

185 ¹¹³²
~~1132~~

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E _____ S _____ D _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad 03-003730- 00008-0000
Fee 2007 03-28 16:04 14 Dep 2020 NUECREACIONES
Tra 11 PATENTE/II
Eve 379 FASENACIONALII
Act 444 RESPUESTAREQUE Folios 19

Ref Expediente No 03 003 730

Solicitud de patente de invención titulada "SECUENCIAS DE VIRUS DE
PAPILOMA CON OPTIMIZACION DE CODONES "

Solicitante GLAXO GROUP LIMITED

RESPUESTA AL OFICIO No 14892 NOTIFICADO POR FIJACIÓN EN LISTA

No 469, DE FECHA 29 DE DICIEMBRE DE 2006.

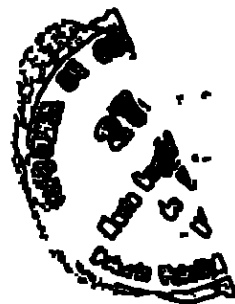
(REQUERIMIENTO ARTÍCULO 46)

J IAN RAISBECK, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de en mi carácter de apoderado sustituto de la sociedad solicitante, como consta en la sustitución de poder adjunta, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal previsto en el artículo 46 de la Decisión 486

1 Presento copia completa de los siguientes documentos exigidos por el Examinador en el examen de búsqueda de anterioridades, según lo previsto en el Artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina

i)

ZHOU JIAN ET AL 'Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability' JOURNAL OF VIROLOGY, vol 73, no 6, June 1999 (1999-06), pages 4972-4982, XP002164427 ISSN 0022-538X





11)

HAN R ET AL. "Protection of rabbits from viral challenge by gene gun-based intracutaneous vaccination with a combination of cottontail rabbit papillomavirus E1, E2, E6 and E7 genes.", J VIROL , , August 1999, vol 73, no. 8, pages 7039 to 7043

Atentamente,

J IAN RAISBECK

C C 79'376 996 de Bogotá D C

T P 135 799 del C S de la J

Anexo

- Artículos mencionados
- Sustitución de poder

NOTARIA EL ANTERIOR ESCRITO DIRIGIDO A

FUE PRESENTADO PERSONALMENTE
ANTL AL SUSCRITO NOTARIO VEINTISIETE
DE LA CIUDAD DE BOGOTA POR

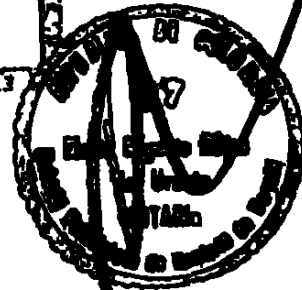
Con CL 75.276156 07

LI. DÉCLARATION

26 MAR 2007

HUELLA

MARIA EUGENIA ROJAS DE URUETA
NOTARIA VEINTISIETE
REPUBLICA DE COLOMBIA



Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E S D

Ref Expediente No 03 003 730

Solicitud de patente de invención titulada "SECUENCIAS DE VIRUS DE
PAPILOMA CON OPTIMIZACION DE CODONES "

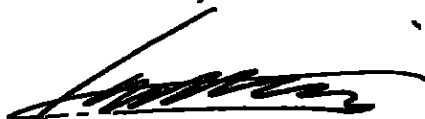
Solicitante GLAXO GROUP LIMITED

SUSTITUCIÓN DE PODER

CARLOS R OLARTE, mayor de edad y vecino de Bogotá D C , identificado como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad de la referencia, respetuosamente me permito manifestar que SUSTITUYO el poder a mi conferido por la mencionada sociedad, en el doctor J IAN RAISBECK LLINAS, mayor de edad, domiciliado en Bogotá D C , identificado con la Cédula de Ciudadanía No 79 376 996 de Bogotá y portador de la Tarjeta Profesional No 135 799 del Consejo Superior de la Judicatura, para que sea él quien en adelante asuma el trámite de la presente acción

El apoderado sustituto conserva las mismas facultades del principal

Atentamente,



CARLOS R OLARTE
C C No 79 782 747 de Bogotá
T P 74 295 del C S de la J

DILIGENCIA PRACTICADA POR EL
RECONOCIMIENTO DE CONTINUIDAD

NOTA: EL INTERIOR ES DE DISEÑO J. A. B.
Industria y Comercio

27

Carlos E. Oarte

56245 P 1060d T P 74295

CONSENTIMENTO DELL'AUTORE

החלטתו של בית דין זה

ALLA PER IL

~~26 OCT 2014~~

MAPIA LUCENA POJAS DE URUETA
VOLUNTARIA VEINTISIETE
REPUBLICA DEL COLOMBIA

Papillomavirus Capsid Protein Expression Level Depends on the Match between Codon Usage and tRNA Availability

JIAN ZHOU,[†] WEN JUN LIU, SHI WEN PENG, XIAO YI SUN, AND IAN FRAZER*

Centre for Immunology & Cancer Research, University of Queensland,
Princess Alexandra Hospital, Brisbane, Queensland 4102, Australia

Received 25 November 1998/Accepted 11 February 1999

Translation of mRNA encoding the L1 and L2 capsid proteins of papillomavirus (PV) is restricted in vivo to differentiated epithelial cells, although transcription of the L1 and L2 late genes occurs more widely. The codon composition of PV late genes is quite different from that of most mammalian genes. To test the possibility that PV late gene codon composition determines the efficiency of PV late gene expression in some cell types, synthetic bovine papillomavirus type 1 (BPV1) late genes were constructed with codon composition modified to resemble the typical mammalian gene. Expression of these genes from a strong promoter in Cos-1 cells was compared with expression of wild-type BPV1 late genes from the same promoter. Both unmodified and modified PV late genes were transcribed in Cos-1 cells, but only the codon-modified genes were translated. In vitro translation of wild-type but not synthetic BPV1 L1 mRNA was markedly enhanced by addition of aminoacyl-tRNAs. Codon composition thus limits BPV1 late gene translation in Cos-1 cells, and this limitation can be overcome by modification of the codon composition of the genes or by provision of excess tRNA. Replacement of codons in the green fluorescent protein (*gfp*) gene with those frequently used in PV late genes did not alter *gfp* transcription in Cos-1 cells but almost abolished translation, supporting the hypothesis that the observed differences in efficiency of translation of modified and unmodified PV capsid genes were related to codon usage rather than mRNA structure. As tRNA populations vary within and between tissues in the same eukaryotic organism, we speculate that matching of tRNA availability to codon usage may be one determinant of the restriction of expression of PV late genes to differentiated epithelia.

Papillomaviruses (PV) infect stratified squamous epithelia, and PV gene expression is linked to the state of differentiation of the epithelial cells. Early genes associated with plasmid replication and with regulation of host cell division and differentiation are expressed at low levels in the replicating basal cells of squamous epithelia, while the genes encoding immunogenic capsid proteins (L1 and L2) are transcribed predominantly in nonreplicating differentiated keratinocytes, and their protein product can be detected only in the most superficial layers of the epithelium (31). Transient translation of these genes follows infection of eukaryotic cells with viral vectors including vaccinia virus (10, 39) and baculovirus (18, 34) and stable transcription and translation of PV late genes from strong constitutive promoters can occur in yeast (14, 24). However, stable expression of authentic sequence late genes from plasmid eukaryotic expression vectors, as seen in yeast, has not been reported for replicating eukaryotic cells in vitro, although expression in nonreplicating differentiated cells in raft culture can be observed (22). Further, L1 translation is lost in vivo as cells dedifferentiate (31–37). Lack of PV late gene expression in undifferentiated cells has been variously attributed to putative differentiation-specific transcriptional enhancers for late promoters (2, 9), to alternate message splicing in differentiated tissues (3), to message-processing sequences (16) or AU-rich instability-promoting sequences (7, 30) in the 3' untranslated sequence of L1 mRNA, to RNA sequences within the late genes inhibitory to transcription (8, 25), or to transcriptional

repressor proteins (33). However, these proposed mechanisms of regulation of PV late gene transcription or translation could not explain the absence of L1 protein observed in some tissues with abundant L1 mRNA (23).

The genetic code shows redundancy: several nucleotide triplets can encode the same amino acid, and several corresponding isoaccepting tRNAs are observed. Differences in the abundance of isoaccepting tRNAs are observed between organisms, and also between the tissues of a given organism, and are postulated as one explanation for why the same gene is more or less efficiently translated in different organisms (27). Virus genomes frequently have a G+C content significantly different from that of their host species, reflecting a different codon usage pattern of unknown significance (32). Analysis of codon usage in the PV genome shows that bovine papillomavirus type 1 (BPV1) late genes use 12 codons more than twice as frequently than the average for mammalian genes (Table 1). Similar observations apply to the late genes of other PV genotypes. We therefore hypothesize that as tRNA availability in a particular species is assumed to reflect codon usage in genes of that species (13, 27), PV late mRNAs may not be efficiently translated in undifferentiated cells in vitro or in vivo due to a mismatch of codon usage and tRNA availability. If this hypothesis were true, two alternate mechanisms could be proposed for the resolution in differentiated cells of the codon usage-tRNA mismatch in undifferentiated cells. tRNA availability might change with keratinocyte differentiation, or a reduction in the range and number of mRNAs competing for the rare tRNAs might allow more effective translation. This latter hypothesis could also explain why PV late gene expression can be induced in cell cultures infected with recombinant virus, such as vaccinia virus or baculovirus (10, 34) in which the virus infection switches off host mRNA synthesis. If our hypotheses are correct, conservative replacement of these codons in PV

* Corresponding author. Mailing address: Centre for Immunology & Cancer Research, University of Queensland, Princess Alexandra Hospital, Brisbane QLD 4102, Australia. Phone: 61-7 3240 5315. Fax: 61 7 3240 2048. E-mail: ifrazer@medicine.pa.uq.edu.au.

[†] Jian Zhou passed away in Hangzhou, China, on 10 March 1999 after a brief illness. He is greatly missed.

