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Transactivation by the E2 Protein of Oncogenic Human Papillomavirus Type 31 Is Not Essential for Early and Late Viral Functions

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The activation of transcription and of DNA replication are, in some cases, mediated by the same proteins. A prime example is the E2 protein of human papillomaviruses (HPVs), which binds ACCN$_6$GGT sequences and activates heterologous promoters from multimerized binding sites. The E2 protein also has functions in replication, where it complexes with the virally encoded origin recognition protein, E1. Much of the information on these activities is based on transient-transfection assays as well as biochemical analyses; however, their importance in the productive life cycle of oncogenic HPVs remains unclear. To determine the contributions of these E2 functions to the HPV life cycle, a genetic analysis was performed by using an organotypic tissue culture model. HPV type 31 (HPV31) genomes that contained mutations in the N terminus of E2 (amino acid 73) were constructed; these mutants retained replication activities but were transactivation defective. Following transfection of normal human keratinocytes, these mutant genomes were established as stable episomes and expressed early viral transcripts at levels similar to those of wild-type HPV31. Upon differentiation in organotypic raft cultures, the induction of late gene expression and amplification of viral DNA were detected in cell lines harboring mutant genomes. Interestingly, only a modest reduction in late gene expression was observed in the mutant lines. We conclude that the transactivation function of E2 is not essential for the viral life cycle of oncogenic HPVs, although it may act to moderately augment late expression. Our studies suggest that the primary positive role of E2 in the viral life cycle is as a replication factor.

DNA replication and gene expression are regulated in higher eukaryotes, in part, through the action of DNA-binding proteins. Studies on eukaryotic viruses have provided important new insights into these mechanisms and have suggested that these two processes are related. Evidence from viral systems has indicated that transcription factors, either of cellular or viral origin, can be directly involved in DNA replication (70). Similar roles for transcription factors have been demonstrated in *Saccharomyces cerevisiae* (70). Transcription factors appear to function in replication either by directly associating with replication proteins or by changing the global structure around origins (70). Interestingly, it is not the process of transactivation itself which mediates replication enhancement as mutational studies of viral proteins, such as the Epstein-Barr virus Zta protein and the papillomavirus E2 protein, have demonstrated that the replication and transactivation functions are separable in transient-transfection assays (termed transient assays) (1, 10, 19, 25, 50, 51). This suggests that both processes may function independently of each other and may be required at different phases of the viral replication cycle. Examination of the replication and transactivation function of the E2 proteins from the oncogenic human papillomavirus (HPV) types has provided important insights into the relationship of these two processes.

HPVs induce benign squamous epithelial tumors which may in some cases progress to carcinomas (72, 73). Following infection of basal cells, HPV genomes are stably maintained as multicopy nuclear plasmids and only early viral genes are expressed. The completion of the viral life cycle requires differentiation of the infected keratinocyte, which results in amplification of the viral copy number and the activation of late viral promoters. These late promoters direct the expression of the capsid proteins L1 and L2 and the abundant viral E1^E4 protein, which may play a role in viral egress (31, 38). The molecular events that trigger these processes have not been characterized due to the lack of a genetic system for analysis of the viral life cycle. Recent success in synthesizing HPV virions from transfected cloned DNA templates now permits a detailed investigation of these mechanisms (22, 23).

Studies by several groups have shown that papillomavirus E2 proteins can act as regulators of both viral gene expression and replication. Initial studies used the bovine papillomavirus type 1 (BPV1) to show that E2 is required for transient replication of the viral DNA and for stable maintenance of the virus in transformed cells (14, 15, 47, 69). The E2 protein forms dimers which specifically bind to palindromic DNA of the sequence ACCN$_6$GGT, which is present in multiple copies in the regulatory regions of papillomaviruses (40, 60). E2 enhances replication of the viral DNA through complex formation with the viral E1 protein, which has characteristics of a replication initiator protein (61). In vitro and in vivo studies have demonstrated that binding of E2 to E1 increases the specificity of the origin recognition by E1 (8, 20, 36, 39, 43, 54, 55, 63, 71). The BPV1 E2 protein was first characterized as an enhancer-binding protein that could stimulate transcription from multimerized E2 binding sites located upstream of heterologous promoters in transient assays (3, 27, 58). Subsequent studies revealed that several early BPV1 promoters were activated through E2 in a binding site-dependent manner (26, 28, 57,

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64). The ability to transactivate was shown to be essential for the transformation of cells by BPV1 (10, 14, 15, 47, 52).

While HPVs have some similarities to the bovine viruses, significant differences exist between the two groups. The oncogenic genital HPVs differ from BPV1 in the tissues they target and in their transcriptional control. The major early promoter of genital HPVs (which is labeled P97 in HPV16 and HPV31) is located in the upstream regulatory region (URR) immediately upstream of the E6 gene. Its activity is solely dependent upon cellular transcription factors and directs the expression of early genes through a variety of alternatively spliced, polycistronic mRNAs (31, 38). The organization of the URR is highly conserved among genital HPVs and contains two E2 binding sites just upstream of the P97 TATA box as well as two other sites upstream (65). In transient expression assays, binding of E2 to the P97 proximal sites can downregulate P97 activity and provide a means to regulate the levels of the oncoproteins E6 and E7 as well as replication proteins (6, 13, 49, 65–67). In addition, low concentrations of E2 have been suggested to weakly activate P97 transcription through promoter-distal E2 binding sites (9, 59). Like the BPV1 E2 protein, HPV E2 proteins can strongly activate transcription from synthetic promoters consisting of multimerized E2 binding sites fused to a minimal promoter (11, 29, 35, 45, 50, 68).

The conservation of the E2 transactivation function suggests that it has an important function in the viral life cycle, but to date no targets for the E2 transactivation function have been identified in oncogenic HPVs. In transient assays, the transactivation and replication functions of BPV1 and HPV16 E2 can be separated by mutations in the amino-terminal domain of E2 (1, 10, 19, 25, 50). Though separable, both activities reside in amino acid residues that are highly conserved among papillomaviruses. In this study, we sought to determine the role of E2 transactivation in the life cycle of oncogenic HPVs through a genetic analysis. We find that viral genomes expressing transactivation-defective but replication-competent E2 proteins can be established in NHKs as episomes and are able to induce late viral functions after differentiation. This suggests that transactivation by E2 is not an essential function in the virus life cycle of oncogenic HPVs.

