HPV16 E7: Primary Structure and Biological Properties

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Introduction

Human papillomaviruses (HPVs) have been found in over 90% of cervical cancers, as well as in other carcinomas [10]. Certain HPV types have been classified as "high risk" types, and others as "low risk", based on the clinical prognosis of lesions which they cause. Only the high risk types generally cause lesions which can progress to cancers [43]. HPV-16 in particular is found in roughly half of cervical cancers [10], and HPV-16 proteins have been extensively studied. The major transforming proteins of the high risk HPVs have been identified as the early proteins E6 and E7; expression of these proteins is maintained in carcinoma cells lines [6], and expression of these two proteins induces immortalization and transformation in a variety of rodent and human cell types [54].

The viral function of E6 and E7 appears to be, at least in part, to control the cellular environment in a fashion favorable for replication of the viral genome, via transcriptional activation and induction of DNA synthesis as well as inhibition of cellular differentiation and promotion of cell growth [93]. In vitro studies have identified several biological properties of HPV-16 E7 which may be relevant to its function(s) in vivo, including: immortalization and transformation, alone or in cooperation with *ras* or HPV-16 E6, of a variety of cultured cells and cell lines; binding to the underphosphorylated form of the retinoblastoma protein (pRb) as well as other "pocket proteins" including p107 and p130; susceptibility to phosphorylation by casein kinase II; zinc-binding; induction of DNA synthesis; transactivation of the Ad E2 promotor and other viral and cellular promoters with shared sequence elements, including E2F responsive elements; dissociation of E2F from pRb, and formation of other complexes with cellular proteins including E2F transcription factor complexes and cyclin A; abrogation of TGF-β induced G1-arrest and transcriptional repression of the c-*myc* promoter; nuclear localization; multimerization; abrogation of growth arrest signals; interaction with and inactivation of cyclin-dependent kinase inhibitors; destabilization of pRB and stabilization of p53; and interaction with other cellular proteins. Several of these properties are shared with other viral oncoproteins, specifically adenovirus E1A and SV40 large T antigen, with which E7 shares extensive sequence similarity in certain regions identified with these biological properties. (See the main text for references concerning these various properties.)

Several reviews discuss the mechanisms and significance of these properties in greater detail [93]. The current review is meant to provide a detailed summary of studies which have mapped the various properties to specific regions of the HPV-16 E7 protein. Point mutations and deletions have been used to map the contribution of particular elements of the protein sequence to a given function. In addition, many of the properties of HPV-16 E7 are not shared, or are only weakly shared by the E7 protein of the low risk HPV-6b, and chimeric HPV16/HPV6b E7 proteins have been constructed to map the regions of the proteins involved in these differences; likewise, chimeric E7/E1A and E7/TAg proteins have been studied. Figure 1 presents a graphical summary of the information which is laid out in more detail in the text and tables of the sections which follow. Figures 2 and 3 illustrate sequence similarities between the E7 proteins of various HPV types, and similarities between the E7 protein and other viral oncoproteins, respectively.