TABLE 1 Frequency of codon usage for individual organisms*

Amino acid	Codon	Frequency (10 ⁻³) of codon usage				
		Human	Cow	Yeast	Wheat	BPV L1 and L2
Arg	CGA	5.4	5.5	2.3	2.3	7.2
	CGC	11.3	12.2	2.0	7.5	4.1
	CGG	10.4	11.2	1.1	4.6	5.1
	CGU	4.7	3.7	7.5	1.1	10.4
	AGA	9.9	9.9	24.0	4.1	14.4
Leu	AGO	11.1	11.4	7.5	7.1	9.5
	CUA	6.2	4.9	11.8	12.1	18.6
	CUC	19.9	21.2	4.1	18.6	6.2
	CUG	42.5	46.6	8.3	15.5	15.5
	CUU	10.7	10.6	9.6	6.5	20.7
Ser	UUA	5.3	4.0	24.5	1.8	14.5
	UUG	11.0	9.6	32.1	15.3	15.5
	UCA	9.3	7.6	15.6	14.6	16.6
	UCC	17.7	17.6	14.4	10.1	11.4
	UCG	4.2	4.5	6.5	9.6	6.2
Thr	UCU	13.2	11.2	24.6	14.8	15.5
	AGC	18.7	18.7	7.1	12.8	12.4
	AGU	9.4	8.6	11.7	12.9	21.7
	ACA	14.4	11.4	15.6	4.6	37.3
	ACC	23.0	21.1	13.9	15.9	19.7
Pro	ACG	6.7	7.8	6.7	4.5	4.1
	ACU	12.7	9.6	22.0	11.8	28.0
	CCA	14.6	12.0	21.4	71.2	22.8
	CCC	20.0	19.2	5.9	11.1	15.5
	CCG	6.5	7.9	4.1	19.4	0.0
Ala	CCU	15.3	14.6	12.8	10.3	33.1
	GCA	14.0	13.1	15.3	11.2	33.1
	GCC	29.1	35.8	15.5	19.5	17.6
	GCG	7.2	9.3	5.1	13.8	4.1
	GCU	19.6	19.1	28.3	9.6	13.5
Gly	GGA	17.1	16.2	8.9	25.9	22.8
	GGC	25.4	28.1	8.9	28.0	12.4
	GGG	17.3	19.2	5.1	28.5	22.8
	GGU	11.2	11.8	34.9	9.6	18.6
	GUA	5.9	5.1	10.0	4.4	15.5
Val	GUC	16.3	18.4	14.9	14.8	6.2
	GUG	30.9	32.9	9.5	12.9	23.8
	GUU	10.4	9.9	26.6	11.6	18.6
	AAA	22.2	21.6	37.7	4.5	37.2
	AAG	34.9	37.1	35.2	17.4	13.5
Asn	AAC	22.6	22.4	25.8	14.2	10.3
	AAU	16.6	12.5	31.4	6.7	24.8
	CAA	11.1	9.7	29.8	171.8	22.8
	CAO	33.6	34.4	10.4	79.4	17.6
	CAC	14.2	14.0	8.2	8.2	6.2
His	CAU	9.3	7.5	12.3	7.1	13.4
	QAA	26.8	24.4	48.9	7.8	36.2
	QAG	41.4	45.4	16.9	19.7	21.7
	GAC	29.0	31.5	22.3	13.0	18.6
	GAU	21.7	19.2	37.0	4.0	33.1
Tyr	UAC	18.8	20.3	16.5	24.5	17.6
	UAU	12.5	10.5	16.5	12.5	18.6
	UGC	14.5	13.9	3.7	14.8	5.2
	UGU	9.9	9.4	7.6	4.9	5.2
	UUC	22.6	25.5	20.0	14.1	7.2
Phe	UUU	15.8	17.0	23.2	15.0	23.8
	AUA	5.8	5.2	12.8	5.4	22.7
	AUC	24.3	25.8	18.4	19.7	8.2
	AUU	14.9	13.1	31.1	10.7	20.7

* The codon usage data for human, cow, yeast, and wheat proteomes are derived from published results (20). The BPV1 data are from the sequences in the GenBank database. Usage of triplets in boldface is more than twice as frequently observed in PV L1 and L2 genes as mammalian consensus codons.

late genes less frequently used in mammalian genes should result in a gene encoding mRNA which can be efficiently expressed in dividing cells in culture. We therefore produced synthetic versions of BPV1 L1 and L2 genes, substituting codons preferentially used by mammalian genes for the codons in the original BPV1 L1 and L2 sequences which are less commonly used in mammalian genes (creating so-called humanized genes). We used these synthetic variants to examine the efficiency of transcription and translation of modified and unmodified BPV1 late genes expressed from strong constitutive and natural promoters in cultured cells in vitro. In further testing of the hypothesis, we examined whether replacing the major codons of *gfp* (encoding green fluorescent protein) with synonymous PV-preferred codons would reduce the level of translation of *gfp* mRNA in cultured cells.

MATERIALS AND METHODS

Codon-replacements in the BPV L1, L2, and *gfp* genes. The DNA and amino acid sequences of the L1 and L2 genes are shown in Fig. 1. To determine whether the presence in the L1 and L2 genes of codons less frequently used in mammalian genes (Table 1) could in part explain the reduced translation of the L1 and L2 genes in some mammalian cells, we synthesized codon-modified L1 and L2 genes by using synonymous substitutions as shown. To construct the synthetic sequences, we synthesized 11 pairs of oligonucleotides for L1 and 10 pairs of oligonucleotides for L2. Each pair of oligonucleotides has restriction sites incorporated to facilitate subsequent cloning (Fig. 1). The degenerate oligonucleotides were used to amplify L1 and L2 sequences by PCR using a plasmid with the BPV1 genome as the template. The amplified fragments were cut with appropriate enzymes and sequentially ligated to the pUC18 vector producing pUCHBL1 and pUCHBL2. The synthetic L1 and L2 sequences were sequenced and found to be error free; they were then subcloned into the mammalian expression vector pCDNA3 containing origin of replication (SV40) and (Invitrogen), giving expression plasmids pCDNA/HBL1 and pCDNA/HBL2. To compare expression of L1 and L2 with that of the original sequences, the wild-type (wt) L1 and L2 genes were cloned into the pCDNA3 vector resulting in pCDNA/BPV1wt and pCDNA/BPV12wt. To construct *gfp*, a modified *gfp* gene with PV-preferred codons, six pairs of oligonucleotides were synthesized. Each pair of oligonucleotides has restriction sites incorporated and was used to amplify *gfp* by using a humanized *gfp* gene (GIBCO) as the template. The PCR fragments were ligated into the pUC18 vector to produce pUCGFP. The *gfp* gene was sequenced and cloned into the *Bam*HI site of the same mammalian expression vector pCDNA3, under the cytomegalovirus (CMV) promoter.

To construct plasmids with the L2 gene of BPV1 in the context of the BPV1 sequences and with only BPV1 promoters, the BPV1 genome was cloned at nucleotides (nt) 4430 and 6858 with *Bam*HI/*Hind*III and cloned into pUC18. In the resultant plasmid, the original L1 (nt 4186 to 5595) and L2 (nt 5066 to 7885) open reading frames (ORFs) were removed. The synthetic humanized L2 gene, together with an SV40 poly(A) sequence to allow mRNA processing and the SV40 ori sequence to allow plasmid replication in eukaryotic cells, was cloned from pCDNA/HBL2 with *Hind*III/*Sma*I and inserted into the *Bam*HI-cleaved and *Klenow*-blunted BPV1 genome lacking L1 and L2 ORF sequences. This plasmid was designated pCICR2. A similar plasmid was constructed with wt rather than synthetic humanized L2 and designated pCICR1.

Immunofluorescence and Western blot staining. For immunofluorescence, Cos-1 cells in six-well plates were transfected with 2 µg of L1 or L2 expression plasmid by using Lipofectamine (Gibco); 35 h after transfection, cells were washed with phosphate-buffered 0.15 M NaCl, pH 7.4 (PBS), and lysed in sodium dodecyl sulfate (SDS) loading buffer. The cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel blotted onto a nitrocellulose membrane. The L1 and L2 proteins were identified by enhanced chemiluminescence (Amersham, Little Chalfont, England), using BPV1 L1-specific (DAKO) or L2-specific (19) antisera. For immunofluorescent staining, Cos-1 cells were grown on eight-chamber slides, transfected with plasmids, and fixed and permeabilized with 85% ethanol 36 h after transfection. The slides were blocked with 5% milk-PBS and probed with L1 or L2-specific antiserum, followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma). For *gfp* or *gfp* plasmid-transfected cells, the cells were fixed with 4% buffered formaldehyde and viewed by epifluorescence microscopy. Data presented are representative of at least three experiments for each transfection.

In vitro translation assay. One microgram of each plasmid was incubated with 20 µCi of [³⁵S]methionine (Amersham) and 40 µl of T7-DNA polymerase coupled rabbit reticulocyte or wheat germ lysates (Promega). Translation was performed at 30°C and stopped by adding SDS loading buffer. The L1 proteins were separated by SDS-PAGE on a 10% gel and visualized by autoradiography.

Northern blotting. Cytoplasmic or total RNA was extracted from Cos-1 cells, transfected 24 h previously with various plasmids, using a Qiagen RNeasy mini