MATERIALS AND METHODS

Recombinant plasmids. Plasmid pUKHPV31 contains the complete genome of HPV31 cloned into the *Eco*RI site of a modified pUC18 plasmid (34). Mutations in the E2 gene were introduced with the Chameleon Mutagenesis Kit (Stratagene) and primers that contain specific mutations in the E2 gene. pHPV31 E2:EN20 contains mutations at nucleotides (nt) 2750 (G to A) and 2752 (A to C), pHPV31 E2:RK37 contains mutations at nt 2801 and 2802 (CG to AA), pHPV31 E2:EQ39 was mutated at nt 2807 (G to C), and pHPV31 E2:IL73 was mutated at nt 2909 (A to C). All mutations were confirmed by sequence analysis. Plasmid pRP742 contains HPV31 nt 678 to 919 cloned into the *Bam*HI site of pcDNAII (Invitrogen) and has been described previously (34). Plasmid pRPA31L1 consists of HPV31 nt 5521 to 5703 cloned into pSP72 (Promega, Madison, Wis.) and has been described previously (62). The HPV31 E1 and E2 expression vectors are based upon pSG5 (Stratagene) and have been described previously (21). To facilitate subcloning of the mutated E2 genes, the E2-containing fragment was released with *Bam*HI from pSG31E2 and then cloned into the *Bgl*II site of pSG5, resulting in pSBE2. E2 mutants EN20, RK37, and EQ39 (see Results) were amplified by PCR from mutant genomes, used to replace the *Bam*HI-*Acc*B7I fragment in pSBE2, and resequenced. E2 mutant IL73 was transferred as an *Acc*B7I-*Eco*RI fragment (HPV31 nt 2865 to 3361) into pSXE2. Plasmid pSXE2 is a derivative of pSG31E2, which was modified by removing the *Eco*RI site in the polylinker through partial digestion and by deleting the *Aat*II-*Nae*I fragment from the vector backbone in pSG31E2. Plasmid pGL31URR contains HPV31 nt 7067 to 107, which were amplified by PCR with primers containing *Mlu*I and *Xho*I restriction sites and inserted into *Mlu*I/*Xho*I-digested pGL3 basic (Promega). The luciferase reporter plasmid p6XE2BS-luc was constructed by inserting three copies of an oligonucleotide, containing HPV31 E2BS3 and E2BS4 (5′GCGTGACCGAAAGTGGTGAACCGTTTTCGGTTG GTGCGC-3′) into the *Mlu*I site of plasmid pGL3 promoter upstream of a minimal simian virus 40 (SV40) early promoter (Promega).

Generation, culture, and induction of differentiation of HPV31-containing human keratinocytes. NHKs purchased from Clonetics (San Diego, Calif.) were grown in KGM (Clonetics) and transfected at passage 2 with religated HPV31 DNA or mutated viral DNA and pSV2neo DNA as described previously (23). Cells were selected with 200 μg of Geneticin (Gibco BRL) per ml for 7 to 10 days in E-medium supplemented with 5 ng of epidermal growth factor on mitomycin-treated fibroblast feeder cells, and resistant clones were expanded. The generation of stable cell lines was repeated three times with cells from two different donors to ensure reproducibility. Organotypic raft cultures were grown without the addition of protein kinase C activators as described previously (23, 41, 42).

Transient luciferase expression assay. SCC-13 cells are derived from a squamous cell carcinoma of the cheek and were maintained in E-medium in the presence of fibroblast feeder cells. Approximately 2.5×10^5 SCC-13 or NHK cells were seeded the day before transfection. The next day, the cells were transfected with 0.5 μg of p6XE2BS-luc and 0.1 μg of pSG5 or the respective E2 expression vectors by using 15 μl of Lipofectamine in OptiMEM (Gibco BRL) or KGM for NHKs (Clonetics) in accordance with the manufacturer's recommendations. The following day, the medium was changed and the cells were incubated for another 24 h. The cells were washed twice with cold phosphate-buffered saline (PBS) and then lysed by adding 350 μl of cold luciferase extraction buffer (0.1 M potassium phosphate [pH 7.8], 1% Triton X-100, 1 mM dithiothreitol [DTT]). Lysates were cleared by centrifugation (Eppendorf Microfuge; $16,000 \times g$, 5 min, 4°C), and 2 to 5 μl was measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratories) as described in the manufacturer's manual.

Gel retardation analysis. SCC-13 cells (6×10^5) were seeded the day before transfection and transfected the next day as described above with 2 μg of pSG5 or E2 expression vectors. The cells were harvested 44 h after transfection, and whole-cell lysates were prepared as described before (33) with minor modifications. Briefly, the cells were washed with cold PBS and then scraped in PBS into a Microfuge tube and collected by centrifugation (Eppendorf Microfuge; $16,000 \times g$, 30 s, 4°C). The cell pellet was resuspended in 20 μl of lysis buffer (10 mM HEPES [pH 7.9], 500 mM KCl, 50 mM NaF, 0.5 mM Na *o*-vanadate, 0.2 mM EDTA, 1 mM DTT, 20% glycerol, protease inhibitor cocktail [Boehringer Mannheim]). Lysates were prepared by incubating the tubes in a dry ice-ethanol bath and then by incubating them at 37°C for 2 min each. Lysates were cleared by centrifugation in an Eppendorf Microfuge ($16,000 \times g$, 5 min, 4°C). The supernatants were diluted in 10 mM HEPES (pH 7.9)–125 mM KCl–50 mM NaF–0.5 mM Na *o*-vanadate–0.2 mM EDTA–1 mM DTT–20% glycerol–protease inhibitor cocktail (Boehringer Mannheim), snap-frozen, and stored at −80°C. Gel retardation analysis was carried out with 20,000 cpm of a ^{32}P-end-labeled double-stranded oligonucleotide containing E2BS4 (HPV31 nt 45 to 70). Binding reaction mixtures received equal amounts of protein as determined by the Bradford assay. Reactions were carried out in 10 mM HEPES (pH 7.9)–100 mM KCl–1.4 mM DTT–10% glycerol–50 μg of heat-denatured herring sperm DNA per ml–100 μg of poly(dI-dC) (Pharmacia) per ml–5 mM NaF–0.2 mM Na *o*-vanadate at room temperature for 15 min. Complexes were separated in a 4% polyacrylamide gel (37.5:1) in 0.25× Tris-borate-EDTA at 200 V. Gels were dried and autoradiographed.