E7 Protein

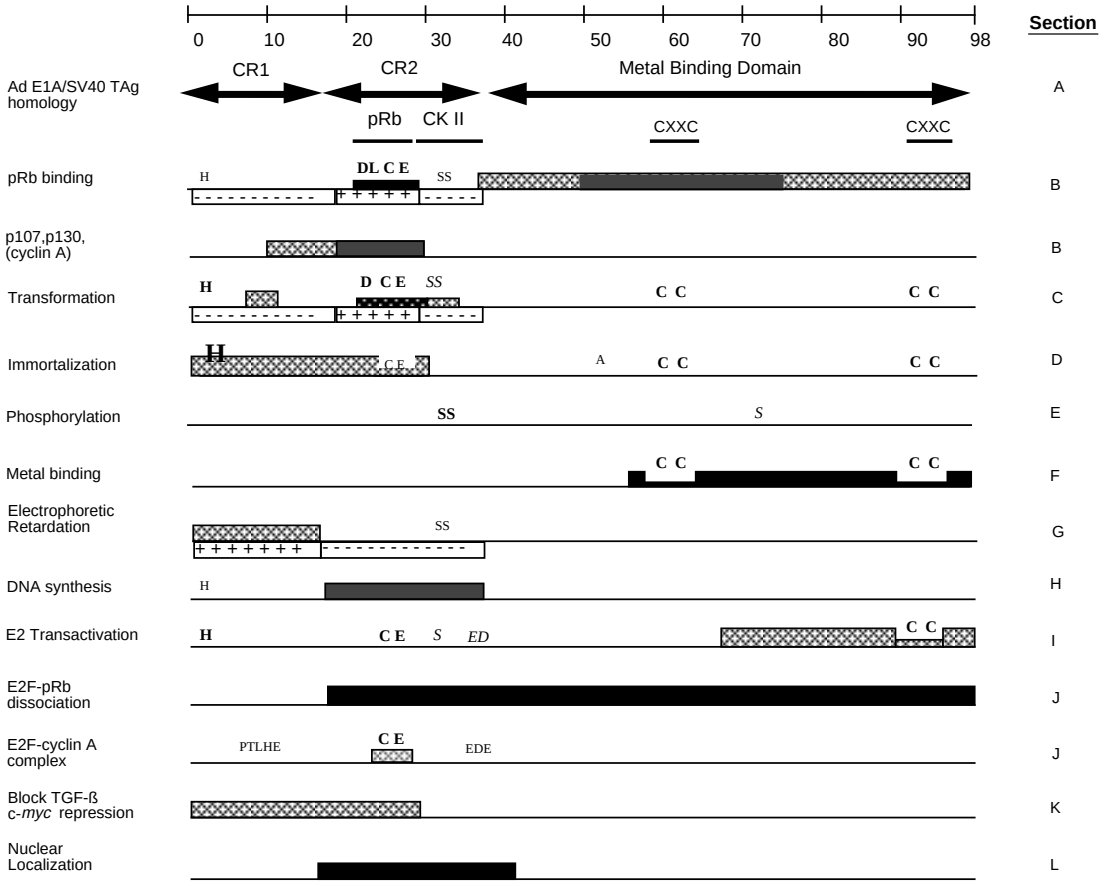

Figure 1. Summary of some of the available information regarding regions of HPV16 E7 whose contribution (or lack thereof) has been assayed for various properties. To the right of the figure is a column of letters indicating which section of the text to refer to for more details. Specific amino acids that have been subjected to mutational analysis are indicated by the single letter code corresponding to the residue present in the wild-type HPV-16 E7. The effects of these mutations are indicated by type style used to print the letter. Mutations that show no effect on the relevant properties are shown in a small type size (ABCD). Mutations which have shown a slight or inconsistent effect are shown in an italic style (*ABCD*), and mutations that have strong effects are printed in bold (**ABCD**). Longer regions which have been examined are represented as rectangles. Gray rectangles indicate regions that have been shown to have an effect on the property, with darker shades of gray indicating stronger effects; black rectangles indicate essential regions. Rectangles containing strings of plus signs (+) indicate regions which, when exchanged with the corresponding region of HPV-6, show loss or reduction of a property not shared by HPV-6 E7, indicating that the region is important to this property; rectangles containing minus signs (-) indicate regions which can be exchanged for the corresponding HPV-6 E7 peptide without reduction of functionality.

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HPV16  MHGDTPTLHEYMLDLQP.ETT...DLYCYEQL....NDSSEEE...DEID.........GPA.GQA.EPDR..AHYNIVTFCCKCDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP   98
HPV33  -R-HK---K--V---Y-.-P-..----------....S---D-D...EGL-.........R-D.---.Q-AT..-D-Y---C-HT-NT-V----N--AS-L--IQQ-----VN----T-A-Q    97
HPV31  -R-E----QD-V-----.-A-...--H-----....P---D--...-V--.........S--.---.---T..SN--------Q-K----------Q----I-QE----SF-----N--TRL   98
HPV52  -R--KA-IKD-I-----.---...--H-----....G---D--D.T-GV-.........R-D.---.-QAT..SN-Y---Y-HS--------IH--AT-L---QQM-L---QV---G-ARL    99
HPV35  ---EIT--QD-V---E-.-A-...----------....C-------..E-T--.........---.---.K--T..SN-----S----EA----------I---K--------F-----G---RA   99
HPV35h --EIT--QD-V---E-.-A-...----------....C-------..E-T--.........---.---.K--T..SN-----S----EA----------I---K--------F-----G---RA   99
HPV58  -R-NN---R--I---H-.-P-...--F-----....C---D-D..EIGL-.........--D.---.Q-AT..-N-Y---C-YT-GT-V---IN--TT-V---QQ-----CT----S-A-Q    98
HPV34  ---KK-SVQDIV---K-TTE-...--T---S-.....-N--D-...--T-.......S.HLE.R--...EQ..-W-R---D-SR-Q--VC-TIE---A-LLV-------A-K----N--RRL   97
HPV6b  ---RHV--KDIV-----PDPV...G-H-----....V----D-..V--V-.........-QD.S-...-LK..Q-FQ---C--G---NV--V--C-ET---EVQQ--L---N------AP-T   98
HPV11  ---RLV--KDIV-----PDPV...G-H-----....E----D-..V-KV-.........KQD.A-...-LT..Q--Q-L-C--G---NV--V-EC-DG---Q-Q---L---N------AP--   98
PCPV1  ---KYT--KDIV---S-.DPV...G-H-N---..........-----DE.V--QA.........TQ-.T--..TFT..Q--Q---C-GQ---NV--V-DC-GS--QH-HK--L-S-N----L-APQT   98
HPV44  ---NYT--K-IV-Q-E-PDPV...G-H-N---..........----D-..V--LA.............T--TQDVT..QP-Q---T-GT-SRKV--V--C-GT--HH-HT--L-S-D-L--V-AP-T   97
HPV55  ---NY---K-IV-E-D-PDPV...G-H-N---..........----D-..V--LA.............T--TQDVT..QP-Q---T-GT-NRNV--V--C-GT--CQ-HT--L-S-E-L--V-AP-N   97
HPV13  ---KY---KDIV-E-T-.DPV...G-H-N---..........----D-..V--QA.........TQ-.T--TQHSTLLQC-Q-L-S-S--C-NV--V-EC-GP--HD-H---L---N----L-AP-S  101
HPV18  ---PKA--QDIV-H-E-QNEI.PV--L-H---.....SD----N..-----.....GVN.HQH.LP-RRAEP..QRHTMLCM----EARIE-V-E-SAD-L-AFQQ-FLN--SF---W-ASQQ  105
HPV59  ---PKA--CDIV---E-QNYE.EV--V------...PDSD--N-K..--P-.....GVN.H-L.LL-RRAEP..QRH---CV----NNQ-Q-V-ETSQDGL-A-QQ-F-D--SF---L-AANQ  107
HPV45  ---PRE--Q-IV-H-E-QNELDPV--L-----.....SE----N..--A-.....GVS.HAQ.LP-RRAEP..QRHK-LCV-----GRIE-T-E-SAE-L---QQ-FLS--SF---W-ATNQ  106
HPV39  -R-PK---Q-IV---C-YNEIQPV--V-H---.....GE--D-I..--P-....HAVN.HQHQLL-RRDEP..QRHT-QCS----NN--Q-V-EASRDTL-Q-QQ-F-DS--F---W-ATANQ  109
HPV70  ---PR---Q-IV---Y-YNEIQPV--V-H---.....ED-DN-T..--P-....HVVN.HQQQLL-RREEP..QRHK-QCM----NT--H-V-EASQENL-S-LQ-F-E--SF---W-ASGTQ  109
HPV68  ---PK--VQ-IV-E-C-CNEIEPV--V-H---.....GD-DD-I..--P-....HAVNHHQHQLL-RRDEQ..QRHT-QCT----NNL-Q-V-EASRENL-NV-L-F-DS-NF---W-ATETQ  110
HPV51  -R-NV-Q-KDVV-H-T-QTEI...--Q----F....---------...--V-.....NMR.DQL.PERRAGQA..TC-R-EAP--R-S-VVQ-A-E-SGDTL-VVQQM---E-SL---C-ANN  101
HPV26  ---NIINIEDVI---V-QPEI...--R-----DYEQF---D-....--T-.....NMR.D...Q--RQAGQ.EVC-R-EAQ--M-N-IVQ-A---SRQNV-V--QM--EDVSL--HQ-AAQ  104
HPV30  ---KVT-IP--I---V-QTEI...--H-----.....N-----D.E--V-.....NLQ.KQP.Q--RQEEQ.HPC-L-N-Q--R-A-AVQ-A---PTKEL-A-QQM---A-EL---L-ATRR  105
HPV53  ---NV---PQ-IIE-I-QTEI...--Q-H---.....N---D-D.E--V-.....HLQ.EQP.Q--RRDEQ.HPC-L-E-Q--R-E-LVQ-A---STKEL-I-QQM----VEL---L-ATRR  105
HPV56  ---KV---QDVV-E-T-QTEI...--Q-N---..........----D-D.E--V-.....HLQ.ERP.Q--RQAKQ.HTC-L-HVP--E-KFVVQ-DI---KE-L-VVQQ----A-TVT--L-ASSN  105
HPV66  ---KV---Q-VI-E-A-QTEI...--Q-N---..........----D-D.E----.....HLL.ERP.Q--RQAEQ.HKC-L-HVP----ELVVQ-DI---KEEL-VVQQ----A-TVT--L-ASSK  105
HPV61  ---QVA-IKDIV-EER-.-VV...--H-N---....L----S-...E-.-.........SVR.E-LV-QAQ..QA-RV--T-GI-KCPV--V--CGDA-LKV-HE--L-D-S----G-A      95
RhPV1  -I-PK---EDIV-----FPQPQPV--M-----....S----D-...--V-HHHNNQQQHHQHARPEVPE-G..DC-R--SD-YS-GKP---V-V-S-EEL-V---------D----S-ASRV  113
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Figure 2: Alignment of predicted protein sequences for the E7 protein of types of HPV from phylogenetic groups which have a primarily mucosal tissue tropism. Dashes ("-") indicate residues identical to those found in HPV-16. Periods (".") indicate gaps inserted to maintain alignment.