A	1	ATG GCG TTT TGG CAA CAA GCG CAG AAG CTG TGT CTC CCT CCA ACC GCV	
		M A L W Q Q G G H L V L F P Y F	
	19	GTG AAG AAG GTG GTT TGC AGT GAA ACC GAT GTG CAA AAG AAA AGC AGT	
		V G K V L G S H T V V G K K S I	
	91	TTT TGT CAT GCA GAA AGG GAG GCG CTG CTA ACT ATA GAA CAT GCA TAT	
		F Y H A S T H R L S V L G H P Y	
	145	TAC CCA GTG TGT ATC GGG GCG AAA ACT GGT GGT AAG GTC TGT GCA AAT	(Rae1)
		Y P V H L G A K T V P K V S A W	
	193	CAG TGT AAG GGA TTT AAA ATA CAA CTA CCT GAT GCG AAT CAA TTT GCA	
		Q V R V P K K G L P D P H Q P A	
	241	CTA GCT GAC AAG ACT GTT CAC AAC CCA AAT AAA GAG GAG CTG GTG TGG	(Rae1)
		P V L G V S V S R S Q P L G S Y	
	289	GCA GTC ATA GGT GTG CAG GTG TGC AAA GAG CAG GCT GTT GAA GGT AGT	
		P V L G V Q V S R G Q P L G S Y	
	337	GGA ACT GAG CAC GCG ACT TTT AAT GGT TTT CTG GAT GCA GAA AAT GTG	
		V T G H P T P H A L L D A S H V	
	385	AAT AAG AAA GTC ACC AGC CAA ACA GAT GAC AAG AAA CAA ACA GAT	(Rae1)
		N H H V V T R V T D R K Q P G	
	433	GTA GAT GGT AAG CAA CAA CAG ATT CTG TGG CTA GCG TGT AGC CCT GCT	
		L D A H Q Q Q I H L L G S V P A	
	481	GAA GAG GAA TGT TGG ACA ACA GCG GAT CCA TGT GGT ACT GAT GGT GTC	(Rae1)
		H G R V V V A L P G V T D R L	
	529	GAA AAT GCG GCG TGC GCT GCT GTT GAA TTA AAA AAG AAG CAC ATA GAA	
		H H G A C P P L H L H H H H H H	
	577	GAT GAG GAT ATG ATG GAA AAT GAG TTT GAT GCA GCG AAC TTC AAA GAA	
		D G D H H H X G P G A A H F A R	
	625	ATT AAT GCA AAT AAA TCA GAT CTA GCT GTT GAC AAT CAA AAT GAG ATC	
		T N A H H H G D L P L D X G H H X	
	673	TGC TTT TAC CCA GAC TAC CTC AAA ATG GCT GAG GAG GAT GAT GAT AAT	(Rae1)
		G L V P D V L K H A H D A A G H	
	721	AGC AGG TTC TTT TTT GCA AAG AAA GAA CAG GTG TGT GGT AAG CAG AGC	
		S H P V V A H K H Q V V V H H F	
	769	TGG AGC GGA GAG GCG TGG GAG AAA GAA GCG CCT ACC ACA GAT TTT TAT	
		W P R G S S H K H A P T T D P V	
	817	TGA AAG AAT AAT AAA GAG GAT GCG ACC GTT AAA ATA GCG AAT GAG CAT	
		L R H H K G D A V L H S V S V H	
	865	TTT GAT AAT GCG AAT GCG TCA TTA GTC GCA ACT GAT AAT CAA AAT TTT	(Rae1)
		V G S P S G A L V S V D H Q L V	
	913	AAT GAG GCG TAC TGG CTA TGC GAT GCG CAG GCG ATG AAC AAT GAA ACT	
		H H P Y W L P H A H G H H H G I	
	961	GCA TGG AAT AAT TTA TTT TTA ACA GTG GAG GAG AAT ACA GAT GAT	(Rae1)
		A S H H L L P L V G D H V T A G	
	1009	ACT AAT GTT AGC ATA AAT GTC GCG TCA GAT GAA AGC CCA CTA ACA GAG	
		T H L P X S V A S D G V P L V H	
	1057	TAT GAT AGC TGA AAA TTC AAT GTC TAC CAT AAG CAT AAG GAA GAA TAT	
		Y D S G H P H V V H H H H S H Y	
	1105	AAG GTC GCG TTT ACA TTA GAG CTA TGC TCT GTG GAA AGC ACA GCT CAA	(Rae1)
		K L A P X L H L G H V H Y V A G	
	1153	ACT GTG TCA CAT CTG CAA GGA CTT ATG GCG TCT GTG CTT GAA AA TGG	
		Y V S H L G G L H P G V L H H H	
	1201	GAA ATA GAT GTG CAG GCT GCT ACC TCA TCG ATA TTA GAG GAG AGC TAT	(Rae1)
		H I G V G P P V S S L H D P Y	
	1249	GCG TAT ATA GAG TGT GCT GCA AAT AAA GAT GCA AGC AAT GTC AAT GCT	
		H Y H H S P A V R C A D H V X P	
	1297	GCA AAA GAA GAC CCT TAT GCA GAG TTT AAG TTT TGG AAG ATA GAT CTT	
		A H S D S Y A S V H F V H X D L	
	1345	AAA GAA AAG CTT TGT TTT GTC TTA GAT CAA TTT GCG TTA GAA AAG AAG	(Rae1)
		H H L L S L D A D G P P L G A R	
	1393	TTT TTA GCA CAG CAA GAG GGA GAA TAT TCA ACT GTG AAG AAA GAG AAG	
		P L A G H G A G S P V H H H H	
	1441	ACT AGC CAA AAA ACT TGC AAT AAG GCT GCA AAA AAA AAA AAA TAA	
		I S G H V S S K P A H K K K K -	

1	ATG AGT GCA CCA AAA AAA ATA AAA CCG GCG AGT GCG TAT GAC CTG TAC	
69	AGG ACC TGC AAG CAA GCG GCG ACA TGT CCA CCA GAT GCG ATA CCA AAG	
97	ATA GAA GAA GAT ACT ATA CCA GAT AAA ATT TGG AAA TTT GCG GGT GTT	
145	GCA AAG TAC TTA GCA GCG CTA GAA ATA GAA ACA TGG TGT ACT GAA AAG	(ApoE)
193	GTA GCG GCA GAT GCA TCA CCA AAG TAC ACA CCA CTC CCA AAG GCA GCG	
241	TCC ACA TCA TCG GTT GCA TCA ATA <u>GAA TGG</u> AAG GCT GAA ACA CCA GCG	(BamHI)
319	ACC GCG GCG AGT AAG GGT GCG GCG ATT GGT TTA GAC ACC GGT GAA ACT	
327	GTT GCG GCG TGG GGT CCA GCG GCG TAT GAG GAC ACT GCG GCA CCA GAG	
365	GCG GGT GCA AAG GCG ACT GGT GAT GGT GGT GGT GCA GAT TCA GCG GTT	(PstI)
433	GAT GCG CTG TCG AAT GAT ACA GAC TCG TCG AAG GAG ACC GCG AGT AGT	
461	CTG CCA GAG GGT GAT GGT GCG GAG GAC ATA GCG GGT GTT GAG GCG GAA	(BamHI)
529	GCG CTG GAC GGT GCA ACT TGG CAA GTG AAG AAT GGT GGT GAT CAG TCG	
577	TCT GCA TAC CAC GCG GGT GCG GCG CTG CAA TCG TCG ATT GCA GAA ACA	
625	TCT GCG TTA GAA AAT ATT TTT GCA GCA GCG TCG GGT TTA GCG GAT ACA	(ApoE)
673	GAA GAA GAA AAC AGT GAA CTG ACA TAC TTC GCG TCG GCG CCA AAG ACC	
721	AGG GCG GCG AGT AGT GCG TCT AAA TCA GCG GCG AGT TTA AAC TGG TTC	
769	AGT AAA GCG TAC TAC ACA CAG GCG GCG GAA GAT GGT GAA GCG TTT	(BamHI)
817	TCA TCG CAA ACA TTT GCA AAG CCA CTG TGT GAA GCA GAA CCA GGT GTG	
865	GTT AAG GAA GGT AGT GAA GGT GGT GCA CTC AGT CAG GGT TAT AAA GGT	
913	GAT ACA CTG ACA CCA GGT GCG GCG AAG GCG GCG GCA CAG GCA CAG	(BamHI)
961	GTC AAG TAC TCA TCG AAT ACT ACA CAG GAA GAT GTA GAA CCA AGC GCG	
1009	TAC ACA GTT GAT GAA AAT ACA CAG GAA CTT GCA TCG GCG GCG TCG GAT	
1057	GAA GAG CAA GCA GGT TTT GAG GAG AAG <u>GAA TGG</u> GAT GAT TTT AGT GCG	(BamHI)
1105	ACA CAG AAG CTG CCG GGT CAG AAG ACC TCT TCT ACA GGT GGT AGT	
1153	GAT GCA GCA AAG AAG CTC AGT CCA ACT GCA GAA TTT AGT GCA ACA GCG	
1201	GCT ACA GGT GTT GTA ACC TAT GCG TCA GGT GAC ACT TAC TCT AGT AAG	(BamHI)
1249	GCA GTT ACT GAC GGT GAT TCT GCG TCT CAG AGT GCA GGT ATC GAT GAC	
1297	ACT ACT ACT ACA CCA AAG AGT ATA AGT GAT GCG GAC AAG GAT GAT TGG	
1345	TAG AAG AGT AAG TAC ACC TGG CAG GCG TCG TGG TGG AAG AAA CCA AAA	
1393	AAA CCG AAA CAG GCG TAA	

FIG. 1. Nucleotide sequences of BPV1 L1 (A), L2 ORF (B) and *gfp* (C) and deduced amino acid sequences. Amino acids (in single-letter code) are presented below the second nucleotide of each codon. Mutations introduced into the genes are indicated above the corresponding nucleotides of the original sequence. The sites and enzymes used for cloning are indicated by horizontal lines. This replacement of nucleotides resulted in a polynucleotide sequence encoding BPV1 L1, L2, and GFP polypeptides with amino acid sequences identical to the original, but having preferred (for L1 and L2 genes) or PV (for the *gfp* gene) codons substituted for the synonymous codons of the wt nucleotide sequences.

8192

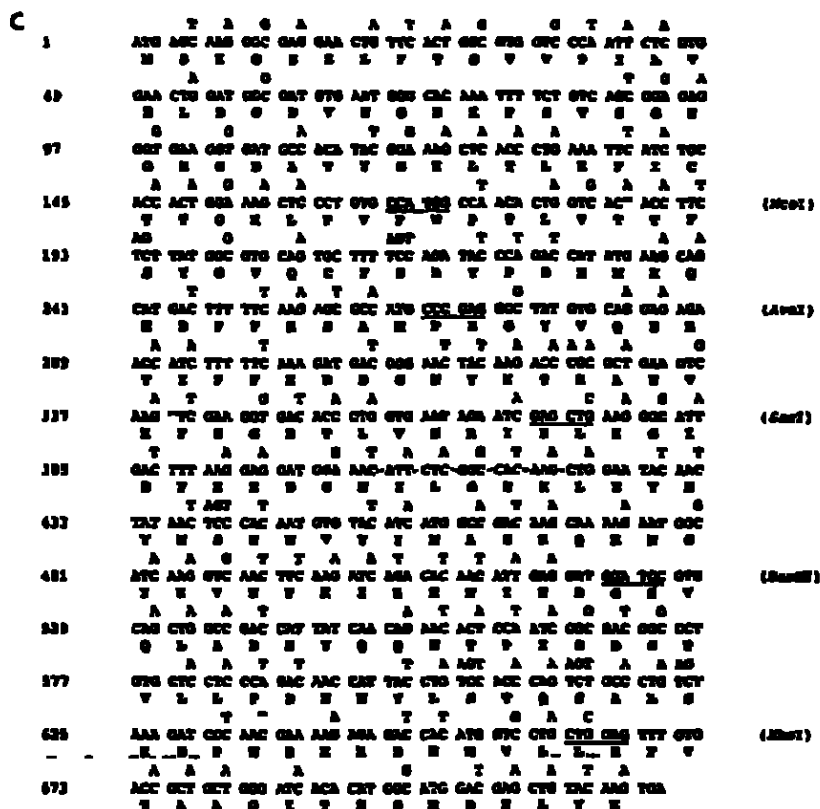


FIG. 1—Continued.

lot as instructed by the supplier. Briefly, for cytoplasmic RNA purification, buffer RLN (50 mM Tris (pH 8.0), 140 mM NaCl, 1.5 mM MgCl₂, and 0.5% Nonidet P-40) was directly added to monolayer cells, and cells were lysed at 4°C for 5 min. After the nuclei were removed by centrifugation, cytoplasmic RNAs were purified by column. For total RNA extraction, the monolayer cells were lysed in buffer RLT supplied with the kit, and RNA was purified on a spin column. Purified RNA (10 µg per lane) was separated on a 1.5% agarose gel in the presence of formaldehyde. The RNAs were then blotted onto nylon membrane and probed with (i) 1.1 kb full-length L1 wt and HBL1 fragments, (ii) 1.1 kb full-length L2 wt and HBL2 fragments, (iii) 1.1 kb full-length L1 wt and HBL1 fragments, or (iv) randomly labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment. The blots were washed extensively at 65°C and exposed to X-ray films for 3 days.

