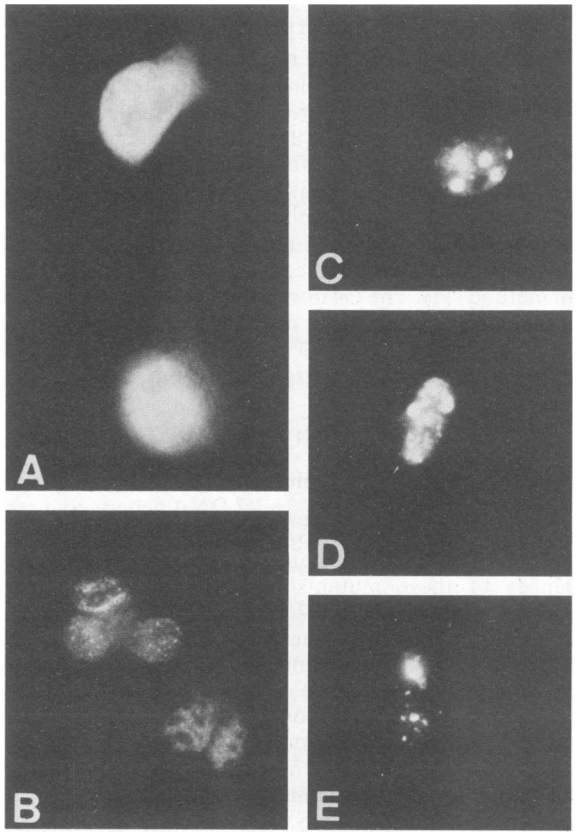

FIG. 3. Immunofluorescence staining of monkey COS-1 cells expressing HPV 16 E7 protein. Cells were transfected with pSV2-E7P (A, B) or pSV2-774 (C, D, E) and stained indirectly with anti-*lac*-E7 serum 2 days after transfection.

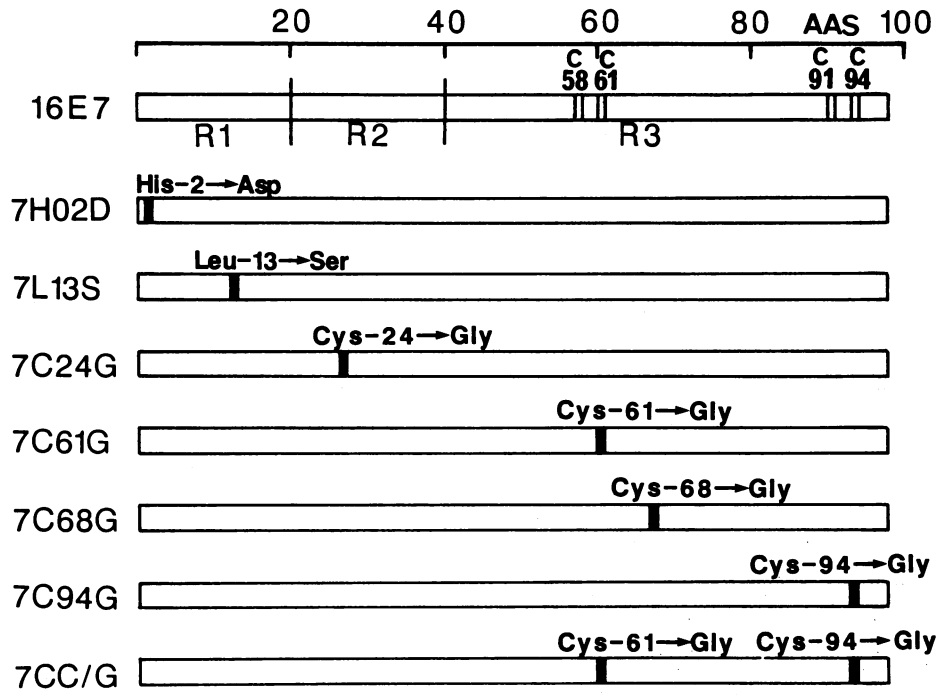

FIG. 4. Schematic representation of AA sequences of HPV 16 E7 single-AA substitution mutants. Wild-type E7 protein (98 AAs long) is as given in the legend to Fig. 2. Vertical bars in open rectangles indicate the positions of replaced AAs. Conversion of AAs is indicated above the rectangles representing mutated E7 proteins.

Single-AA substitution mutants of HPV 16 E7. Replacement of the single nucleotide at nt 565, 598, 631, 742, 763, or 841 by site-directed mutagenesis was intended to generate the AA substitution mutants shown in Fig. 4. His-2 was replaced with Asp in the protein encoded by 7H02D DNA. Leu-13 was replaced with Ser in 7L13S protein. Cys-24, Cys-61, Cys-68, and Cys-94 were each replaced with Gly in 7C24G, 7C61G, 7C68G, and 7C94G proteins, respectively. The mutant 7CC/G DNA was constructed from 7C61G and 7C94G DNAs.