A. Similarity to other proteins

Based on conservation between and within the adenoviruses and polyomaviruses, three conserved regions in adenovirus E1A and SV40 TAg have been identified, known as CR1, CR2, and CR3 [50]. HPV16 E7 can be separated into three corresponding domains, consisting of aa 1–15, aa 16–37, and aa 38–98; see Figure 1. Sequence similarity between E7 and E1A or SV40 is notable in CR1 and CR2; in the carboxyl terminus of E7, no extended similarity is observed but the proteins do all contain twin CXXC elements which are capable of zinc binding [65, 21]. The similarity in CR2 is commonly thought to contain two independent, nonoverlapping functional domains involved in binding pRb and CKII phosphorylation [8, 23, 24]. Although in functional assays there has been little dependence between the two subparts of CR2, the conserved arrangement, also found in some cellular pRB binding proteins [16, 82], suggests that they may indeed be functionally related in an as-yet poorly understood fashion.

Functionally, HPV-16 E7 (16-E7), TAg, and the E1A protein encoded by the 12S-form mRNA share many properties including tranformation in cooperation with *ras*, induction of DNA synthesis, transcriptional control, all of which have been related to CR2 [65]. The E7 CR1 domain does not include sequence elements similar to those in E1A or TAg CR1 involved in binding to the "pocket proteins" (pRb, p107, p130), nor dissociation of pRb/E2F [65]; intriguingly, some of these functions may be conferred by the carboxyl terminus of E7 [61, 89].

	CR1		CR2		Metal Binding	
	* * ***** **		* * ** *** **** **		* *	* *
Ad 5-E1A	HFEPPTLHE.LYDL		..VP.E.VIDLTCHEAGFPPSDDE.DE		CRSC	CSLC
aa	37	49	116	137	154-7	171-4
HPV 16 E7	HGDTPTLHEYMLDL		..QP.E.TTDLYCYEQLNDSSEEE.DE		CCKC	CPIC
aa	2	15	16	37	58-61	91-94
HPV 18 E7	HGPKATLQDIVLHL		EPQN.EIPVDLLCHEQLSD.SEEENDE		CCKC	CPWC
HPV 6 E7	HGRMVTLKDIVLDL		..QPPD.PVGLHCYEQLVDSSEDEVDE		CCGC	CPIC
HPV 11 E7	HGRLVTLKDIVLDL		..QPPD.PVGLHCYEQLEDSSEDEVDK		CCGC	CPIC
SV40 TAg	REESLQLMD.LLGL		NAFN.E.E.NLFCSEEM.PSSDDEATA		CLKC	HEKH
aa	7	19	99	116	301-4	316-9

Figure 3: Similarity in CR1 and CR2 and metal binding motifs between Adenovirus E1A, SV40 large T antigen (TAg) and HPV E7 proteins. (After Figure 1 of Phelps et al. 1992). Asterisks note positions at which Ad 5-E1A and HPV-16 E7 show identical or similar residues.

B. Binding to the retinoblastoma protein and other "pocket proteins".

Like E1A and TAg, HPV16 E7 binds to the unphosphorylated form of the retinoblastoma protein [8, 20, 28, 36]. E7 proteins of other types (HPVs 6, 11, 18) have also been shown to bind to pRb, although the affinity is substantially weaker for some (HPVs 6, 11) [8, 28, 36, 55].