Production of antisense-RNA. (RNA (2.5 × 10⁻⁴ M Boehringer) was added to a 20-µl reaction mixture containing 10 mM Tris-acetate (pH 7.8), 44 mM KCl, 12 mM MgCl₂, 9 mM β-mercaptoethanol, 38 mM ATP, 0.25 mM GTP, and 7 µl of rabbit reticulocyte extract. The reaction was carried out at 35°C for 20 min, and 20 µl of H₂O was added to the reaction to dilute the RNAs to 10⁻⁴ M. The antisense-RNAs were then aliquoted and stored at -70°C.

RESULTS

Expression of synthetic L1 and L2 protein in cultured cells. To test the hypothesis that the codon composition of the genes encoding the L1 and L2 capsid proteins of PV contributes to their restricted expression in undifferentiated epithelial cells, we produced synthetic BPV1 L1 and L2 genes substituting codons preferentially used in mammalian genes for the codons frequently present in the wt BPV1 L1 and L2 sequences (Fig. 1). Codons where the ratio of usage in BPV1 late genes to mammalian genes was >2, shown in boldface in Table 1 were replaced, additionally most codons where the ratio was >1.5 were replaced. For the L1 gene, a total of 202 base substitutions were made in 196 codons, without changing the encoded

amino acid sequence (Fig. 1A). This synthetic humanized BPV1 L1 gene was designated HBL1. In a similarly modified BPV1 L2 gene designated HBL2, 303 bases were changed to replace 290 less frequently used codons with the corresponding preferentially used codons. Using the synthetic HBL1 and HBL2 genes, we constructed two eukaryotic expression plasmids based on pCDNA3, designated pCDNA/HBL1 and pCDNA/HBL2, respectively. Similar expression plasmids, constructed with the wt BPV1 L1 and BPV1 L2 genes, were designated pCDNA/BPV1L1wt and pCDNA/BPV1L2wt, respectively. In each of these plasmids, the SV40 ori allowed replication in Cos-1 cells, and the L1 or L2 gene was driven by a strong constitutive CMV promoter.

To compare the expression of the synthetic humanized and wt BPV1 L1 and BPV1 L2 genes, we separately transfected Cos-1 cells with each of the L1 and L2 plasmids described above. Transfected cells were analyzed for expression of L1 or L2 protein by immunofluorescence 36 h after transfection (Fig. 2). Cells transfected with the pCDNA3 expression plasmid containing the synthetic humanized L1 or L2 gene produced large amounts of the corresponding protein. About 80% of cells showed significant L1 or L2 staining, within the cell nucleus as expected from the nuclear localization of these proteins (38). Cells transfected with expression plasmids with the wt L1 or L2 sequences produced no detectable L1 or L2 protein (Fig. 2). To compare more accurately the expression of the different L1 and L2 constructs, L1 and L2 protein expression was assessed by immunoblot analysis of Cos-1 cells transfected with the wt or synthetic humanized BPV1 L1 or L2 pCDNA3 expression construct. Immunoreactive L1 and L2 proteins were

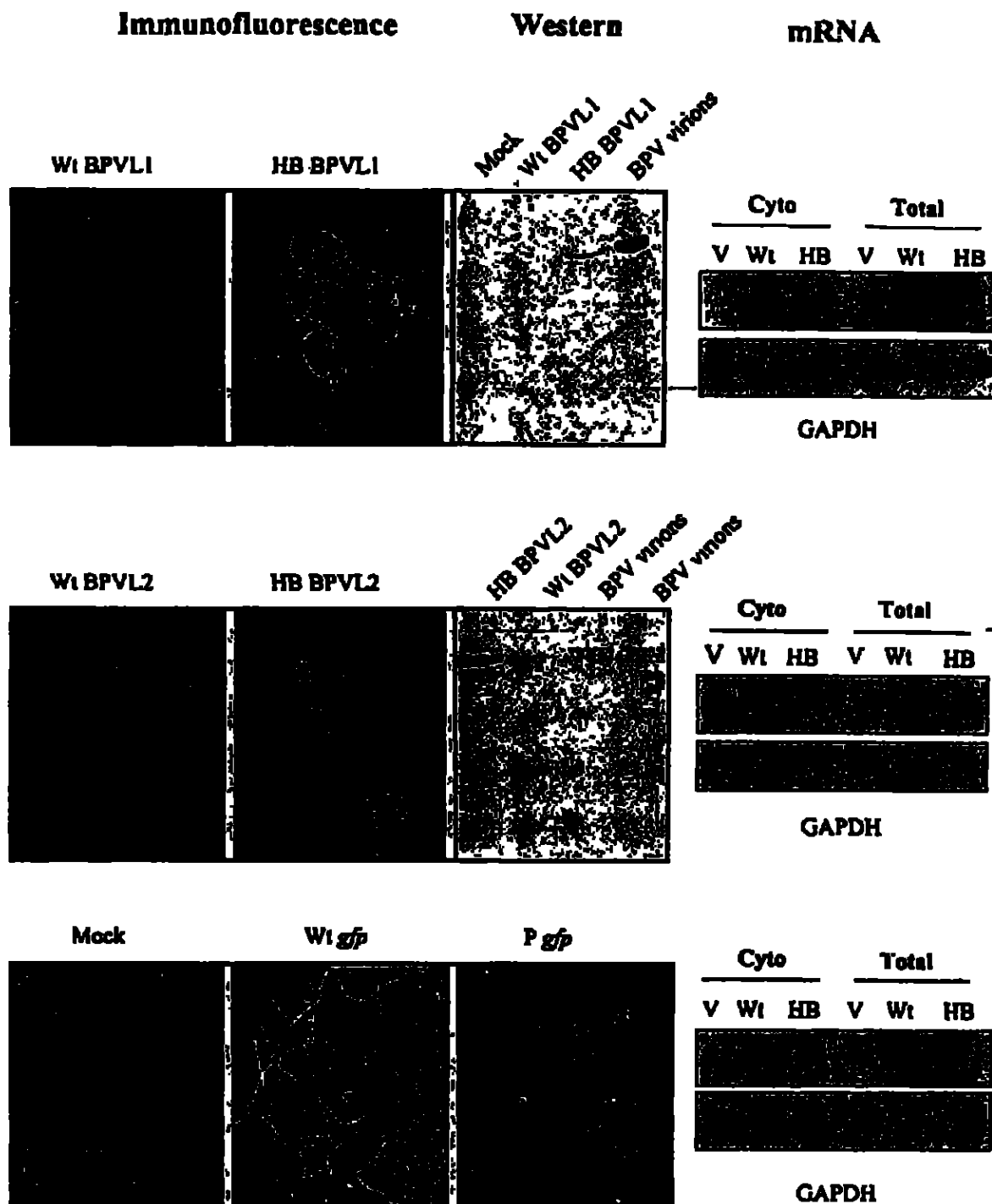


FIG. 2. Expression of L1 and L2 proteins and GFP and of the corresponding mRNAs from wt or codon-modified genes. Cos-1 cells were transfected with wt or modified L1 and L2 expression plasmids pCDNA/BPVL1wt plus pCDNA/HBL1 (row 1), pCDNA/BPVL2wt plus pCDNA/HBL2 (row 2), and pWt.gfp plus P.gfp (row 3). Cells were fixed after 36 h and incubated with rabbit anti-BPV1 L1 (row 1) or L2 (row 2) antisera, followed by FITC-conjugated goat anti-rabbit IgG antibody, and protein was visualized by immunofluorescence. L1 and L2 proteins were visible in the nuclei of cells transfected with HBL1 (row 1, panel 2) and HBL2 (row 2, panel 2) plasmids. The cells transfected with wt L1 (row 1, panel 1) or L2 (row 2, panel 1) sequences failed to produce detectable protein. Cells transfected with humanized *gfp* (row 3, panel 2) produced GFP whereas cells transfected with the *P.gfp* produced little GFP (row 3, panel 3). Immunoblotting of cell lysates of HBL1-transfected cells, and of cells transfected with purified BPV1 but not wt BPV1 L1 (row 1, panel 3), was positive for L1 protein. L2 protein appeared as a 78-kDa band in cells transfected with HBL2 and BPV1 but not in cells transfected with wt L2 (row 2, panel 3). mRNA extracted from the cytoplasm (Cyto) or whole-cell preparation (total) of cells transfected with control pCDNA3 (V), with pCDNA with wt or humanized (HB) L1 or L2 gene inserts, or with wt *gfp* or *P.gfp* was probed with ³²P-labelled probes made with wt L1 (row 1, panel 4) or L2 (row 2, panel 4) or *gfp* (row 3, panel 4) sequences. The total amount of mRNA in these samples was examined by hybridization of the mRNA sample with a GAPDH probe (panel 4, lower).

expressed from the synthetic humanized L1 and L2 sequences but no L1 or L2 protein was expressed from the wt L1 and L2 sequences (Fig. 2).

To establish whether the alterations to the primary sequence of the L1 and L2 mRNAs which resulted from the codon alterations also affected steady-state expression of the corresponding message, total and cytoplasmic mRNAs were prepared from Cos-1 cells transfected with the various capsid protein gene constructs. Using *GAPDH* as an internal standard, we established by Northern blotting that two to three times more modified than wt L1 mRNA and similar levels of wt and modified L2 mRNAs were present in the cytoplasm of transfected cells (Fig. 2, right panel). The total amount of L1 or L2 protein expressed in Cos-1 cell cultures per arbitrary unit of L1 or L2 mRNA expressed was assessed by densitometry as at least 1,000-fold higher for the humanized gene constructs than for the natural gene constructs, confirming that the majority of the difference in level of expressed PV late protein between the two constructs was a consequence of increased L1 or L2 translation, rather than increased gene transcription.