Transient replication assay. Approximately 6×10^5 SCC-13 cells were seeded the day before transfection into 60-mm-diameter dishes. The cells were transfected as described above by using 0.5 μg of pGL31URR, 1 μg of pSG31E1, and 0.1 μg of the respective E2 expression vectors. The following day, the transfected cells were divided into 100-mm-diameter dishes and grown for an additional 48 h. Low-molecular-weight DNA was purified by using the Hirt procedure (30) with the following modifications: cell pellets were digested with 50 μg of proteinase K per ml in 400 mM NaCl–10 mM EDTA–10 mM Tris-HCl (pH 7.5)–0.2% sodium dodecyl sulfate (SDS) at 55°C for 3 h; NaCl was added to 1 M, and high-molecular-weight DNAs were precipitated at 4°C overnight and then centrifuged (60 min, 4°C, $16,000 \times g$). Supernatants were extracted once with phenol-chloroform-isoamyl alcohol and once with chloroform before precipitation with isopropanol. Each sample was digested with 5 U of *Dpn*I, 15 U of *Hpa*I, and 50 μg of RNase A per ml for 5 h prior to Southern analysis. Southern blots were probed with a fragment from pGL31URR that contains the HPV31 fragment linked to the luciferase gene. Transfections were repeated four times with different DNA preparations.

Southern blot analysis. Total cellular DNA from cell lines was isolated by proteinase K and RNase A digestion followed by phenol-chloroform extractions and ethanol precipitation. Digested DNAs were separated in 0.8% agarose gels and transferred to GenescreenPlus membranes (NEN Dupont). Specific fragments were detected with random-primed DNA (HiPrime kit; Boehringer Mannheim). Hybridizations were carried out in 50% formamide–4× SSPE–5× Denhardt's solution–1% SDS–20 μg of salmon sperm DNA per ml at 42°C overnight (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]). Blots were washed at room temperature twice in 2× SSC–0.1% SDS, followed by two washes in 0.1× SSC–0.1% SDS, and then twice in 0.1× SSC–1% SDS at 50°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Blots were visualized by autoradiography and quantitated by phosphorimaging (Molecular Dynamics Inc.).

RNase protection analysis. Total cellular RNA was isolated with Trizol reagent (Gibco BRL) from HPV31 containing keratinocytes. Precipitated RNA pellets were hybridized to 250,000 cpm of antisense ^{32}P-labeled riboprobe tran-

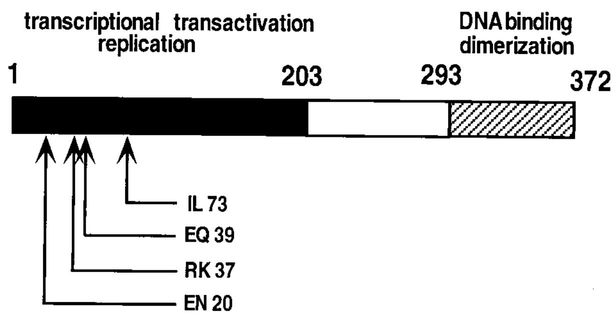

FIG. 1. Linear representation of the HPV31 E2 protein. The highly conserved amino- and carboxy-terminal domains of E2 are presented as black and striped boxes, respectively. The less-conserved hinge domain is shown in white. The arrows indicate mutations resulting in amino acid exchanges of highly conserved residues (residues 20, 37, 39, and 73) among E2 proteins.

scribed from linearized pRP742 or pRPA31L1 in 10 μl of 40 mM PIPES (pH 6.4)–400 mM NaCl–1 mM EDTA–80% formamide overnight at 37°C [PIPES is piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]. RNase digestion was performed by the addition of 300 μl of 10 mM Tris-HCl (pH 7.5)–300 mM NaCl–5 mM EDTA containing 10 μg of RNase A per ml and 30 U of RNase T1 per ml; digestion proceeded for 60 min at 37°C. The RNase reaction was stopped by adding SDS to 0.2% and 400 μg of proteinase K per ml, followed by digestion for 15 min at 37°C. Samples were then extracted with phenol-chloroform and precipitated with ethyl alcohol prior to resuspension in formamide loading buffer and resolution on a 5% denaturing polyacrylamide gel. Dried gels were visualized by autoradiography and quantitated by phosphorimaging.

In situ hybridization. DNA in situ hybridization analysis was performed on 5-μm-thick sections of paraffin-embedded raft tissue cross sections that had been fixed in 4% paraformaldehyde. Hybridization analysis was carried out with the Pathogene HPV in situ screening assay (Enzo Diagnostics) in accordance with the manufacturer's instructions. Quantitation of cells that have amplified DNA was done as follows: positive cells in 10 randomly chosen fields (magnification, ×200) were counted and are expressed as an average number per microscopic field.

Immunohistochemistry. Paraffin-embedded raft tissue cross sections (5-μm-thick) that had been fixed in 4% paraformaldehyde were deparaffinized with xylenes and ethanol gradients. Tissue sections were digested with 100 μg of pronase (Boehringer Mannheim) per ml in PBS for 5 min at 37°C. Sections were then blocked for 1 h at room temperature in 1× PBS containing 1% Triton X-100 and 1% bovine serum albumin. The HPV31 E1^E4 protein was detected by incubating the sections with an affinity-purified rabbit polyclonal antibody (46), diluted to 1:100 in blocking solution for 1 h at room temperature. A fluorescein isothiocyanate-linked donkey anti-rabbit polyclonal antibody (Amersham) was used for secondary antibody detection. Results were visualized with an Olympus BH-2 microscope with a fluorescein microscopy filter set.

RESULTS

The transient replication and transactivation functions of HPV31 E2 can be separated by single amino acid mutations. To study the role of transactivation and replication functions of the oncogenic HPV31 E2 protein, we generated mutations at conserved amino acids 20, 37, 39, and 73 in the E2 gene product of HPV31 (mutants EN20, RK37, EQ39, and IL73) (Fig. 1). Previous reports demonstrated that the transient transactivation and replication functions of BPV1 and HPV16 E2 can be separated by single amino acid exchanges in conserved residues 37 and 73 in the amino-terminal domain of E2 (1, 10, 19, 25, 50) (Fig. 1). The mutant HPV31 E2 genes were first cloned into SV40 expression vectors and analyzed in transient assays for DNA-binding activity, stimulation of transcription, and enhancement of E1-dependent origin replication. Since specific DNA binding by E2 is essential for many of its properties, expression vectors for HPV31 wild-type (wt) E2 and mutants EN20, RK37, EQ39, and IL73 were first transfected into SCC-13 cells and cell lysates were analyzed for binding activity. Equal amounts of lysates were subjected to a gel retardation analysis with a ^{32}P-labeled oligonucleotide that contains HPV31 E2 binding site 4 (Fig. 2A). Similar specific retarded complexes were detected either in cells transfected with wt E2 or the mutant genes. All mutant E2 proteins were observed to bind the E2BS probe to similar levels as wild-type E2, which indicated that there were no major differences in protein levels.