Many studies have shown that amino acids in the portion of the protein corresponding to the second conserved region (CR2) of adenovirus E1A and SV40 large-T antigen (TAg), especially residues 20–29, contribute strongly to pRb-binding [55, 8, 31, 56, 41, 35, 40]. These residues bind to a portion of pRb known as the binding pocket, and rely specifically on portions of pRb known as domains A and B, found between aa 379–772 [87]. The motif XLXCXE (aa 21–26) contains the core for pRb pocket binding. Mutations in this region, especially changes to C_{24} and E_{26}, result in substantial losses of affinity for pRb [8, 40, 41, 55]. Differences in binding affinity between 16-E7 and 6-E7 appear to be restricted to CR2, and may largely be accounted for by the difference at the residue before the leucine of LXCXE, namely the aspartic acid at aa 21 of 16-E7 (. . . DLYCYE. . .) versus the glycine at aa 20 of 6-E7 (. . . GLHCYE. . .), which has been shown to account for much of the difference in transformation activity between 6-E7 and 16-E7 [31, 71]. Comparison of binding by N-methylated peptides, peptides with D-amino acid replacements, or substitution of wild-type residues with alanine or glycine suggest that aa 23, 26 and 27 are largely involved in establishing the correct conformation of the Rb-binding

region, while aa 21,22,28 and more weakly 29 may be involved in direct contact with pRb; data regarding the Cys at aa 24 were more ambiguous [40]. Although not conserved in Ad E1A nor SV40 TAg, the Tyr residue at aa 25 is highly conserved among the papillomavirus types and mutation of this residue results in substantially weaker pRb binding [41].

The pRb-binding domain of E7 may have a protease-like fold since two serine protease inhibitors, tosyl-L-lysine chloromethyl ketone (TLCK) and tosyl-L-phenylalanine chloromethyl ketone (TPCK), reacted with the cysteine residue in the pRb-binding core (LXCXE)and abolished its ability to bind pRb [76]. Addition of the inhibitors to the culture medium of keratinocytes yielded modification of E7 in vivo [76] and interfered with the immortalization capacity of HPV-18 [75].

Full pRb affinity also requires at least a portion of the metal binding domain containing the first C-X-X-C of E7 [35, 61]. Furthermore, disruption of the pRb/E2F complex requires this cysteine doublet [35, 89], and a fragment containing aa 31–98 is sufficient, at least weakly, to disrupt the pRb/E2F complex [61].

The CR1 region (aa 1–15) does not appear to be significantly involved in pRb-binding, in contrast to Ad E1A CR1 [7, 12, 31, 35, 55]. In particular, mutation of $H_2 \rightarrow$ P, which substantially reduced immortalization and transformation, had little effect on pRb binding or DNA synthesis [7]. CKII phosphorylation of Serine residues at aa 31 or 32 also does not seem to affect pRb-binding [8, 24].

As for binding to other pocket proteins, Ad E1A forms complexes with pRb, p107, p130 and cyclin A. 16-E7 peptides containing aa 2–30 compete with E1A to bind to these proteins, while peptides containing aa 2–20 do not, suggesting competition between the pRb (or, more generally, pocket protein) binding elements in CR2 of E7 and E1A [15, 20, 79].

The HPV E7 protein can also interact with a cyclin E/cdk2 complex. This interaction is mediated by p107 [48].

LOCATION	EFFECT	REF
CR1	No substantial effect on pRb binding	[12, 31, 35, 55]
$H_2 \rightarrow$ P	No substantial effect on pRb binding	[7]
CR2 pRb		
aa 20–29	Minimal peptide maintaining (near) full pRb binding	[41]
	Peptides corresponding to aa 2–30, but not 2–20, bind pRb, p107, p130, and cyclin A, suggesting that CR2 pRb is necessary for binding to other pocket proteins as well.	[20]
$D_{21} \rightarrow$ G,N	Substantial reduction in pRb binding	[31, 41, 71]
L_{22} XCXE$_{26}$	Core conserved pRb binding motif	
$Y_{23} \rightarrow$ F	Little effect on binding by aa 20–29 peptide	[41]
$C_{24} \rightarrow$ G	Substantial loss of pRb binding	[41]
$C_{24} \rightarrow$ S	Partial loss of pRb binding	[55, 64]
$Y_{25} \rightarrow$ F	Substantial loss of binding by aa 20–29 peptide	[41]
$E_{26} \rightarrow$ G,Q	Substantial loss of pRb binding	[41, 55, 64]
$Q_{27} \rightarrow$ N	Little effect on binding by aa 20–28 peptide	[41]
CR2 CKII		
$S_{31} \rightarrow$ R	Partial loss of pRb binding	[8]
$S_{32} \rightarrow$ W	Little effect on pRb binding	[8]
S_{31} $S_{32} \rightarrow$ RP,CC,AA,DD	Little effect on pRb binding	[8, 24, 31]
S_{31} $S_{32} \rightarrow \phi$	No effect on pRb binding	[64]
E_{35} $D_{36} \rightarrow$ DH	Little effect on pRb binding	[8]

D_{30} SSEEEDE$_{37}$ → QSSQQQQQ	Partial loss of pRb binding	[24]
CR3	Necessary for efficient interaction with pRb; low affinity pRb interaction domain	[61]
aa 31–98	Necessary for disruption of pRB/E2F-1 complex	[35, 61, 89]

C. Transformation

HPV16 E7 has been found to induce cellular transformation in various assays, including induction of anchorage-independent growth in NIH 3T3 cells, and focus formation in cooperation with activated *ras* of baby rat kidney (BRK) cells, rat embryo fibroblasts (REFs), C127, 3Y1, and No. 7 cells [54].

Low-risk HPV6 E7 does not share the transforming potential of HPV16 E7. This difference is largely confined to the N-terminal portion of E7 [56, 78], and particularly to the primary pRb binding region of HPV16 E7 [31, 71]. Chimeric E7 proteins containing HPV6b CR1 or the CKII recognition sequence in place of the corresponding portion of HPV16 E7 cooperate with *ras* to transform BRK cells at approximately the level of wt HPV16 E7 [31]. Replacing aa 16–30, containing the primary pRb binding site of HPV16 E7, with the corresponding portion of HPV6b E7 resulted in near total loss of tranforming potential [31]. The primary difference in this region between high-risk types and low-risk types is the change between the aspartic acid at aa 21 in HPV16 and the corresponding glycine at aa 20 in HPV6; replacing D_{21} of HPV16 E7 with G results in substantial but not total loss of transforming potential, while replacing G_{20} of HPV6b E7 with D results in an E7 protein with transforming potential at near the level of the wild type HPV16 E7 [31, 71].