PV late protein translation in vitro. As the major limitation to expression of the wt BPV L1 and L2 genes in dividing cells in vitro appeared to be translational, we wished to test whether this limitation reflected a limited availability of the appropriate tRNA species for gene translation. As transient expression of the synthetic genes within intact cells may be regulated by many factors, we tested our hypothesis in a cell-free system using rabbit reticulocyte lysate (RRL) or wheat germ lysate to examine gene translation. Similar amounts of plasmids expressing the wt or synthetic humanized BPV L1 gene were added to a T7-DNA polymerase coupled RRL transcription translation system in the presence of [35 S]methionine. After 20 min translated proteins were separated by SDS-PAGE and visualized by autoradiography. Efficient translation of the modified L1 gene was observed (Fig. 3A, lane 2), while translation of the wt BPV L1 sequence resulted in a weak 55-kDa L1 band (lane 1). We reasoned that although the wt sequence was not optimized for translation in RRL, some translation would occur since there would be no cellular mRNA species competing for the rare codons present in the wt L1 sequence. These data suggest that the observed difference in efficiency of translation of the wt and synthetic humanized L1 genes is a consequence of limited availability of the tRNAs required for translation of the rare codons present in the wt gene. We therefore expected that addition of excess tRNA to the in vitro translation system would enhance translation of the wt L1 gene to a greater extent than that of the synthetic gene. To address this question 10^{-5} M aminoacyl-tRNA from yeast or bovine liver was added to the RRL translation system and L1 protein synthesis was assessed. Introduction of exogenous tRNAs resulted in a dramatic improvement in translation of the wt L1 sequence, which now gave a yield of L1 protein comparable to that observed with the synthetic humanized L1 sequence (Fig. 3A, right lanes). Enhancement of translation of the wt L1 gene by aminoacyl tRNA was dose dependent, with an optimum efficiency at 10^{-5} M tRNA. As addition of exogenous tRNA improved the yield of L1 protein translated from the wt L1 gene sequence we assessed the speed of translation of wt and humanized L1 mRNAs. Samples were collected from the translation mixture every 2 min, starting at min 8. Translation of L1 from the wt sequence was much slower than that from the humanized L1 sequence (Fig. 3B), and the retardation of translation could be completely overcome by adding exogenous liver tRNA. Addition of a comparable amount of exogenous liver tRNA to the humanized L1 gene expression mixture somewhat enhanced expression of this gene, as might be

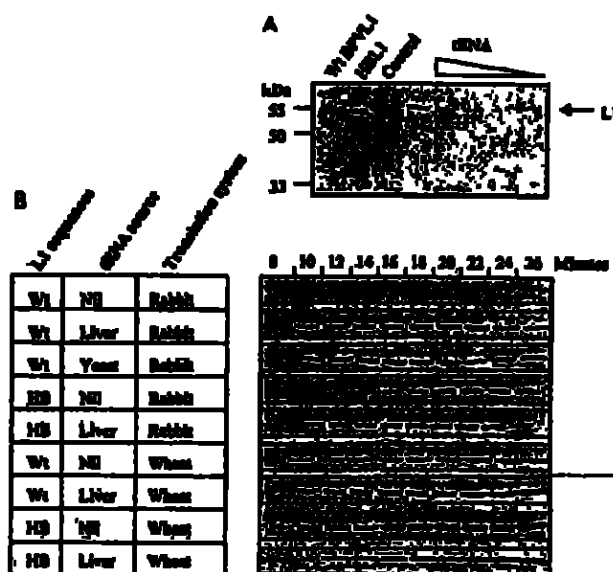


FIG. 3. For in vitro translation of BPV L1 sequences, wt BPV L1 (wt) or synthetic L1 (HB) sequences were translated in vitro, using RRL or wheat germ extract in the presence of [35 S]methionine. (A) wt L1 or HB L1 plasmid DNA was added to the T7-DNA polymerase coupled in vitro translation system (Promega), and L1 protein was translated for 20 min at 30°C. L1 protein was detected by PAGE and autoradiography. The BPV L1 wt gene produced a weak L1 band at 55 kDa (lane 1). The synthetic HB L1 gene made a larger amount of L1 protein (lane 2). The translation reaction where only vector was added produced no visible band (lane 3). When 10^{-5} to 10^{-1} M (lanes 4 to 8) bovine liver aminoacyl-tRNA was added to the translation system, the protein translation efficiency from wt L1 sequence was improved in a tRNA dose-dependent manner. (B) Comparison of the translation efficiencies of wt L1 and HB L1 sequences in the presence and absence of tRNA. Translation was carried out in RRL (rabbit) or wheat germ extract (wheat), and samples were collected every 2 min starting from min 8. Addition of 10^{-5} M bovine liver or yeast tRNA is indicated at the left.

predicted from a mass action effect on the kinetics of protein translation. Translation of the wt gene in the absence of exogenous tRNA was too slow to allow exact determination of the increased rate of translation following addition of exogenous tRNA but was greater than 10-fold in contrast to the 1.3-fold increase observed with the synthetic gene upon addition of exogenous tRNA (Fig. 3B). In separate experiments, we established that wt L1 translation could be similarly enhanced by yeast tRNA (Fig. 3B) and by tRNAs extracted from bovine skin epidermis which presumably constitutes a mixture of tRNAs from differentiated and undifferentiated epithelial cells (data not shown).

Translation of wt L1 is efficient in wheat germ extract. To further test our hypothesis that tRNA availability is a determinant of expression of the wt BPV L1 gene, we examined the translation of L1 in a cell type in which a quite different set of tRNAs would be available. In a wheat germ translation system wt L1 mRNA was translated as efficiently as humanized L1 mRNA, and addition of exogenous aminoacyl-tRNAs did not improve the translation efficiency of either wt or humanized sequences (Fig. 3B). This finding suggested that in wheat germ, in contrast to RRL, there are sufficient of the tRNAs which are limiting for translation of wt L1 sequence in RRL to allow efficient L1 translation.

Modified late genes can be expressed from constructs with only the natural BPV promoters. While the data presented above indicate that translation is limiting for the production of BPV capsid proteins in our test system, these experiments

77
195

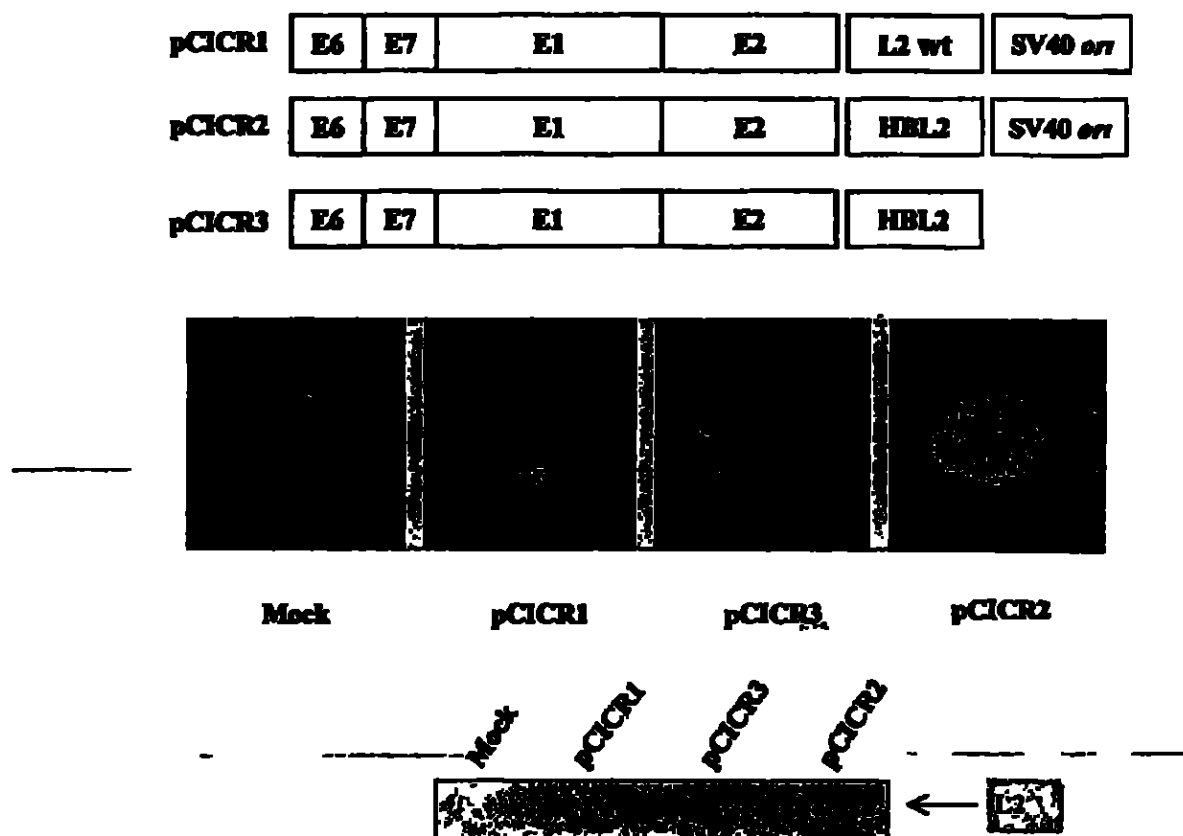


FIG. 4. Expression of L2 protein from the native BPV promoter. (Top) Schematic representation of plasmids used to determine L2 expression from the BPV cryptic promoter(s). The original L1 and most of the L2 sequences were deleted from the BPV1 genome by *Bam*HI and *Hind*III digestion, and the remaining BPV1 sequence was cloned into pUC18. Wild-type or synthetic humanized L2 sequences were inserted into the *Bam*HI site of the BPV1 genome. The position of the inserted SV40 *ori* sequence is indicated. The plasmid where modified L2 was used but without the SV40 *ori* sequence was also used as a control. (Middle) The plasmids were used to transfect Cos-1 cells, and the expression of L2 protein was determined by using BPV1 L2-specific polyclonal antiserum followed by FITC-linked anti-rabbit IgG. Expression of L2 was observed only in cells transfected with a plasmid (pCICR2) containing the SV40 *ori* and a synthetic humanized L2 gene. Cells transfected with the plasmid with the wt L2 sequence (pCICR1) with SV40 or a modified L2 sequence without the SV40 *ori* (pCICR3) failed to produce detectable L2 protein. A mock transfection in which the cells did not receive a plasmid was used as a control. (Bottom) Transfected cells were subjected to SDS-PAGE and immunoblotting with a rabbit polyclonal antibody to L2, and L2 was detected only in cells transfected with pCICR2.

were conducted in systems which are not truly representative of the viral late gene transcription from the BPV genome, in part because the genes were driven by a strong CMV promoter. We therefore wished to establish whether synthetic humanized BPV capsid protein mRNA would be translated more efficiently than the wt mRNA if transcribed from a plasmid containing only the natural BPV1 promoters. This would establish whether translation was indeed one of the limiting factors for expression of BPV1 late genes in the context of the BPV1 genome and in an undifferentiated cell. Cos-1 cells were transfected with plasmid pCICR1 or pCICR2 (Fig. 4, top panel) in which the wt or synthetic humanized L2 gene was inserted into the BPV1 genome cloned into pUC18. L2 protein expression was examined by immunofluorescence of transfected cells and by immunoblotting. Synthetic humanized L2 was efficiently expressed, whereas the wt L2 sequence, driven from a similar construct, produced no immunoreactive L2 protein (Fig. 4, bottom panel). As undifferentiated cells supported the expression of the humanized L2 gene but not the wt L2 gene expressed in the context of the BPV1 genome without an additional promoter, the results confirmed our earlier observations from experiments using the CMV promoter. The plas-

mids tested here contained the SV40 *ori*, designed to replicate the DNA in Cos-1 cells. The increased copy number of the BPV1 L2 plasmids or the transcription-enhancing activity of the SV40 *ori* might explain in part the increased efficiency of expression of L2 in this experimental system compared with infected skin. However, the marked difference in expression between the natural and humanized genes seen with a CMV promoter construct was still observed with a plasmid containing only the natural promoter, suggesting that the variation in L2 expression efficiency observed was independent of the transcriptional promoter used in our system.