Some of the E7 deletion mutants (774, 54/61, and 791) and the single-AA substitution mutants shown in Fig. 4 (all mutants were placed under the control of the SV40 promoter in pSV2-0) were transfected into monkey COS-1 cells, in which production of E7 mRNA and E7 protein was examined 48 h after transfection. Total RNA from the transfected cells was electrophoresed and subjected to Northern blot analysis. E7-specific RNA of 1.4 kilobases in length was detectable in all cases (data not shown). The transfected cultures were labeled with ^{35}S-AAs for 1 h and subjected to subcellular fractionation. Cytoplasmic fractions, in which the radiolabeled E7 protein is found almost exclusively (33, 37), were allowed to react with anti-*lac*-E7, and the resulting immunocomplexes were electrophoresed in sodium dodecyl sulfate gels (Fig. 5A). Except for 774 and 54/61, 19S E7 protein bands were detected in all cases. The level of radioactive E7 (darkening of the negatives) was significantly lower in pSV2-7C94G-transfected cells than in the other positive cases. Chase experiments (Fig. 5B) revealed that the 7H02D protein was significantly more stable than *wt* E7. The 791 protein seemed to be slightly less stable than *wt* E7 protein. The low levels of 7C94G and 791 proteins probably reflect the instability of these proteins, because E7 mRNA levels were all similar to one another (data not shown).

Table 2 summarizes the E7 functions of the single-AA substitution mutants shown in Fig. 4. The steady-state nuclear E7 protein expressed in COS-1 cells and detected by immunofluorescence staining tended to be less affected in the mutations in the N-terminal (regions 1 and 2) than in the C-terminal (region 3) half. The capacity to transactivate the adenovirus E2 promoter in CV-1 cells was lowered in all of the mutants. It virtually disappeared upon changing Cys-24 to Gly, but was less affected by changes of Leu-13 and Cys-68 than by other changes. The ability to induce focal transformation of rat 3Y1 cells was eliminated by changing His-2 to Asp and Cys-24 to Gly. The transforming capacity was somewhat lowered in the mutants when Leu-13 or Cys-68 was changed to Ser or Gly, respectively. The activity was greatly lowered by the other changes (Cys-61 and Cys-94 to Gly).

Two mutants behaved differently in transformation function. The ability to transform human WI38 cells (in cooperation with E6) survived the change of His-2 to Asp, which eliminated the ability to transform rat 3Y1 cells. The transforming function for WI38 cells was abolished by the change of Leu-13 to Ser, which lowered the transforming activity for rat 3Y1 cells only slightly. The change of Cys-24 to Gly, however, abolished the transforming activity for both rat and human cells. The transforming function of E7 for both rat and human cells survived the mutation at Cys-68. The transforming activities for human cells in the other mutants (Cys-61 and Cys-94) were lowered to undetectable levels. Mutant 7CC/G seemed to be weakly transforming for WI38 cells.

DISCUSSION

The 98-AA-long HPV 16 E7 protein consists of three regions (Fig. 1). Regions 1 and 2 show high homology to the