Mutants of HPV16 E7 CR1 indicate that this region does contribute to tranformation, in some way not related to pRb-binding, nor DNA synthesis [7, 12, 64]. Deletion of aa 6–10 in particular results in substantial loss of transformation [64, 12]. Mutations to H_2 results in reduced transformation [7, 64, 84]. However, in addition to CR1 from HPV6b E7, CR1 from Ad E1A or TAg from SV40 may be substituted for the HPV16 CR1 domain without substantial loss of transforming potential [12].

Expression of HPV-16 E7 under control of the human keratin 14 promoter in transgenic mice is sufficient to induce epidermal hyperplasia and epithelial tumors. Studies with mutant E7 proteins in this system have also shown that amino acid sequences in both the CR1 homology domain (deletion of aa 6-10) as well as the pRB-binding site (deletion of aa 21-24) contribute to E7-mediated cellular transformation in vivo [29].

HPV16 E7 induction of anchorage-independent growth of NIH 3T3 cells is abolished in G_{24} or G_{26} mutants [22, 8]. In contrast to the results of replacing D_{21} with G (see above), replacing D_{21} with S results in little loss of transforming potential [22]. Similar results confirming the importance of the CR2-pRb region have been reported [8, 64].

Loss of CKII phosphorylation (various substitutions at S_{31} or S_{32}) has been reported to substantially reduce tranformation by some groups [8, 24], but others have not found such a strong effect [22, 31, 64, 77].

The metal binding domain of HPV16 E7, in particular the integrity of the CXXC motifs, has been repeatedly shown to be important for transformation [22, 37, 49, 64, 77, 84].

LOCATION	EFFECT	REF
CR1	HPV16 and HPV6 CR1 interchangeable.	[31]
	Ad E1A CR1 and SV40 TAg CR1 also can replace HPV16 CR1.	[22]
	Various deletion mutants lead to small reductions in transformation.	[12]
$H_2 \rightarrow$ D,P	Reduced transformation	[84, 7]
aa 6–10	Deletion of aa 6–10 leads to substantial reduction in transformation.	[7, 12]
CR2 pRb	Replacing aa 16–30 with corresponding HPV6b peptide leads to loss of transforming potential	[31]
$D_{21} \rightarrow$ G	Large reduction in transformation. This corresponds to the major difference between low- and high-risk E7s. Replacing the corresponding G in HPV6B E7 with D results in a protein with transforming capacity near that of HPV16 E7.	[31, 71]
$D_{21} \rightarrow$ S	Little effect on transformation	[22]
$C_{24} \rightarrow$ G,S	Loss of tranformation	[22, 8, 64]
$E_{26} \rightarrow$ G,Q	Loss of tranformation	[22, 8, 64]
CR2 CKII phos		
$S_{31} \rightarrow$ R	Little loss of transformation	[22]
$S_{31} \rightarrow$ G	Little loss of tranformation	[77]
$S_{32} \rightarrow$ W	Little loss of transformation	[8]
$S_{32} \rightarrow$ A	Little loss of tranformation	[77]
$S_{31}\ S_{32} \rightarrow$ RP	Reduction in transformation	[8]
$S_{31}\ S_{32} \rightarrow$ CC,AA,DD	No loss of transformation	[31]
$S_{31}\ S_{32} \rightarrow \phi$	No loss of transformation	[64]
$E_{35}\ D_{36} \rightarrow \phi$ DH	No loss of transformation	[8, 22]
$E_{35}\ D_{36}\ E_{37} \rightarrow \phi$	No loss of transformation	[64]
CR3	Integrity of the CXXC motifs, especially the one at aa 91–94, has repeatedly been shown to be important to in vitro transformation and protein stability	[22, 37, 49, 64, 77, 84]

D. Immortalization and cell growth

HPV16 E7 causes cell growth and extended proliferation of primary rat embryo fibroblasts (REFs) and human keratinocytes (HKs) [54]. Stimulation of cell growth in REFs is also conferred by 6 E7 and 6/16 or 16/6 E7 chimeras containing approximately the first 30 amino acids from one type and the remaining amino acids of the other, but extended proliferation is conferred only by 16 E7 and 16/6 E7, indicating that the relevant difference between 16-E7 and 6-E7 is contained within the CR1 or CR2 pRb regions [78].

Given the importance of the pRb binding site in CR2 for transformation, it is of interest that immortalization seems to be at least somewhat independent of pRb binding, suggesting involvement

of other cellular factors [37]. Some mutants with much reduced pRb-binding ($C_{24} \rightarrow G$; $E_{26} \rightarrow G$) retained significant (although less than wild type) immortalization of rat embryo fibroblasts (REFs), but showed little ability to cooperate with *ras* to transform REFs. Similarly, $C_{24} \rightarrow G$ and $E_{26} \rightarrow G$) mutations did not interfere with immortalization of HKs [37]. If the mechanisms of E7-induced REF and HK immortalization are the same, this suggests the crucial difference between 16-E7 and 6b-E7 is contained in CR1. In support of this, mutation of $H_2 \rightarrow P$ resulted in reduced immortalization in cooperation with activated Ha-*ras* of baby rat kidney (BRK) cells [7], although little effect on pRb binding or DNA synthesis was observed.

Immortalization by HPV16 E7 also appears to require elements in the metal binding domain (CR3), especially the CXXC motif at aa 91–94. REF immortalization was abrogated by mutation of $C_{91} \rightarrow G$ [78]; similarly, immortalization of BRK cells by E7 requires both of the E7 CXXC motifs [84]; and, the CXXC element at aa 91–94 of 16-E7 appears to be essential for HK immortalization [37].

HPV E7 can independently immortalize a subset of human mammary epithelial cells [86]. This activity may be related to the ability of E7 to overcome a proliferation block in early passage human mammary epithelial cells [25]. The retinoblastoma protein or a related "pocket protein" has been implicated in controlling this block [25].