Substitution of PV-preferred codons prevents translation but not transcription of a non-PV gene in undifferentiated cells. To further confirm that codon usage can alter gene expression in mammalian cells, we made a further variant of a synthetic *gfp* gene modified for optimal expression in eukaryotic cells (40). In our variant, codons optimized for expression in eukaryotic cells were replaced with those preferentially used in PV late genes. Of 240 codons in the humanized *gfp* gene, which expresses high levels of GFP in cultured cells, 156 were changed to the corresponding PV late gene-preferred codons to produce a new *gfp* gene designated *Pgfp*. Expression of *Pgfp*

42
196

in cells *in vitro* was compared with that of humanized *gfp*. Cos-1 cells transfected with the humanized *gfp* produced a bright fluorescent signal after 24 h, while cells transfected with *Pgfp* produced only a faint fluorescent signal (Fig. 2). Thus, modification of codon usage in other genes to match that of PV late genes can reduce expression of those genes in cell lines, as is observed for PV late genes.

DISCUSSION

In this study we have confirmed that one determinant of the efficiency of expression of three different genes in dividing mammalian cells *in vitro* is their codon composition. This observation has commonly been made when genes from prokaryotic organisms have been expressed in eukaryotic cells (27), and these data are now extended to apply to two PV genes and one eukaryotic gene modified to resemble a PV gene in codon composition. The differential expression of genes utilizing different codons to express the same protein appears predominantly to reflect different efficacy of gene translation, and tRNA availability is apparently rate limiting for translation when genes use codons rarely used by consensus genes from the expression host.

Alterations to mRNA secondary structure or protein binding (29) as a consequence of the changes to the primary sequence of the genes might contribute to the observed differences in efficiency of translation between natural and modified genes in cultured cells. However, the marked enhancement of translation of the natural but not the modified mRNA that was observed after addition of tRNA in a mammalian *in vitro* translation system strengthens the argument that tRNA availability is rate limiting for translation of the natural gene in mammalian cells. A shortage of critical tRNAs could result in slowed elongation of the nascent peptide or premature termination of translation (21). Slowed elongation appears to be the major consequence for the PV late gene. Analysis of codon usage in the PV genome shows that PV late genes use many codons that mammalian cells rarely use. For example, PV frequently uses UUA for leucine, CGU for arginine, ACA for threonine, and AUA for isoleucine, whereas these codons are significantly less often used in mammalian genes. In contrast, PV late genes can be translated efficiently when stably expressed in yeast (14, 24), and the codon compositions of yeast and PV genes are similar (Table 1). The correlation of codon usage in yeast genes and PV late genes is puzzling and may simply represent chance convergent evolution. An apparent exception to the inefficient expression of PV late genes in dividing cells is that PV L1 genes can be expressed in insect cell lines (18) by using recombinant baculovirus or in mammalian cell lines by using recombinant vaccinia virus (39). As infection with these viruses down regulates cellular protein synthesis, the efficient expression of the L1 capsid proteins under these circumstances may occur because less cellular mRNA is available in a virus-infected cell to compete with the L1 mRNA for the rarer tRNAs.

Codon composition could be a more general determinant of gene expression within different stages of differentiation of the same tissue. Although the genetic code is essentially universal, different organisms show differences in codon composition of their genes, while the codon compositions of genes tend to be relatively similar for all genes within each organism and matched to the population of isoaccepting tRNAs for that organism (13). However, populations of tRNAs in differentiating and neoplastic cells are different (15, 35, 36), and the tRNA populations also vary in cells growing under different growth conditions (6). Together these observations suggest

that codon composition and tRNA availability may provide a primitive mechanism for spatial or temporal regulation of gene expression. It is well recognized that the G+C content of many double-stranded DNA viruses, a crude marker for viral gene codon composition, is markedly different from the G+C content of the DNA of the cells they infect (32). Viruses may therefore have evolved to take advantage of codon composition to regulate their own program of gene expression, perhaps to avoid expression of lethal quantities of viral proteins in undifferentiated cells where the virus utilizes the cellular machinery to replicate its genome. Similar alteration of efficiency of expression of at least one gene from human immunodeficiency virus type 1 upon alteration of codon usage has recently been described (1), although the mechanism of increased efficacy of expression was related in that study to release of translation from dependence on human immunodeficiency virus type 1 *rev*. The early genes of PV, with the notable exception of E4, have codon usage patterns similar to those of the late genes, which could explain the very low levels of expression of these proteins observed in dividing cells *in vivo* and *in vitro* (26, 28). In keeping with our hypotheses, it has recently been shown that the translation of at least one PV early gene, E7, can be enhanced *in vitro* by addition of exogenous tRNA (5).

As our current observations may represent an apparently novel mechanism of regulation of gene translation within a single tissue, it is relevant to consider how this relates to previously proposed hypotheses for the restriction of expression of PV late genes to differentiated epithelium. It has been suggested that reduced late gene expression may reflect dependence of expression from the late promoter on transcription factors expressed only in differentiated epithelium or may alternatively be due to suppression of late promoter transcription by viral (33) or cellular gene products expressed in undifferentiated cells. The late promoters of human papillomavirus types 31b and 5 (11, 12) are described as differentiation dependent, although the search for relevant transcription control factors in differentiated keratinocytes by conventional footprinting and DNA binding studies has been unrewarding. Our data show that capsid proteins are not translated from PV L1 and L2 mRNAs in cells transfected with CMV promoter-based expression vectors (Fig. 2), suggesting that in addition to any transcriptional controls that may exist, there is also a posttranscriptional block to capsid protein synthesis in undifferentiated cells. Within L1 or L2 mRNA or within flanking untranslated message are sequences resembling 5' splice donor sites which are inhibitory to transcription of genes with which they are associated (8, 17). Other AU-rich sequences in L1 or L2 mRNA promote mRNA degradation (30). Consistently, moderately decreased levels of mRNA for the wt L1 compared with the sequence of modified L1 were observed in the present study following expression of these genes in Cos-1 cells. This observation confirms that transcription or stability of wt L1 may be diminished in comparison with other genes. However, the overall contribution of reduced transcriptional efficacy to PV L1 expression in our systems was small in comparison to the effect of codon usage on translational efficiency and no similar effect on transcription was seen for L2 although the translational difference with the modified L2 gene was equally large. Because untranslated inhibitory sequences were not included in our test system, we cannot determine the roles of inhibitory sequences and codon mismatch in suppression of PV late gene expression *in vivo*. However, regulatory sequences promoting RNA degradation or inhibiting translation are presumed to act through interaction with nuclear or cytoplasmic proteins (29) and inefficient translation of native sequence L1

25
197

mRNA was observed in a cell free translation system from anucleate cells, demonstrating that codon composition of the PV late genes must play some role in regulation of PV late gene translation. Further, the mechanisms shown to inhibit L1 and L2 mRNA production or increase message instability in undifferentiated cells have yet to be shown to be inactive in differentiated epithelium as would be required to explain by these mechanisms the successful translation of PV late genes in this tissue.

Further evidence supporting the hypothesis that codon composition may be an important determinant of PV capsid gene expression was gathered from an analysis of the 84 PV L1 sequences currently available in GenBank. The codon composition of the L1 genes, and particularly the frequency of usage of the rarer codons, was essentially the same across all the published sequences (data not shown) as would be expected given the similar G+C contents of the PV genomes. The PV L1 gene is relatively conserved at the amino acid level showing 60 to 80% amino acid homology between PV genotypes, as might be expected given the constraints on capsid protein function. There are, however, no obvious constraining influences on the codon composition of the PV late genes beyond those of our hypothesis, as the late gene region does not code for other genes, either in other reading frames or on the complementary DNA strand, and has no known cis-acting regulatory functions other than those intrinsic to L1 or L2 gene expression. If codon composition of the capsid genes were not important for PV function, a considerable heterogeneity of codon usage might therefore be expected given the evolutionary diversity of PVs (4).

Taken together, the data presented in this report makes a case that codon usage should be considered as a possible determinant of differential expression of PV late genes between undifferentiated and differentiated epithelial cells, in addition to many proven regulation mechanisms, and suggests that this observation may be generalizable to other genes. The relative role of message instability and codon mismatch in determining PV late gene expression in differentiated tissues will require comparisons of transcriptional activity and translation of the L1 and L2 genes driven from strong constitutive promoters in differentiated and undifferentiated epithelium. Such work using either transgenic technology or keratinocyte raft cultures should now be feasible. The hypothesis that expression of other genes may be regulated by tRNA availability in vivo is now testable, as methods for fingerprinting tRNA distributions in tissues have recently been developed (15).

ACKNOWLEDGMENTS

We thank Mark Williams for helpful discussions throughout the study.

This work was funded in part by grant RO1-CA 57789 from NIH and by grants from the NH&MRC, the Queensland Cancer Fund, the Mayo Bequest, and the Princess Alexandra Hospital Research and Development Foundation.