We next investigated whether the HPV31 E2 mutants were altered in their ability to transiently activate transcription from an E2-dependent reporter plasmid, as has been reported for BPV1 E2 and HPV16 E2 (Fig. 2B). For these studies, we used a luciferase reporter plasmid that contains six E2-binding sites upstream of a minimal SV40 early promoter. In SCC-13 cells, cotransfection of the HPV31 wt E2 expression vector stimulated basal luciferase expression from the reporter plasmid at levels which ranged from 40- to 80-fold depending upon the experiment. In each experiment, expression vectors containing mutants EN20 and EQ39 stimulated transcription to the same extent as wild-type E2, whereas mutants RK37 and IL73 failed to stimulate luciferase expression (Fig. 2B). Raising the amount of transfected HPV31 E2:IL73 expression vector 10-fold did not increase the level of stimulation of the reporter plasmid above background (data not shown). E2 mutants RK37 and IL73 also failed to transactivate E2-dependent expression in NHKs (data not shown).

To determine the influence of the mutant E2 proteins on the stimulation of the E1-dependent replication of the HPV31 origin, we performed transient replication assays with plasmid pGL31URR, which contains the viral origin in the context of the HPV31 URR (Fig. 2C). SCC-13 cells were transfected with an HPV31 E1 expression vector and expression vectors for wt E2 or the mutant derivatives together with pGL31URR. After 72 h, low-molecular-weight DNA was prepared from transfected cells, digested with the restriction enzymes *Hpa*I and *Dpn*I to distinguish replicated from nonreplicated DNA, and analyzed by Southern hybridization (44). No replicated DNA was detected in transfections with the origin plasmid by itself or with the HPV31 E1 expression vector alone. Cotransfection of both E1 and E2 expression vectors with the pGL31URR plasmid led to high levels of replicated origin plasmid, as shown in Fig. 2C. The E2 mutants EN20, RK37, and IL73 induced replication levels comparable to that of wt E2. In contrast, transfection of the E2:EQ39 expression vector showed a reduction in the transient replication of the origin plasmid to 30% of the wt E2 levels. Taken together, these data indicate that all mutant proteins were stably expressed and functioned in at least one assay as well as wt E2. Furthermore, we determined that it was possible to genetically separate the transactivation function from the replication function of HPV31 E2, as has been described for the E2 genes of BPV1 and HPV16 (1, 10, 19, 25, 50).

HPV31 genomes containing the transactivation-negative E2 IL73 mutant gene can be stably maintained as episomes in NHKs. The activities of E2 which are recorded in transient-transfection assays may not accurately reflect the function of E2 in the viral life cycle. To investigate the role of the replication and transactivation functions of E2 in the viral life cycle, HPV31 genomes that contain the above-described mutations in the E2 gene were constructed. All mutations are in the 5′ part of the E2 gene and do not overlap with any known open reading frame nor do they alter *cis*-acting sequences known to be involved in replication or transcription. The viral genomes containing wt or mutant E2 genes were released from the plasmid sequences by restriction digestion, gel purified, and religated. The religated viral genomes were then transfected into early-passage NHKs together with the pSV2neo plasmid, and following drug selection, stable cell lines were isolated (22, 23).