LOCATION	EFFECT	REF
CR1	May distinguish immortalizing 16-E7 from 6-E7	[78]
$H_2 \rightarrow P$	Reduced BRK immortalization	[7]
CR2 pRb		
$C_{24} \rightarrow G$	Not essential for HK immortalization	[37]
$E_{26} \rightarrow G$	Not essential for HK immortalization	[37]
CR3		
C_{91} XXC_{94}	Necessary for HK immortalization	[37]

E. Phosphorylation

HPV16 E7 is phosphorylated at serine residues, as are HPVs 18 and 6b E7 [8, 23, 74, 72]. The CR2 region contains a CKII recognition site shared with CR2 of Ad E1A and SV40 TAg [65]. Phosphorylation of 16-E7 is observed in keratinocytes (human and murine), but not fibroblasts (human and murine), consistent with levels of CKII activity [30]. The rate of phosphorylation is highest for 18-E7, intermediate for 16-E7, and lowest for 6-E7, agreeing with the levels of phosphorylation observed [8]. The difference in rates between 6-E7 and 16-E7 seems to be determined by the sequences in the CKII recognition site itself [31].

Mutation of either S_{31} or S_{32} resulted in less efficient but still significant CKII phosphorylation of 16-E7, indicating that either of these positions is a possible target, although it is not clear whether both may be phosphorylated in vivo; mutation of both serines (S_{31} S_{32}) resulted in loss of CKII phosphorylation [8, 64]. Mutations to other serine residues (S_{63}, S_{71}, S_{95}) have been assayed, with some suggestion that S_{71}, which is conserved in the E7 proteins of other types, also is phosphorylated [77], although this mutation also resulted in lower levels of detectable E7 protein; this result for S_{71} was not confirmed in a separate study [64].

The low risk HPV-6 E7 but not the high risk HPV-16 E7 protein is a substrate for phosphorylation by protein kinase C (PKC) in vitro. The threonine residue at sequence position 7 has been mapped as the main target of phosphorylation by PKC [2].

LOCATION	EFFECT	REF
S_{31} $SEEEDE_{37}$	CKII recognition site	[8, 23]
S_{31} ,S_{32}	Possible CKII targets	[8, 24, 64]
E_{35} D_{36} $\rightarrow$ DH	No effect. Not all acidic residues are necessary for CKII phosphorylation.	[8]
E_{35} DE_{37} $\rightarrow \phi$	Substantial loss of phosphorylation	[64]
S_{71}	Mixed results.	[77, 64]

Comparison of oncoprotein CKII sites	
16-E7	DSSEEEDE
18-E7	DSEEENDE
6-E7	DSSEDEVD
TAg	PSSDDE
E1A	PSDDEDE

F. Metal Binding and Complex Formation

The E7 proteins of various PV types are capable of binding zinc, as might be expected from the presence of the two CXXC elements in the carboxyl terminus [9]. Binding of zinc appears to take place at a 1:1 ratio of E7 molecules to Zn(II) ions [62, 69]. This may not, however, involve a classical zinc finger structure in which both CXXC elements are coordinated to the same zinc ion, since a peptide corresponding to aa 67–98, which contains only one CXXC element, also binds zinc; mutation of C_{68} $\rightarrow$ A in this peptide does not interfere with zinc binding, establishing that this property is not dependent on the use of C_{68} $XXXXH_{73}$ as an alternative coordinating element [68]. Moreover, the distance between the two CXXC elements is too large for a classical zinc finger.

Several findings have hinted that the CXXC elements may be involved in protein-protein interactions [61]. One indication is that E7 mutants or fragments which contain only the C-terminal CXXC element can still bind zinc [68]. Other implications come from the loss of binding to other proteins when the CXXC elements are disrupted or deleted [35, 37, 61, 89].

Integrity of the zinc binding sites is important for protein stability [64].

LOCATION	EFFECT	REF
C_{58} XXC_{61}	Protein stability	[64]
aa 67–98	Sufficient to bind zinc	[68]
C_{68} $\rightarrow$ A	Not essential in aa 67–98 peptide	[68]
C_{91} XXC_{94}	Protein stability	[64]

G. Electrophoretic retardation

HPV16 E7 migrates more slowly on a gel than expected for a protein of its predicted mass; this property is not shared with HPV6-E7. Mutations eliminating CKII phosphorylation (S_{31} S_{32}) do not affect the mobility of 16-E7 [8, 31, 64]. Chimeric E7 peptides made from 16-E7 and 6-E7 indicate that the determinants of reduced mobility are contained in CR1 (aa 1–15) [31]. The acidic character of E7 is a factor in the mobility [4]; the residue D_4 is particularly important [3, 71]. Homologous sequences from E1A, but not TAg, also lead to retardation [12].

LOCATION	EFFECT	REF
CR1	Primary determinant of reduced mobility	[3, 4, 31]
$D_4 \rightarrow R$	Increased mobility	[3]

H. Induction of DNA synthesis

HPV16 E7 can induce DNA synthesis in quiescent rodent cells. Peptides corresponding to aa 1–40 and aa 16–98 were both induction-competent, albeit less efficiently with aa 1–40 roughly half as efficient and aa 16–98 roughly 10% as efficient, suggesting that the core elements for this function are contained within CR2 (aa 16–40 specifically) with additional elements in CR1 necessary for efficient induction [68]. HPV6b E7 also is capable of inducing DNA synthesis, but at a lower level [85]. Integrity of the pRB binding site is important for induction of DNA synthesis [7]. Interaction with pRb and deregulation of E2F may be necessary but not sufficient for induction of DNA synthesis [53].

LOCATION	EFFECT	REF
CR1	Necessary for efficient induction	[68]
$H_2 \rightarrow P$	Little effect	[7]
CR2	Core elements for induction	[7, 68]
CR3	Limited effect	[68]

I. Ad E2 transactivation.

HPV16 E7 transactivates the Ad E2 promotor [65]. This transactivation does not require additional protein synthesis, implying that the transactivation involves preexisting cellular factors [68]. The E7 protein does not appear to transactivate all Ad E1A-responsive promotors [63, 68].

This transactivating function is shared with E7 of other HPV types, including the low-risk HPV-6b. Substitution of any or all of HPV16 E7 CR1, CR2 pRb, and CR2 CKII with the corresponding peptides from HPV6b retained E2 transactivation in CV-1 monkey kidney cells [31, 56].