REFERENCES

- Andre, B., B. Seed, J. Eberlin, W. Schram, A. Bittmann, and J. Haas. 1993. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J. Virol.* 72:1497-1503.
- Baker, C. C., and P. M. Hawley. 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissues. *EMBO J.* 6:1027-1033.
- Barbante, S. K., and C. C. Baker. 1995. Differentiation-specific alternative splicing of bovine papillomavirus late mRNAs. *J. Virol.* 69:6553-6556.
- Chen, S.-Y., H. Delius, A. L. Halpern, and H.-U. Bernard. 1993. Analysis of genomic sequences of 95 papillomavirus types: linking typing, phylogeny and taxonomy. *J. Virol.* 69:3074-3083.
- De Paquale, C., and D. Kamb. 1998. Modulation of HPV16 E7 translation by tRNAs in eukaryotic cell-free translation systems. *Biochem. Mol. Biol. Int.* 45:1005-1009.
- Del, R. H., L. Kandel, and R. T. Ignotz. 1968. Pattern of valine transfer ribonucleic acid of *Escherichia coli* under different growth conditions. *J. Biol. Chem.* 243:945-951.
- Firth, P. A., and C. C. Baker. 1991. An element in the bovine papillomavirus late 3' untranslated region reduces polyadenylated cytoplasmic RNA levels. *J. Virol.* 65:5806-5812.
- Firth, P. A., W.-T. Chen, J. H. Rex, J. C. Byrne, and C. C. Baker. 1994. Sequences homologous to 5' splice sites are required for the inhibitory activity of papillomavirus late 3' untranslated regions. *Mol. Cell. Biol.* 14:5278-5289.
- Graessmann, K., B. Rapp, H. Maschek, K. U. Pater, and T. Illner. 1996. Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV 16) in cell cultures of a new cell line containing high copy numbers of episomal HPV-16 DNA. *J. Virol.* 70:2359-2369.
- Hagena, M. E., N. Yangstul, and D. A. Galloway. 1993. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J. Virol.* 67:315-322.
- Haller, K., F. Stabele, and H. Pfister. 1995. Differentiation-dependent transcription of the epidermolytic human papillomavirus type 3 in benign lesions. *Virology* 214:245-255.
- Hanriot, M., J. B. Hudson, and L. A. Laimins. 1992. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *J. Virol.* 66:6070-6080.
- Hennrich, T. 1981. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes. *J. Mol. Biol.* 146:1-21.
- Jansen, K. H., M. Rastvorova, L. D. Schultz, H. Z. Marini, J. C. Cook, J. J. Donnelly, D. Martins, R. W. Ellis, and A. R. Shaw. 1995. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. *Vaccine* 13:1309-1314.
- Kamb, D. 1997. Changes of tRNA population during compensatory cell proliferation: differential expression of methionine-tRNA species. *Arch. Biochem. Biophys.* 343:1-6.
- Kennedy, L. M., J. K. Hadden, and J. B. Clements. 1990. Analysis of human papillomavirus type 16 late mRNA 3' processing signals in vitro and in vivo. *J. Virol.* 64:1825-1829.
- Kennedy, L. M., J. K. Hadden, and J. B. Clements. 1991. A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability. *J. Virol.* 65:2093-2097.
- Kirnbauer, R., F. Brey, N. Cheng, D. R. Levy, and J. T. Schiller. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* 89:12180-12184.
- Liu, W. J., L. Glusman, X. Y. Sun, A. Kengharubethal, M. Müller, J. Duerber, and J. Zhou. 1997. Sequence data to the N-terminus of L2 protein is displayed on the surface of bovine papillomavirus type 1 virions. *Virology* 217:474-483.
- Nakamura, Y., K. Wada, H. Del, S. Kanaya, T. Gajburi, and T. Ignotz. 1996. Codon usage tabulated from the international DNA sequence database. *Nucleic Acids Res.* 24:214-215.
- Oda, T., Y. Andachi, A. Mats, and T. Obara. 1991. Translation in vitro of codon UGA as tryptophan in *Mycoplasma capricolum*. *Biochimie* 73:1109-1112.
- Ostun, M. A., and C. Meyers. 1997. Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. *J. Virol.* 71:5161-5172.
- Rasoch, M. N., F. Stabele, and L. A. Laimins. 1998. Activation of papillomavirus late gene transcription and genome amplification upon differentiation in acellular medium is coincident with expression of involucrin and transglutaminase but not keratin-18. *J. Virol.* 72:5016-5024.
- Sengupta, T., P. Pustina, G. Steers, S. E. Gschwend, M. A. V. Rajbhandari, J. Finch, L. Crawford, and M. Tummato. 1993. Synthesis and assembly of virus-like particles of human papillomavirus type 6 and type 16 in fusion yeast *Schizosaccharomyces pombe*. *Virology* 204:126-135.
- Schwartz, S. 1998. Cis-acting negative RNA elements on papillomavirus late mRNAs. *Senes. Virol.* 8:291-300.
- Schuy, L. A., L. A. Dunn, B. Murray, R. W. Tindis, and L. H. Frazer. 1992. An ELISA capture assay for the E7 transforming proteins of HPV16 and HPV18. *J. Virol. Methods* 37:119-127.
- Smith, D. W. 1996. Problems of transcribing heterologous genes in expression systems: the role of tRNA. *Biochem. Prog.* 12:417-422.
- Smolnik, D., and F. O. Wettstein. 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA* 83:4680-4684.
- Sokolowski, M., W. Tan, M. Jeffers, and S. Schwartz. 1998. mRNA instability elements in the human papillomavirus type 16 L2 coding region. *J. Virol.* 72:1504-1515.

30. Saksela-Ojala, M., C. P. Zhao, W. Tan, and S. Schwartz. 1997. AU-rich mRNA instability elements on human papillomavirus type 1 late mRNAs and *c-fos* mRNAs interact with the same cellular factors. *Oncogene* 15:2303-2319.
31. Stoler, M. H., C. R. Rhoads, A. Whitbeck, S. M. Wolfmeyer, L. T. Chow, and T. R. Broker. 1992. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. *Hum. Pathol.* 23:117-128.
32. Strauss, E. G., J. H. Strauss, and A. J. Levine. 1995. Virus evolution, p. 153-171. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Virology*. Lippincott-Raven, Philadelphia, Pa.
33. Szebenyach, F., L. M. Leigh, and H. Pfister. 1996. E2 represses the late gene promoter of human papillomavirus type 8 at high concentrations by interfering with cellular factors. *J. Virol.* 70:119-126.
34. Volpers, C., P. Schirmacher, R. E. Strech, and M. Sapp. 1994. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. *Virology* 200:504-512.
35. Yang, S. S., and D. G. Comb. 1988. Distribution of multiple forms of hypoxanthine transfer RNA during early embryogenesis of sea urchin, *Lytechinus variegatus*. *J. Mol. Biol.* 21: 138-142.
36. Yang, W. K., and G. D. Novelli. 1968. Inactivating +RNA's in mouse plasma cell tumors that synthesize different myeloma proteins. *Biochem. Biophys. Res. Commun.* 31:534-539.
37. Zainier, R., L. A. Borensztein, F. O. Weinstein, and T. Iltner. 1994. Changes in RbA expression pattern during the malignant progression of cutaneous rabbit papillomavirus-induced tumors in rabbits. *J. Virol.* 68:3620-3630.
38. Zhou, J., J. Doerber, X. Y. Sun, L. V. Crawford, C. S. McLennan, and L. H. Frazer. 1991. Identification of the nuclear localization signal of human papillomavirus type 16 L1 protein. *Virology* 185:625-632.
39. Zhou, J., X. Y. Sun, D. J. Stenzel, and L. H. Frazer. 1991. Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* 185:251-257.
40. Zlotolow, A. S., M. Potter, W. W. Renswirth, J. Gay, and N. Maryucha. 1996. A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J. Virol.* 70:4646-4654.

ATG
199

Protection of Rabbits from Viral Challenge by Gene Gun-Based Intracutaneous Vaccination with a Combination of Cottontail Rabbit Papillomavirus E1, E2, E6, and E7 Genes

RICAI HAN,¹ NANCY M. CLADEL,² CYNTHIA A. REED,¹ XUWEN FENG,²
AND NEIL D. CHRISTENSEN^{1,3*}

*John Gittlen Cancer Research Institute, Department of Pathology,¹ Department of Comparative Medicine,²
and Department of Microbiology and Immunology,³ Pennsylvania State University
College of Medicine, Hershey, Pennsylvania 17033*

Received 4 February 1999/Accepted 12 May 1999

In this study, cottontail rabbit papillomavirus infection of domestic rabbits was used as an animal model to develop papillomavirus early gene-based vaccines. Groups of rabbits were intracutaneously vaccinated with single papillomavirus early genes E1, E2, E6, and E7 or with a combination of these four genes. Only a fraction of rabbits were protected from subsequent viral challenge when vaccinated with the E1 or E6 gene. Viral tumor growth in those rabbits vaccinated with the E1 or E2 gene was suppressed compared to that in controls. In contrast, seven of nine rabbits vaccinated with the combination of the E1, E2, E6, and E7 genes were completely protected against viral challenge. These data indicated that intracutaneous genetic vaccination with the combination of the E1, E2, E6, and E7 genes can be an effective strategy for immunoprophylaxis of papillomavirus infection.

Human papillomavirus (HPV) infection induces mucosal and/or cutaneous hyperproliferative lesions which persist for months or years. Certain HPV types are linked to the development of skin cancer (21) tumors of the head and neck (8), and anogenital carcinomas (30). Development and testing of papillomavirus vaccines have been conducted extensively in animal models, such as bovine papillomavirus (BPV) infection of cattle (3) and cottontail rabbit papillomavirus (CRPV) infection of domestic rabbits (16). Currently several strategies have been utilized to develop papillomavirus vaccines. One strategy involves the induction of neutralizing antibodies by immunization with the viral structural protein L1 or L2, particularly, virus-like particles (VLPs) assembled from L1 or L1/L2. Another strategy involves the induction of cell-mediated immunity by papillomavirus early gene/protein-based vaccination. Recently VLPs containing L1 and chimeric molecules of L1 and early proteins were used as immunogens. This strategy has been applied to elicit concurrent viral neutralizing antibodies and cell-mediated immunity specific for viral early proteins (11).

A number of studies have demonstrated that VLP immunization protected animals against experimental virus challenge in the CRPV-infected rabbit model (2, 6, 13), in the BPV-infected cow model (15), and in the canine oral papillomavirus (COPV)-infected beagle dog model (28). Furthermore, genetic vaccination with CRPV L1 (9, 26) or immunization with CRPV L1 proteins expressed as bacterial fusion proteins (18) also protected rabbits from viral challenge. Protection has also been achieved by immunization with L2 proteins (5, 10, 19). One caveat of these animal model systems is that protection from natural papillomavirus infection has not been determined. In experimental infection models, sites to be infected

are vigorously scarified or wounded, resulting in damage to local blood vessels and the release of circulating neutralizing antibodies at the sites of infection. In contrast, natural infection may occur following microtrauma to the epithelium without significant damage to blood vessels and subsequent direct exposure of virus to circulating neutralizing antibodies. Thus, circulating neutralizing antibodies may be unable to protect against natural papillomavirus infection. Protective vaccines targeting virus-infected epithelium via cell-mediated immunity would overcome these potential limitations.

Induction of protective cell-mediated immunity by immunization with papillomavirus early gene/proteins is expected to prevent the establishment of new lesions (immunoprophylaxis) as well as to eliminate existing lesions (immunotherapy). However, early studies disclosed variable results. In the CRPV-infected rabbit model, different papillomavirus early antigens and several methods of antigen delivery have been applied in an attempt to elicit protective antipapillomavirus immunity: (i) immunization with bacterial fusion proteins of CRPV E1 and/or E2 (24); (ii) immunization with recombinant *Listeria monocytogenes* expressing CRPV E1 (14); (iii) intracutaneous genetic vaccination of rabbits with CRPV E6 (27); and (iv) intramuscular injection of plasmid DNA encoding CRPV E1, E2, E6, or E7 (12). The immunity so induced stimulated papilloma regression in a fraction of vaccinated rabbits (14, 24) and partially protected rabbits from subsequent virus challenge (27). However, none of these studies revealed complete protection. In the BPV-infected cow model, immunization with BPV E6 and E7 proteins delayed papilloma formation, reduced papilloma size, and promoted papilloma regression but also did not lead to complete protection (3, 4). BPV E2 immunizations were ineffective (4). In the present study, we immunized rabbits by gene gun-mediated intracutaneous vaccination with individual CRPV E1, E2, E6, and E7 genes or with a combination of all four genes. We report that vaccination with the combination of CRPV E1, E2, E6, and E7 genes provided strong and complete protection of outbred rabbits against CRPV challenge.