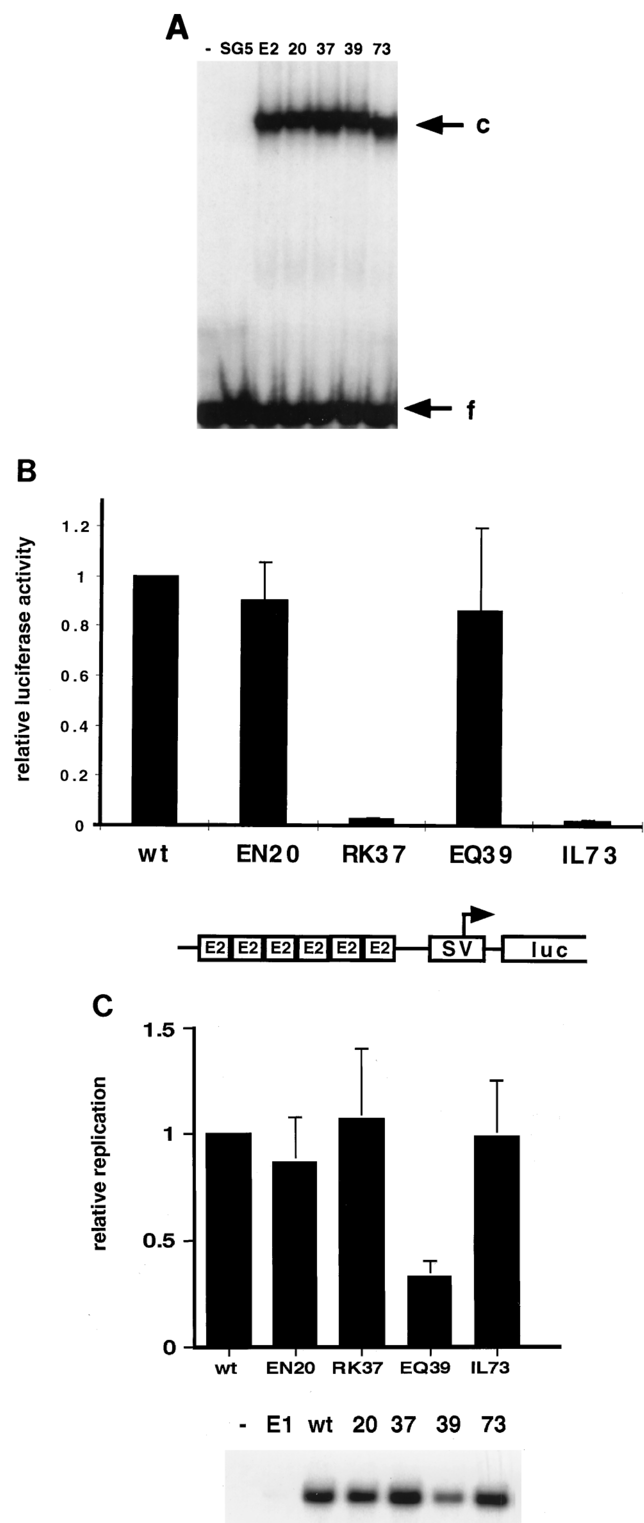

FIG. 2. (A) Gel retardation analysis of HPV31 wt E2 and E2 mutant proteins EN20, RK37, EQ39, and IL73 expressed in SCC-13 cells. Control lanes received no protein extract (lane −) or extract from cells transfected with the parental pSG5 vector (lane SG5). Lanes E2, 20, 37, 39, and 73 received identical amounts of extracts from cells transfected with the respective expression vectors. The retarded band corresponding to the E2-DNA complex is indicated with an arrow labeled c. The free ^{32}P-labeled oligonucleotide containing an E2 binding site is indicated by an arrow labeled f. (B) Transient luciferase expression assays. SCC-13 cells were transfected with expression vectors for HPV31 wt E2 or E2 mutant protein EN20, RK37, EQ39, or IL73 and the E2-responsive reporter plasmid p6XE2BS-luc and analyzed for luciferase activity. The reporter plasmid consists of six E2-binding sites (E2) upstream of the minimal SV40 early promoter (SV) that drives the expression of the luciferase gene (luc) and is diagrammed below the graph. The luciferase activity obtained by cotransfection of the E2 mutant expression plasmids is given relative to the activity of wt E2-transfected cells, which was set to 1. The standard deviations are indicated by error bars. (C) Transient replication assay. SCC-13 cells were transfected with plasmid pGL31URR alone (−) or together with an expression vector for HPV31 E1 or both vectors together with expression vectors for HPV31 wt E2 or E2 mutant proteins EN20, RK37, EQ39, and IL73. Transient replication of pGL31URR was analyzed by Southern hybridization. A representative autoradiograph is shown below the graph. Replication levels of pGL31URR were quantitated by phosphoimaging analysis and are represented relative to the replication levels induced by HPV31 wt E2, which was set to 1. The standard deviations are indicated by error bars.

HPV-transfected cell lines were viable for at least 12 passages in culture. This exceeded the life span of normal keratinocytes in tissue culture, which typically senesced at passage 3 to 5. The state of the viral DNAs was determined after three cell passages by Southern analysis of total cellular DNA (Fig. 3). Analysis of DNA from cell lines obtained after transfection of the HPV31 wt, E2:EN20, E2:RK37, and E2:IL73 genomes demonstrated three prominent species that are consistent with supercoiled, open-circle, and concatemer forms of viral DNA (Fig. 3, lanes N) (5, 22, 23). This indicated that the viral DNA in these cell lines is present primarily as episomes. In contrast, the HPV31 E2:EQ39 cell line contained only high-molecular-weight hybridizing DNA consistent with exclusively integrated forms of viral DNA. Analysis of DNA from the HPV31 E2:EN20 transfection identified DNA fragments with sizes that were different from that of the linear form of the viral genome (Fig. 3, EN20, lane S), indicating that integration of the viral DNA into the cellular chromosomes had occurred. The linearized, supercoiled, and open-circle forms of viral DNA of the HPV31 wt, E2:RK37, and E2:IL73 cell lines were analyzed by phosphorimager analysis to determine the viral copy number. This revealed that the copy number of the E2:IL73 cell line in this experiment was 85% of wt levels, whereas the copy number in the E2:RK37 cell line was reduced to 15% of wt levels. Cell lines established in three independent transfections of NHKs isolated from two different donors confirmed that E2:IL73 genomes are maintained as episomes at copy numbers similar to those of the HPV31 wt genome, whereas E2:RK37 genomes are maintained episomally at a significantly reduced copy number. To confirm that the mutations in E2 were retained in the cell lines, the 5′ part of the E2 gene was amplified by PCR from total cellular DNA. The amplified products were then sequenced, which demonstrated that the introduced mutations were present (data not shown).

Our studies indicate that the stable maintenance of HPV31 episomes in human keratinocytes requires the replication function of E2 defined by residue 39 but not the transactivation function defined by residues 37 and 73. The reduced copy number of the E2:RK37 cell lines compared to those of the HPV31 wt and E2:IL73 cell lines cannot be explained by differences in DNA-binding, transactivation, or replication activities of E2:RK37 as measured in transient assays (Fig. 2). We suspect that the severe reduction in copy number in E2:RK37 cell lines is due to an additional unidentified function of E2.

P97 transcript levels are similar in the HPV31 wt and the E2:IL73 cell lines. Since E2 has been implicated in both the positive and negative regulation of the major early promoter P97 (6, 9, 13, 49, 59, 65–67), we investigated whether the levels of P97-initiated transcripts differed between the HPV31 wt, the E2:IL73, and the E2:RK37 cell lines. For these studies, total RNA was isolated from the various cell lines grown in mono-

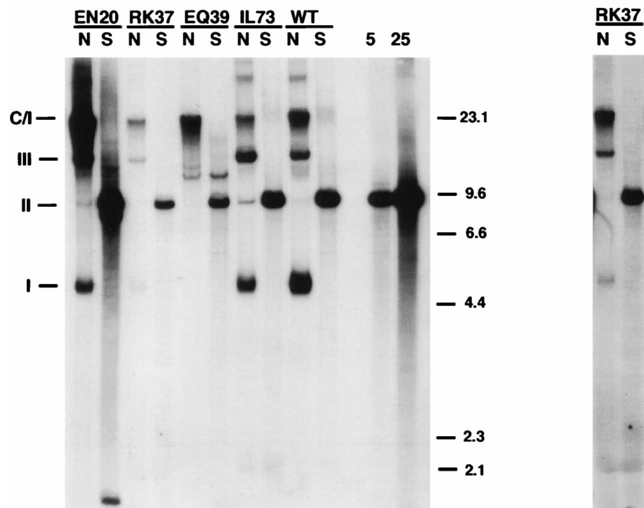

FIG. 3. Southern analysis of DNA from human keratinocyte cell lines obtained after stable transfection of HPV31 wt, E2:EN20, E2:RK37, E2:EQ39, and E2:IL73 genomes. Ten micrograms of total cellular DNA was digested with either restriction enzyme *Bam*HI (lanes N), which does not cut HPV31 DNA, or *Eco*RV (lanes S), which recognizes one site in the HPV31 genome, and then subjected to Southern analysis. As size markers, *Eco*RI-linearized HPV31 DNA equivalent to 5 and 25 viral copies per cell was used (lanes 5 and 25). Specific DNAs were detected with ^{32}P-labeled HPV31 genomic DNA. To the left of the autoradiograph, the positions of concatemeric or integrated (C/I), open-circle (III), linear (II), and supercoiled (I) forms of viral DNA are indicated. To the right, sizes of *Hin*dIII-digested phage lamba DNA are shown in kilobases. On the far right, the results of a longer exposure of the lanes containing DNA from E2:RK37 cells are presented.

layer culture and analyzed by an RNase protection assay with an antisense probe that spans nt 678 to 919. This probe allows the detection of P97 transcripts that are unspliced or spliced at a donor site at nt 877. In HPV31 wt and E2:IL73 mutant lines, similar levels of spliced and unspliced P97 transcripts were detected (Fig. 4). In Fig. 4, the transcript levels in the E2:IL73 cells appeared to be slightly higher than those in the HPV31 wt; however, these differences were not reproducibly detectable in other assays. In contrast, P97 levels in the E2:RK37 cell line were consistently found to be reduced, and this may be due to the lower viral copy number in these cells.