Mutation of the pRb binding site in CR2 ($C_{24} \rightarrow G$; $E_{26} \rightarrow G$) substantially reduced transactivation [84, 22, 12, 63, 64].

In one study, mutation of one target of CKII phosphorylation ($S_{31} \rightarrow R$) resulted in substantial loss of E2 transactivation, and mutation of acid residues in the CKII recognition sequence (E_{35} E_{36} $\rightarrow$ D_{35} H_{36}) resulted in a lesser decrease of E2 transactivation [22], but a subsequent study found little effect of mutations in this region, including loss of phosphorylation [64].

Mutation of $H_2 \rightarrow D$ results in decreased transactivation [84]. Microinjection of E7 peptides into HPV-18 expressing HeLa cervical carcinoma cells suggested that C-terminal fragments of E7 (aa 67–98, 39–98, 16–98) retained significant E2 transactivation, but not the N-terminal fragment consisting of aa 1–40 [68]; similarly, most deletions in CR1 (aa 1–15) were found to have only small effects [12], although deletion of aa 6–10 (PTLHE) was found to have a larger effect [12, 64]. Additionally, disruption of the CXXC motif at aa 91–94 substantially reduces, but does not abolish, transactivation [77, 84]. Analysis of *cis* elements in the Ad E2 promotor showed that the E2F sites and the ATF site are important for activation by E7 [63]. This suggests a functional similarity between HPV E7 and the protein encoded by the 12S mRNA of Ad E1A.

E7 can also activate expression of E2F-dependent cellular genes, including b-myb [42] and cyclins A and E [91]. Mutational analysis of cyclin E-activation showed that domains in CR1 as well as the pRB-binding site are required for activation of these promoters by E7 [91].

LOCATION	EFFECT	REF
CR1		
P_6 TLHE$_{10} \rightarrow \phi$	Reduction in transactivation	[12, 64]
CR2 pRb		
D_{21} LYC$_{24} \rightarrow \phi$	Substantial reduction in transactivation	[12, 63, 64]
$C_{24} \rightarrow$ G,S	Substantial reduction in transactivation	[22, 84, 64]
$E_{26} \rightarrow$ G,Q	Substantial reduction in transactivation	[22, 64]
CR2 CKII	Mixed results	[22, 63, 64]
CR3		
aa 67–98	Sufficient for transactivation when microinjected into HeLa cells	[68]
C_{91} XXC$_{94}$	Necessary for efficient transactivation	[77, 84]

J. Interactions with E2F

HPV16 E7 can disrupt the E2F/pRb complex, a property it shares with adenovirus E1A and SV40 TAg [13]. A complex between E2F and cyclin A appears not to be disturbed by E7 [13, 58], and in fact E7 associates with this complex in S phase [5].

E2F/pRb dissociation. CR1 of E7 appears not to be involved in E2F/pRb dissociation, in contrast to CR1 of E1A. This coincides with the involvement of CR1 with binding to pRb: CR1 of E1A has pRb binding activity, while that of E7 does not [20, 35]. The CR2 element of E7, especially the pRb binding portion, is required for efficient dissociation of E2F and pRb. Mutations in this domain can reduce or eliminate the dissociating function [13]. On the other hand, this region is not sufficient [35, 61, 89] for dissociation. In the presence of CR2, peptides which do not include the second CXXC motif for CR3 are sufficient for dissociation, but the presence of the second CXXC substantially increases dissociation [35, 61, 89].

LOCATION	EFFECT	REF
CR1	Not necessary.	[35]
CR2 pRb	Necessary but not sufficient for efficient dissociation, in the context of the full protein.	[13]
CR3	Necessary; apparently competes with E2F for binding to C-terminal elements of pRb.	[35, 61]

Association with E2F/cyclinA complex. A complex involving HPV-16 E7, E2F, and cyclin A is formed in S phase, in a manner dependent on elements in CR2 which are required for binding of E7 to pRb [5]. This may be contrasted with the behavior of Ad E1A, which dissociates the E2F/cyclinA complex [13, 57]. The ability to associate with E2F/cyclinA partially correlates with transforming potential, and 6b-E7 does not bind to E2F/cyclinA as efficiently as 16-E7. Deletion of 16-E7 E_{35} DE_{37} , which affects CKII phosphorylation, did not affect formation of the E2F/cyclinA complex, pRb binding, nor transformation. A 16-E7 mutant ($C_{24} \rightarrow$ S) bound the E2F/cyclinA complex, but had reduced pRb affinity and transforming activity. 16-E7 $E_{26} \rightarrow$ Q did not bind the complex, nor pRb, nor did it have transforming activity.

LOCATION	EFFECT	REF
CR1		
P_6 TLHE$_{10} \rightarrow \phi$	Able to bind to E2F/cyclinA complex	[5]
CR2 pRb		
D_{21} LY$C_{24} \rightarrow \phi$	Unable to bind E2F/cyclinA complex	[5]
$C_{24} \rightarrow$ S	Able to bind E2F/cyclinA complex	[5]
$E_{26} \rightarrow$ Q	Unable to bind E2F/cyclinA complex	[5]
CR2 CKII		
E_{35} $DE_{37} \rightarrow \phi$	Able to bind E2F/cyclinA complex	[5]
CR3	?	

K. Abrogation of TGF-β repression of c-myc promotor

HPV16 E7 abrogates TGF-β repression of the c-myc promotor [67]. This property is not shared by 6-E7. The N-terminal half of 16-E7 determines this property, as a 16/6 E7 chimera (16-E7 aa 1-50 plus 6-E7 aa 51-98) shares the activity, while a corresponding 6/16 chimera does not [56].

LOCATION	EFFECT	REF
aa 1–50	Contains elements abrogating TGF-β repression	[56]
D_{21} LYC_{24}	Required for abrogation	[67]

L. Nuclear localization

A peptide consisting of aa 16–41 of 6-E7, like full length 16-E7, is localized to the nucleus [26]. Mutation of $C_{24} \rightarrow G$ or $E_{26} \rightarrow G$ does not affect localization, indicating that the nuclear localization is not due to nuclear localization of pRb nor the other pocket proteins.