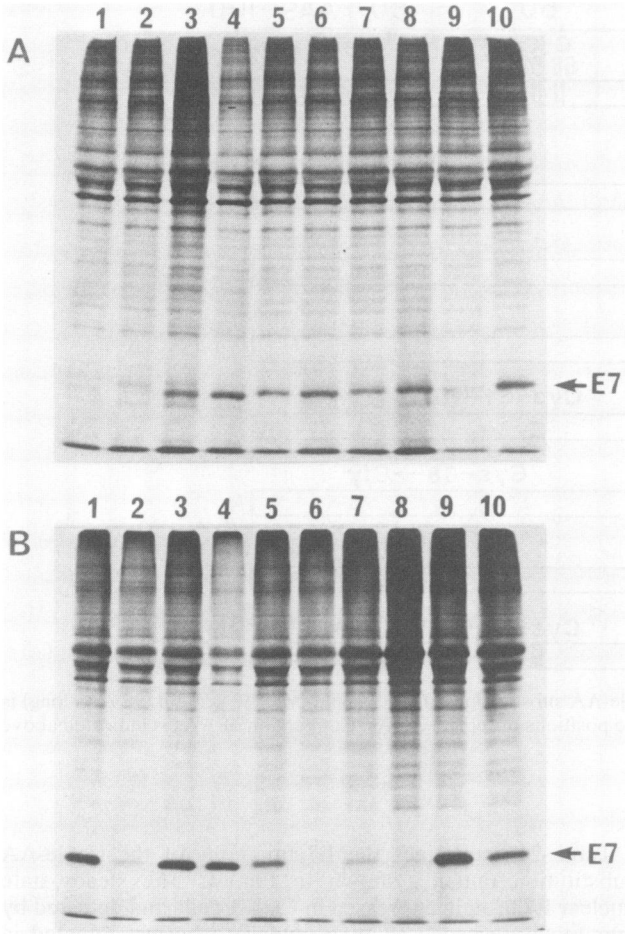

FIG. 5. Immunoprecipitation of E7 proteins from COS-1 cells transfected with HPV 16 E7 mutants. Plasmid pSV2-E7P expresses wild-type E7 protein. AA sequences of mutant E7 proteins are shown in Fig. 2 and 4. (A) Cells transfected with pSV2-774 (lane 1), pSV2-54/61 (lane 2), pSV2-791 (lane 3), pSV2-7H02D (lane 4), pSV2-7L13S (lane 5), pSV2-7C24G (lane 6), pSV2-7C61G (lane 7), pSV2-7C68G (lane 8), pSV2-7C94G (lane 9), and pSV2-E7P (lane 10) were labeled with ^{35}S-AAs for 1 h at 48 h after transfection. Labeled cells were subjected to subcellular fractionation (2), and the cytoplasmic fractions were mixed with anti-*lac*-E7 serum. Immunocomplexes were electrophoresed in a sodium dodecyl sulfate–12% polyacrylamide gel. (B) Duplicate transfected cultures were prepared: one (lanes 1, 3, 5, 7, and 9) was processed as described for panel A; the other (lanes 2, 4, 6, 8, and 10) was chased for 60 min and then processed in the same way. Cultures were transfected with pSV2-791 (lanes 1 and 2), pSV2-7H02D (lanes 3 and 4), pSV2-7C94G (lanes 5 and 6), pSV2-7CC/G (lanes 7 and 8), and pSV2-E7P (lanes 9 and 10).

AA sequences of conserved domains 1 and 2, respectively, of adenovirus E1A, both of which are essential for E1A transformation functions (21, 26, 27, 45). Region 3 contains two metal-binding motifs, Cys-X-X-Cys. Among the three regions, the AA sequences of region 2 in HPV 16, 18, and 6 are most highly conserved (Fig. 1). In this study, we constructed frameshift mutants, deletion mutants (Fig. 2), and single-AA substitution mutants of HPV 16 E7 (Fig. 4) and examined their biological functions expressed under control of the SV40 promoter (Tables 1 and 2; Fig. 3 and 5). Our study revealed some essential and nonessential AAs in the E7 protein for its functions.

TABLE 2. Functions of single-AA substitution mutants of HPV 16 E7

Plasmid[a]	Steady-state E7 protein in COS-1 cells (no.)[b]	Transactivation (%) for adenovirus E2 promoter in CV-1 cells[c]	Transforming activity for:	
			Rat 3Y1 (no. of foci)[d]	Human WI38 (transformation efficiency)[e]
pSV2-0	0	1.0	0	0/2
pSV2-E7P	1.00	9.2	1.00	4/4
pSV2-7H02D	0.98	1.8	0	2/4
pSV2-7L13S	0.43	5.5	0.59	0/4
pSV2-7C24G	0.48	1.2	0	0/4
pSV2-7C61G	0.15	2.7	0.02	0/4
pSV2-7C68G	0.16	5.2	0.32	3/4
pSV2-7C94G	0.11	2.5	0.02	0/4
pSV2-7CC/G	0.11	1.7	0.01	1/4

[a] AA sequences of the proteins encoded by these mutants are shown in Fig. 4.
[b] Average of three experiments. Same as footnote *b*, Table 1.
[c] Average of two experiments. Same as footnote *c*, Table 1.
[d] Average of three experiments. Same as footnote *d*, Table 1.
[e] Human fibroblasts were cotransfected with mutated E7 and pSVneo-E6. Transformation efficiency was expressed as a ratio of cultures with transformed cells to cultures initially replated (42).

Stability of the E7 protein expressed in monkey cells appeared to be affected by some of the mutations. Because the mRNA levels in the transfected cells were not much different among the cultures transfected with various mutants, it is likely that the mutants with lowered efficiency in inducing steady-state nuclear E7 (Tables 1 and 2) and a lowered level of labeled E7 protein (Fig. 5) produce mutant proteins that are less stable than *wt* E7 protein. The mutations in region 3 appeared to decrease the stability of the E7 protein more than those in regions 1 and 2 did. Clearly, the mutations affecting Cys-X-X-Cys motifs decreased the probable stability of E7 protein and its activities. It may be that the metal-binding motifs, especially that at AAs 91 to 94 of HPV 16, are important for the E7 protein to form a stable structure by binding zinc. Unlike the other mutations, the change of His-2 to Asp caused extension of the half-life of E7 protein (Fig. 5). It is unclear at present whether or not the stability of mutant protein varies with the species from which the cells expressing E7 protein originate.