It has been suggested that E2 may transactivate the P97 promoter in transient assays at low levels of E2 expression vector but repress it at higher levels (9, 59). To directly measure the influence of HPV31 wt E2 as well as E2:RK37 and E2:IL73 mutants on P97 activity, transient reporter assays were performed. Increasing amounts of wt E2, E2:RK37, or E2:IL73 expression vectors were cotransfected together with a P97 luciferase reporter plasmid. Both E2 mutants behaved similarly to wt E2 and did not significantly stimulate P97 activity. In contrast, at higher concentrations of E2 expression vectors, all were found to repress P97 transcription to the same extent (data not shown). These results indicate that P97 is not a significant target for the transactivation function of HPV31 E2.

E2:IL73 cells induce differentiation-dependent viral functions. In monolayer cultures, E2:IL73 cell lines did not show significant differences from wt cell lines with respect to viral copy number or levels of early gene expression. It remained possible that the transactivation function of E2 played a role in the differentiation-dependent stages of the viral life cycle. Following differentiation of keratinocytes which maintain episomal copies of HPV, amplification of the viral DNA is induced together with activation of the viral late promoter P742 and expression of the E1^E4, L1, and L2 proteins (5, 12, 16–18, 22, 23, 32). To examine whether E2 transactivation was essential for any of these activities, HPV31 wt, E2:RK37, and E2:IL73 cell lines were grown in organotypic raft cultures. Following stratification, tissue sections from raft cultures were fixed and analyzed by in situ DNA hybridization for amplification of the viral genomes or by immunohistochemistry for the differentiation-dependent expression of the viral E1^E4 protein (Fig. 5). Tissue sections from cells grown in rafts were first analyzed by DNA in situ hybridization for amplification of HPV31 wt, E2:RK37, and E2:IL73 cell lines (Fig. 5A). In raft cultures of HPV31 wt and E2:IL73 cells, similar numbers of cells were observed to have amplified viral DNA. A quantitation of 10 random raft sections revealed that HPV31 wt raft cultures had on average 15.8 $\pm$ 2.9 (mean $\pm$ standard error of the mean) positive cells per field as compared to 11.8 $\pm$ 0.8 positive cells in E2:IL73 raft sections. Since it is not possible to accurately determine the levels of amplification in individual cells, the possibility that amplification may be modulated by E2 transactivation remains. Our major finding, however, is that amplification still occurs in E2:IL73 cells. Only very low levels of amplification were detected in raft cultures of E2:RK37 cells. It is possible that the low basal levels of viral DNA in the E2:RK37 cells prevented us from detecting amplification, which was still occurring but at a reduced rate.

Staining of raft tissue sections with a polyclonal serum specific for the HPV31 E1^E4 protein showed similar patterns of distribution in both the HPV31 wt and E2:IL73 cell lines (Fig. 5B). No E1^E4 immunoreactivity was detected in E2:RK37

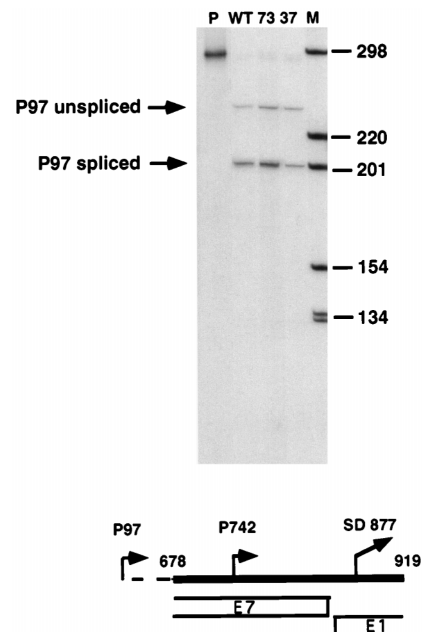

FIG. 4. RNase protection analysis of RNA from HPV31 wt (lane WT), E2:RK37 (lane 37), and E2:IL73 (lane 73) cell lines grown in monolayer culture. Total cellular RNA (10 μg) was hybridized to a ^{32}P-labeled antisense probe transcribed from plasmid pRP742 and subjected to RNase protection analysis. The positions of P97 transcripts, which are unspliced or spliced at a donor site at nt 877 (SD 877), are indicated by arrows to the left of the autoradiograph. Lane P contains undigested probe. A ^{32}P-end-labeled 1-kb ladder was used as a size marker (lane M), and the sizes are indicated in nucleotides to the right. The structure of the antisense probe is depicted below the autoradiograph. The start sites for the P97 and P742 promoters as well as the splice donor site at nt 877 (SD 877) are indicated by arrows. The dotted line indicates that the start site for P97 is not included in the probe. Parts of the E7 and E1 genes that are covered by the probe are shown below the structure diagram.

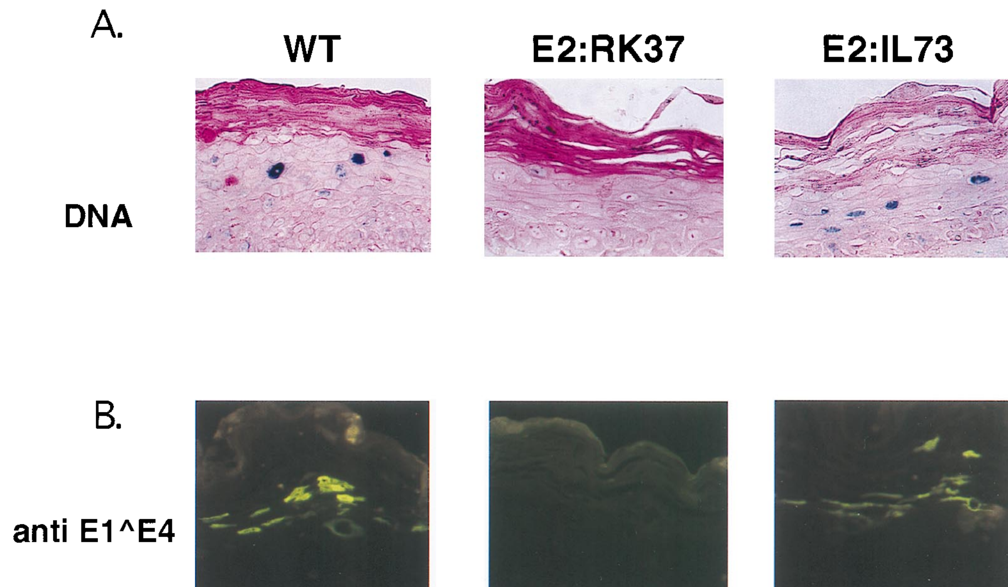

FIG. 5. (A) DNA in situ hybridization analysis of cross sections of HPV31 wt, E2:RK37, and E2:IL73, raft cultures. HPV31 DNA was detected with an HPV31/33/35-specific probe, and positive cells display a blue stain. (B) Immunohistochemical analysis for the differentiation-dependent expression of the HPV31 E1^E4 protein in raft cultures. Tissue cross sections (5-μm thick) of HPV31 wt, E2:RK37, and E2:IL73 cell lines grown in raft culture were incubated with a polyclonal antibody generated against the HPV31 E1^E4 protein. Specifically bound secondary antibodies (fluorescein isothiocyanate-conjugated) were detected by immunoflourescence.