Immunofluorescence and electron microscopy immuno-gold labelling studies have shown that HPV-16 E7 is localized to the nucleolus in cervical carcinoma cell lines. Interestingly, pRB was mapped to the same compartment. Since E7 is also localized in the nucleolus when overexpressed in fission yeast which does not contain pocket proteins, nucleolar localization of E7 is independent of pRB[90].

LOCATION	EFFECT	REF
aa 16–41	A peptide consisting of these residue is localized in the nucleus.	[26]
$C_{24} \rightarrow G$	Did not affect localization.	[26]
$E_{26} \rightarrow G$	Did not affect localization.	[26]

M. Dimer/Multimer formation

E7 can form dimeric and multimeric complexes in vivo. The carboxyl terminal zinc-binding domain is necessary for this biochemical property of E7 [49]. Studies with the two-hybrid system in yeast have been used to further map the determinants of dimer/multimer formation [14, 94]. Specific amino acid residues in the carboxyl terminus of HPV-16 E7 were identified (C58; C59; L67; C91) that are necessary for dimer/multimer formation [14]. These studies also showed that while the carboxyl terminus of E7 is sufficient for dimerization, amino terminal sequences may further stabilize such complexes [14].

N. Abrogation of Growth arrest signals

The HPV-16 E7 protein can overcome p53-mediated growth arrest signals in a number of cell systems [81, 18, 73, 33, 32, 70, 52, 39]. This is not readily predictable since E7 does not directly target p53. Dephosphorylation of pRB is linked to the action of the cdk-inhibitor p21cip1 which is induced by p53, and thus pRB is viewed as an important modulator of p53-mediated growth inhibition. The ability of E7 to interact with and abrogate the growth suppressive activity of pRB was proposed to be the basis for the ability of E7 to counter p53-mediated growth-suppression. A careful analysis of E7 mutants, showed that this hypothesis was not correct [17]. While pRB-binding is necessary, it is not sufficient and several mutants of E7 that can bind to pRB and activate E2F-dependent promoters, are unable to abrogate p53-mediated growth suppression. This suggests that multiple cellular targets of E7 may contribute to the ability to overcome these growth arrest signals. In view of a study that showed that E7 can destabilize pRB and that sequences outside of the pRB-binding site of E7 are necessary for pRB-destabilization it is likely that the ability of E7 to overcome p53-mediated growth inhibition correlates with the ability of E7 to destabilize pRB [39]. While E7 can abolish p53-mediated growth inhibition, it does not inhibit p53-mediated apoptosis [83].

O. Interaction with and inactivation of cdk inhibitors

Many inhibitory signals of cellular growth are mediated by inhibitors of cyclin dependent kinases. The inhibitor p21cip1 is an important modulator of the p53-mediated growth arrest and p27kip1 contributes to TGFβ-mediated growth suppression. As described in more detail in previous sections E7 can overcome these and other growth inhibitory signals.

Like E1A [44], E7 can interact with and abrogate kinase inhibition by p27kip1. The carboxyl-terminal domain of E7 is important for the interaction with p27kip1, but not necessarily for the abrogation of p27kip1-mediated cdk-inhibition [92].

E7 can also interact with and abrogate p21cip1-mediated inhibition of cdk-activity and of PCNA-dependent DNA replication [38, 27]. This activity may be particularly relevant for E7 to allow for papillomavirus replication in otherwise growth-arrested, terminally differentiated keratinocytes. The low risk HPV E7 proteins have a decreased potential to interact with p21cip1. Mutagenic analyses showed that the amino terminal pRB-binding site as well as sequences in the carboxyl-terminus of E7 contribute to p21cip1-binding [38, 27].

P. Destabilization of pRB and stabilization of p53

E7 expressing cells contain decreased levels of pRB and increased levels of p53 [80, 18, 19, 11, 39]. It has been shown that these changes are a consequence of altered protein stabilities [11, 39]. The ubiquitin-dependent proteolysis pathway is involved in the E7-mediated destabilization of pRB [11]. Mutational analysis showed that mutations outside of the core pRB-binding site are also required for pRB-degradation and that the sequences required for pRB-destabilization correlate with those required for cellular transformation. These results strongly suggest that E7-mediated inactivation of pRB involves molecular steps in addition to protein-binding [39].

Interestingly, E7-mediated destabilization is coupled to stabilization of p53 [39]. p53 is accumulated in a wild type form as p21cip1 is highly expressed in E7 expressing cells [39]. Since E7 alleviates p53-mediated growth inhibition but not apoptosis [83], this may, at least in part, account for the observation that E7-expressing cells are predisposed to undergo programmed cell death (apoptosis) [59, 60, 34].

Q. Interaction with other cellular proteins

The E7 protein can also interact with several other cellular proteins. Interactions with the basal transcription factor machinery, including TATA box-binding protein (TBP) [47, 66, 46] and TBP-associated protein associated factor-110 (TAF-110) [47] have been documented. These interactions are likely to be mediated by the amino terminal domain of E7. Of particular interest is the observation that the phosphorylation state of the CKII site adjacent to the pRB-binding site modulates interaction with TBP [46]. It has also been shown that interaction of E7 with TBP can repress the transcriptional activation activity of p53 [45]. In apparent contrast with this observation, it was demonstrated that transcriptional targets of p53 are induced to similar if not higher levels in E7 expressing cells in response to DNA damage [32, 70, 39]. The E7 protein can act as a transcriptional activator when fused to a DNA binding domain. This activity of E7, however, is restricted to yeast cells and is mediated by the amino terminal domain of E7 [14, 94].

E7 can also interact with and activate the AP-1 family of transcription factors [1]. Mutational analysis suggested that the carboxyl terminal domain is important for this interaction although it is possible that additional determinants are also important [1]. The E7 protein can also elevate transcription of c-fos. This effect is mediated by the cAMP-responsive element [51]. The functional interaction of E7 with other transcription factor has been illustrated in a study where it was shown that E7 can complement the CR1-dependent transactivation of adenoviral early genes by E1A by increasing the DNA-binding activity of ATF and oct-1 transcription factors [88].

With the availability of rapid screening methods to select for protein-protein interactions the list of E7-associated proteins is likely to significantly increase in the years to come.

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