The E7 transactivating function for the adenovirus E2 promoter in monkey CV-1 cells was lowered to various degrees by the mutations examined in this study. Expression of the full activity (of *wt*-E7) seemed to require the entire length of E7. In region 1, the His-2 mutation markedly lowered the transactivating function, but the Leu-13 mutation did so less markedly. In region 2, the Cys-24 mutation eliminated the transactivating function, which survived (with lowered activity) the deletion of Asp-Ser-Ser (AAs 30 to 32). In region 3, the Cys-68 mutation, which is unrelated to the metal-binding motif, lowered the function less markedly than the other Cys mutations in the metal-binding motif did (in mutants 790, 791, 7C61G, and 7C94G). Thus, all regions are required for efficient transactivating function, and in region 3 the metal-binding motifs seem to be important.

The transactivating function of the mutants for the E2 promoter was also tested in rat 3Y1 cells (data not shown). The activity in 3Y1 cells was about 1/10 of that in CV-1 cells. Although the data fluctuated from experiment to experiment (especially for mutants with low activity), the activities in 3Y1 cells tended to parallel those in CV-1 cells.

All three regions of E7 were found to contain AAs susceptible to the mutations that eliminate or greatly lower

the capacity to transform rat 3Y1 cells (Tables 1 and 2). In region 1, His-2 was essential, but Leu-13 was nonessential for transformation of rat cells. In region 2, Cys-24, which was essential for E7 transactivation function, was also essential for transformation of rat cells. Asp-Ser-Ser (AAs 30 to 32) and Ser-Ser (AAs 31 to 32) were nonessential, but deletion of these amino acids (7DSS and 7SS) lowered efficiency of transformation of rat cells. In region 3, the transforming capacity seemed to be especially susceptible to destruction of metal-binding motifs. Mutations involving Cys-61 and Cys-94 (790, 791, 7C94G, 54/61, and 7C61G) drastically lowered or abolished transformation of rat cells, but the transformation function survived mutation between the two metal-binding motifs (7C68G). Thus, all three regions of E7 protein appear to be required for efficient transformation by E7.

From the AA sequence homology, regions 1 and 2 of E7 are expected to function like adenovirus E1A domains 1 and 2, respectively. Like E1A (21, 26, 27, 45), both regions 1 and 2 of E7 seem to be required for transformation. In fact, the results obtained with the mutants in region 2 are similar to those obtained with the E1A mutants in domain 2 (45). Cys-24 of E7 and Cys-124 of E1A are both essential for the respective transformation function. Like Ser-132 of E1A, Ser-31 and Ser-32 of E7 are nonessential but necessary for efficient transformation. Since E1A domains 1 and 2 are known to contain the binding sites for cellular target proteins, including retinoblastoma gene product (12, 46), E7 regions 1 and 2 are expected to play similar roles (6), which should be examined with the mutants in future studies.

Whereas the immortalizing function (transformation in conjunction with activated *ras*) for primary rat cells paralleled the transforming function for rat 3Y1 cells, the transformation function (in cooperation with the E6 gene) for human WI38 cells did not agree with that for rat cells in some mutants in region 1. Changing His-2 to Asp abolished the transforming function for rat cells, but not the transforming function for human cells. Changing Leu-13 to Ser, on the other hand, did not affect the transforming function for rat cells much, but eliminated the function for WI38 cells. It may be that the difference in host range of these mutants results from the difference between the two species of cellular target proteins binding to region 1 (6, 12, 46). Transformation of WI38 cells was barely affected by the change of Cys-68 to Gly (7C68G) and survived deletion of 54 to 61 AAs (mutant 54/61). Mutants 791 and 7CC/G seemed to be weakly transforming (Tables 1 and 3). The stability of the mutant proteins (as to the role of the metal-binding motif) in human fibroblasts remains to be investigated.

Unlike 289-AA-long E1A, there was no clear separation of transactivating and transforming functions between the regions within 98-AA-long E7. Probably because the E7 protein is small, the functional sites may be closely distributed or overlapping on an E7 molecule, or a mutation may readily affect the entire molecular structure. Region 3 of E7 is probably required to maintain a stable or functional structure of the protein. Mutations in region 3 tended to lower the E7 protein level detected by immunofluorescence staining and immunoprecipitation. The biological functions were reduced when the metal-binding motifs in region 3 were modified or removed. Although the present study and the recent study by Edmonds and Vousden (7) have revealed a few of the essential and nonessential AAs in each region of the E7 protein for its functions, more studies are needed for full elucidation of the relation between the structure and function of this interesting protein.

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