cells. These data suggest that the loss of the E2 transactivation does not significantly interfere with the differentiation-dependent amplification of the viral genome or expression of the E1^E4 protein. Neither of these assays is highly quantitative, but based on the signal intensities, it appears that viral DNA amplification and the expression levels of the E1^E4 protein in E2:IL73 cells may be modestly reduced from that seen in HPV31 wt cells.

We next investigated whether there were differences in the induction levels of the differentiation-dependent late viral P742 promoter, whose activity correlates with the onset of E1^E4 expression. The RNase protection assay is a quantitative assay which allows for a direct comparison of induction levels in the various cell lines. Total RNA was isolated from HPV31 wt, E2:RK37, and E2:IL73 cell lines differentiated in the organotypic raft system and analyzed by RNase protection assays with the probe described above that allows simultaneous detection of both P97- and P742-initiated transcripts. Transcripts initiated at P97 that were either unspliced or spliced at the donor at nt 877 were detected at similar levels in the HPV31 wt and E2:IL73 cell lines (Fig. 6). Consistent with the previous analysis shown in Fig. 4, the P97 transcript levels were found to be reduced in the E2:RK37 lines. Upon differentiation, the late HPV31 promoter at nt 742 is induced (32). The P742 promoter lacks a TATA box and as a result directs heterogeneous start sites. RNA from differentiated organotypic raft cultures of the HPV31 wt and E2:IL73 cell lines contained transcripts that correspond to initiation at the P742 promoter. In the experiment shown in Fig. 6, the levels of P742 transcripts in the E2:IL73 cell line were reduced to 70% of the levels found in the wt line. In other assays, the level of P742 induction was found to vary from 40 to 73% of that of the wt. In contrast, no P742 transcripts could be detected in E2:RK37 cells grown in raft cultures. We conclude that the E2 transactivation function that is dependent upon residue 73 is not required for induction of the differentiation-dependent P742 promoter. However, we have consistently observed that the induction levels of P742 were lower in the E2:IL73 cell lines than in the HPV31 wt cell lines, suggesting that E2 transactivation may act to augment the levels of P742 expression.

It was important to determine whether transcription of the viral structural genes L1 and L2 was affected by the E2 IL73 mutation. RNA from cell lines allowed to differentiate in the raft system was analyzed by an RNase protection assay with an antisense RNA probe that spans the splice acceptor site at nt 5552 at the beginning of the L1 gene and allows detection of L1- as well as L2/L1-specific transcripts (Fig. 7). No late capsid gene transcripts were found in monolayer cultures of HPV31 wt, E2:IL73, or E2:RK37 cells nor in differentiated E2:RK37 cells. Differentiation of both the HPV31 wt and the E2:IL73 cells gave rise to spliced and unspliced late gene transcripts. However, the amount of L1 and L2/L1 transcripts were consistently reduced to 20 to 30% of the wt levels in the E2:IL73 cell line. We conclude that the transactivation ability of E2 is not essential for induction of early or late viral functions; however, it is possible it may act to augment the levels of these activities.

DISCUSSION

Our studies demonstrate that the transactivation ability of the E2 proteins from the oncogenic HPVs is not essential for the stable maintenance of episomes, the expression of early or late genes, or for differentiation-dependent genome amplification. While the stable copy number of viral episomes and the levels of late transcripts may be slightly reduced in the E2:IL73 mutant cell lines, the ability to induce all the late functions is maintained. This suggests that E2 transactivation is not required for the induction of early or late viral functions, although it may act to modulate the levels of late functions. While our studies failed to demonstrate an essential role for E2 in activating the early P97 or late P742 promoters, we cannot exclude the possibility that other as-yet-unidentified minor viral promoters are activated by E2. However, our stud-

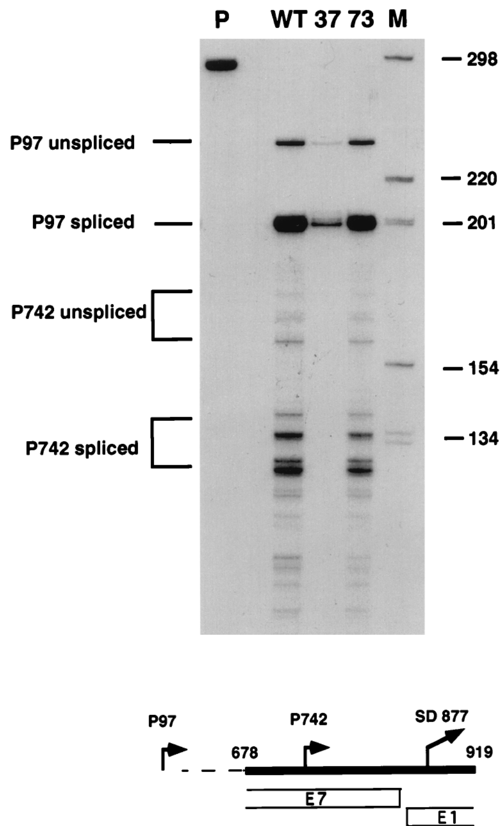

FIG. 6. RNase protection analysis of RNA from HPV31 wt (lane WT), E2:RK37 (lane 37), and E2:IL73 (lane 73) cell lines grown in raft cultures. Total cellular RNA (10 μg) was hybridized to a ^{32}P-labeled antisense probe as described in the legend to Fig. 4. The positions of P97 and P742 transcripts, which are unspliced or spliced at a donor site at nt 877 (SD 877), are indicated to the left of the autoradiograph. Lane P contains undigested probe. A ^{32}P-end-labeled 1-kb ladder was used as a size marker (lane M), and the sizes are indicated in nucleotides on the right. See the legend to Fig. 4 for a description of the diagram at the bottom of the figure.

ies demonstrate that activation of these promoters by E2 is not essential for induction of early or late viral functions.

The observation that E2 transactivation is not essential for the activation of early functions in the oncogenic papillomavirus types is in contrast to studies of BPV1. Several significant differences exist between the human genital papillomaviruses, which target squamous epithelia, and BPV1, which induces fibropapillomas. In BPV1, numerous promoters have been shown to be E2 responsive and mutation of the E2 gene leads to a loss of transforming ability (10, 14, 15, 26, 28, 47, 52, 57, 64). In the human viruses, mutation of E2 does not diminish the immortalization capacity of human keratinocytes by HPV genomes (48). In the genital HPV types, early viral transcription is activated by enhancer elements located in the URR which bind only cellular factors (7, 37). These differences indicate that the two types of papillomaviruses utilize distinct mechanisms for regulating viral gene expression. In agreement with this idea, BPV1 mutant genomes expressing E2 proteins which were replication competent but transactivation defective did not transform mouse C127 cells, whereas in our study, the corresponding HPV31 genome (E2 IL73) was readily able to immortalize keratinocytes (10).

Two major viral promoters have been identified in the high-risk HPV types: the major early promoter upstream of nt 97 and the late promoter centered around nt 742 in HPV31 (4, 24, 32, 53, 56). In transient assays, expression of the early promoters of HPV16 and HPV18 has been shown to be activated by low concentrations of cotransfected E2 expression vectors and repressed at high concentrations (9, 59). In our studies, we failed to detect any activation of the HPV31 early promoter and have only observed the repression of P97 by E2, which we previously hypothesized to be part of a mechanism to regulate stable copy number in infected basal cells (62). Our studies do not exclude the possibility that E2 can augment expression of the late P742 promoter since cells immortalized by E2:IL73 mutant genomes were reduced in this activity. However, it is clear that E2 transactivation is not essential for activation of late expression. Similarly, it is possible that E2 transactivation modulates the level of genome amplification, which appears to be slightly reduced in the E2:IL73 mutant. Again, since genome amplification still occurs, we conclude that E2 transactivation is not essential for this process.

It has been suggested that the episomal state of the viral DNA is essential for high-level activation of the late promoter (23). One explanation for this dependency is that genome amplification acts to increase genome copy number, resulting in increased late expression due to higher numbers of templates. If the loss of E2 transactivation diminishes even slightly the copy number of episomes in basal cells, this could result in reduced levels of late gene expression. Analysis of a BPV1 genome with a temperature-sensitive E2 mutant, which has properties comparable to the HPV31 E2:IL73 mutant, suggested that the E2 transactivation function is not required for the amplification of viral genomes in postmitotic cells, which is

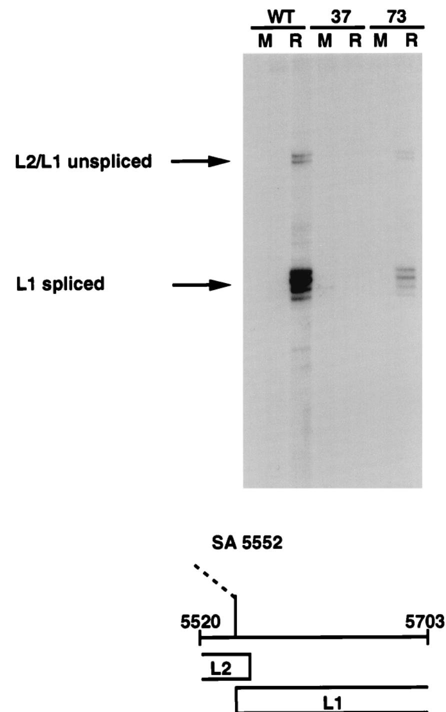

FIG. 7. RNase protection analysis of RNA from HPV31 wt (lanes WT), E2:RK37 (lanes 37), and E2:IL73 (lanes 73) cell lines grown in monolayer (M) or raft (R) cultures. Total cellular RNA (20 μg) was hybridized to a ^{32}P-labeled antisense probe transcribed from plasmid pRPA31L1. The positions of late gene transcripts, which are unspliced or spliced at an acceptor site at nt 5552 (SA 5552), are indicated to the left of the autoradiograph. The structure of the antisense probe is shown below the autoradiograph. The parts of the L2 and L1 genes that are covered by the probe and the splice acceptor site (SA 5552) are indicated.

thought to resemble the amplification process in differentiating epithelium (2). The levels of amplification in the E2 temperature-sensitive cell lines appeared slightly reduced compared to BPV1 wt cell lines, consistent with the idea that E2 transactivation is not essential for the amplification process but may modulate the extent (2). Finally, our studies do not exclude the possibility that amino acid 73 of E2 plays a role in DNA packaging or capsid protein assembly (12a).

We also examined a second E2 mutant at amino acid 37 which was defective for transactivation and found that it behaved similarly to the E2 IL73 mutant in transient assays. In contrast to the E2:IL73 mutant, E2:RK37 mutant genomes exhibited a severely reduced copy number in monolayer cultures and nondetectable levels of late functions in organotypic rafts, which could be a consequence of the low copy number. For example, if a negative factor regulates late gene expression, the extent of amplification of the E2:RK37 genome could be unable to titrate out this protein. We suspect that the E2:RK37 mutant is defective for additional functions which are essential for genome copy number control and induction of late expression. Support for this idea has been provided by Sakai and coworkers, who demonstrated that mutation of residue 37 from arginine to alanine in HPV16 E2 decreases the transient replication function of E2 (50). The HPV16 E2 RA37 mutant protein retained the ability to complex with the viral E1 protein, suggesting that residue 37 in HPV E2 may be part of an additional replication activity of E2. Furthermore, BPV1 genomes containing the E2 K37 mutation were not able to induce focus formation on transfected C127 cells despite the ability of the mutant E2 protein to transactivate, transiently replicate viral DNA, and form complexes with the E1 protein (10). Both studies suggest that amino acid 37 of E2 is critical to additional activities besides transactivation.

The question remains as to whether transactivation of the oncogenic HPV E2 protein plays any physiologically important role in the viral life cycle or whether it is an artifact of transient-transfection assays. Our studies suggest that if E2 transactivation has any role in the viral life cycle, it is a modest one which augments the levels of late functions. In infections in vivo, it is possible that the transactivation function becomes more important. For instance, our studies have not addressed the possibility that E2 activates a cellular gene whose activity is not recorded in our tissue culture system. Alternatively, the primary role of E2 in the human viruses could be as a replication factor acting in conjunction with E1 and as a negative regulator of early gene expression.

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