* Corresponding author. Mailing address: John Gittlen Cancer Research Institute, Pennsylvania State University College of Medicine, 500 University Dr., Hershey, PA 17033-2390. Phone: (717) 531-4700. Fax: (717) 531-5834. E-mail: ncd1@psu.edu.

A28
200

TABLE 1 Papilloma growth in vaccinated and control rabbits

Group ^a	Vaccination dose (μg)			No. of sites with papillomas ^b					% Sites with papillomas
	1st	2nd	3rd	E1	E2	E3	E4	E5	
First exp ^c									
VJmsE1	20	20	10	4	0	2	0	ND	37.5
VJmsE2	20	20	10	2	4	4	4	ND	67.5
VJmsE6	20	20	10	0	4	2	3	ND	56.3
VJmsE7	20	20	10	3	4	4	4	ND	93.8
Combined	10	10	ND ^d	2 ^e	4 ^e	0	0	ND	37.5
Vector	20	20	10	4	4	4	4	ND	100
Second exp ^c									
Combined	10	10	6	0	0	0	0	0	0.0
Vector	10	10	6	4	4	4	4	4	100

Rabbits in each group were vaccinated with one type of plasmid DNA, as indicated, except that rabbits in the combined group were vaccinated with four genes, VJmsE1, VJmsE2, VJmsE6, and VJmsE7.

^a E1 through E5 denote rabbit numbers in each group. E3 was not used in the first experiment.

^b Papillomas regressed in 3 weeks.

^c ND, not done.

Preparation of DNA vectors and gene gun-mediated immunization. CRPV E1, E2, E6, and E7 DNA genes were amplified by PCR and cloned into VJms expression vector (generous gift of M. A. Lin, Merck & Co, Westpoint, Pa.) at the *Bgl*II site individually (20). The resultant recombinant plasmids were referred to as VJmsE1, VJmsE2, VJmsE6, and VJmsE7 respectively. Plasmid DNA was precipitated onto 1.6-μm-diameter gold microparticles at a ratio of 1 μg of DNA/0.5 mg of gold particles as described by the manufacturer (Bio-Rad, Hercules, Calif.). Outbred New Zealand White rabbits (Covance Research Products Inc., Denver, Pa.) were immunized by gene gun-mediated intracutaneous delivery of DNA/gold particles onto dorsal skin sites at 400 lbf/in².

Protection of rabbits against challenge with cloned CRPV viral DNA. In our laboratory we currently use a CRPV stock which was obtained originally from cottontail rabbit viral papillomas and then expanded by using the nude mouse xenograft system. It is possible that several CRPV variants exist in this virus stock (23). In this study the E1, E2, E6, and E7 genes were cloned from a single clone or a variant. Theoretically immunity to these early proteins will generate protection against the matching CRPV variant but not to other variants if critical epitopes are antigenically unrelated. Rabbits were thus challenged with the same cloned viral DNA (17) from which the E1, E2, E6, and E7 genes were amplified. In the first experiment, 24 rabbits were divided into six groups (Table 1). For the groups vaccinated with a single gene or vector rabbits received three immunizations at 3-week intervals: 20 μg of DNA for each of the first and the second immunizations, and 10 μg of DNA for the final booster immunization. For the group vaccinated with the combination of the four genes, rabbits received only two immunizations at 3-week intervals, 10 μg of each E1-, E2-, E6-, and E7-encoding plasmid DNA. Numbers of challenged sites in each rabbit that produced papillomas are shown in Table 1. Papillomas grew at all challenged sites in control rabbits that were vaccinated with VJms vector indicating a high efficiency of papilloma induction by viral DNA (100%) in this experiment. Among the groups vaccinated with single genes, 63% and 44% of challenged sites had no papilloma growth for E1-immunized rabbits and for E6-immunized rabbits, respectively. In contrast, only 13% of challenged sites for E2-vaccinated rabbits and 7% of sites for E7-vac-

inated rabbits had no papilloma growth (Table 1). Two of four rabbits vaccinated with the E1 gene were completely protected, and one of four E6-vaccinated rabbits was also completely protected. These results demonstrated that individual E1 or E6 vaccination provided protection in a subset of rabbits following infection with cloned viral DNA. In contrast, E2 and E7 vaccinations did not induce protective immunity. Among four rabbits vaccinated with the combination of E1, E2, E6, and E7 genes, two rabbits had no papillomas, and on the remaining two rabbits, there were only small papillomas which completely regressed within three weeks. Systemic regression of papillomas was not observed on rabbits in the other groups, indicating that vaccination with all four genes induced the strongest protective antiviral immunity in this experiment. It is important to note that the rabbits in the combination vaccine group received two immunizations with a total of 20 μg of DNA of each gene in contrast to the rabbits in the other groups, which received three immunizations with a total of 50 μg of DNA for individual gene vaccination.

In the second experiment (Table 1) 10 rabbits were divided into two groups. One group was vaccinated with the combination of the E1, E2, E6, and E7 genes. The second group received vector DNA only as a control. In addition to the first and second immunizations, rabbits also received a third, booster immunization containing 6 μg of DNA of each gene (Table 1). Papillomas grew at all challenged sites in all five control rabbits. In contrast, all five rabbits vaccinated with the combination of E1, E2, E6, and E7 genes were completely protected (Table 1). These results demonstrated that intracutaneous genetic vaccination with the combination of E1, E2, E6, and E7 protected all rabbits from challenge with cloned viral DNA.

Protection of rabbits against challenge with infectious virions. To investigate whether these protected rabbits were also resistant to challenge with virus particles, we infected all protected rabbits with infectious virions (two E1-vaccinated, one E6-vaccinated, and nine combination gene-vaccinated rabbits; Table 1). Five rabbits that were previously vaccinated with VJms vector were also challenged with virus particles as a control. All control rabbits grew papillomas at virion-challenged sites, and all protected rabbits were resistant to the viral challenge except one rabbit which was vaccinated with the E1 gene alone. Papillomas on the latter rabbit appeared at 4 weeks after virus challenge and persisted without regression until the termination of this experiment (4 months). Vaccination with the combination of E1, E2, E6, and E7 genes therefore completely protected rabbits from virus particle infection.

Papilloma growth in nonprotected and partially protected rabbits. Papilloma size was measured for a total of 14 weeks following viral DNA challenge in those nonprotected and partially protected rabbits. Mean papilloma sizes per site for rabbits vaccinated with the E1 or E2 genes were significantly smaller than those in the vector-injected rabbits (vector group versus E1 group, *t* test, *P* < 0.05; vector group versus E2 group, *t* test, *P* < 0.05). Papilloma size per site for E6-vaccinated rabbits was somewhat smaller than that for vector-vaccinated control rabbits, but size differences were not statistically significant at 14 weeks postchallenge with virus (Fig. 1). E7 vaccination has little effect on papilloma growth rates (Fig. 1).

Systemic regression of papillomas was observed only for two rabbits that were vaccinated with the combination of the E1, E2, E6, and E7 genes in the first experiment (rabbits 1 and 2). For one partially protected rabbit in the E2 group (rabbit 1, Table 1) papillomas at one challenged site rapidly regressed and papilloma growth at another site was significantly suppressed (9 mm² at 14 weeks) but did not regress. One papil-

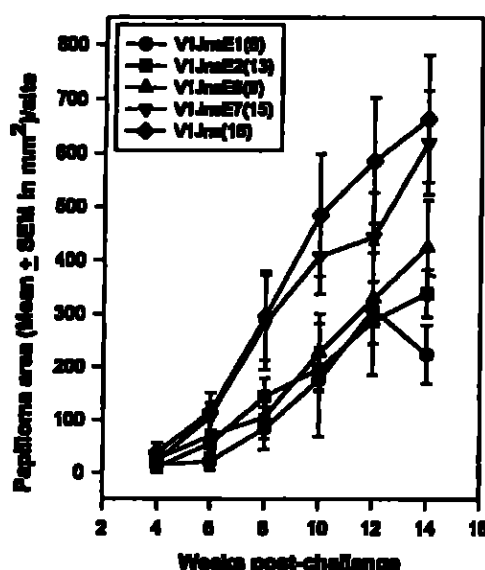


FIG. 1. Papilloma growth for nonvaccinated and control rabbits in the first experiment. Data represent the mean $\pm$ standard errors of the means of papilloma areas that were calculated from the total numbers of papillomas, which are indicated in parentheses, for each group. Individual papilloma size was calculated by multiplying length (millimeters) and width (millimeters). Mean papilloma areas were significantly different in the VJmE1 group and VJmE2 group (t test, $P < 0.05$) and in the VJmE1 group and VJmE6 group and in the VJmE1 group and VJmE7 group at 14 weeks postchallenge.

loma on one E6-vaccinated rabbit (rabbit 3; Table 1) appeared at week 4, then regressed, and reappeared at week 8. However this papilloma regressed again, regrew at 14 weeks, and then became persistent. These observations indicated that immunity

induced by single gene vaccinations by using the protocol described for the first experiment had an impact on papilloma growth but was insufficient to completely eliminate all viral papillomas. It is possible that additional booster vaccinations with single genes prior to viral DNA challenge may have led to an increased level of protection.

T-cell-mediated immune responses. In vitro peripheral blood mononuclear cell (PBMC) proliferation assays were conducted to evaluate T-cell-mediated immune responses (12). PBMCs were isolated from ear arterial blood one week after the final booster immunization and stimulated with the specific antigens, CRPV E1, E2, E6 and E7 as histidine-tagged fusion proteins. Control antigens for all rabbits were CRPV L2 histidine-tagged fusion proteins. All fusion proteins were prepared by using recombinant baculoviruses and purified by using nickel columns under denaturing conditions (Invitrogen, San Diego, Calif.) as previously described (12). All four E2-vaccinated rabbits showed strong antigen-specific proliferative responses (Table 2). Three of four rabbits in each of the E1, E6, and E7 groups also showed antigen-specific proliferative responses (Table 2). These data indicated that genetic vaccination with CRPV E1, E2, E6, and E7 genes induced T-cell-mediated immunity. Interestingly, in vitro antigen-specific proliferative responses were strongest in the E2- and E7-vaccinated rabbits despite a lack of protection against viral DNA challenge in these two groups (Table 2). Furthermore, papilloma sizes in rabbits vaccinated with E7 were substantially larger than the papillomas of the E6-vaccinated group (Fig. 1). These data suggest that in vitro antigen-specific proliferative responses do not correlate with in vivo protective immunity.

Humoral immune responses. Rabbit serum was collected before vaccination and after the first, second, and third vaccinations (Fig. 1). Western blot analysis did not detect specific anti-E1, -E2, -E6, or -E7 antibodies in these vaccinated rabbits (data not shown).

Papillomavirus-induced lesions are maintained by hyperproliferation of virus-infected, undifferentiated cells in the basal and suprabasal layers of the epithelium. Immune-mediated

TABLE 2. PBMC proliferative responses to in vitro stimulation with antigens

Rabbit	Vaccine	Time ^a	PBMC proliferation (cpm $\times 10^{-3} \pm$ SEM) ^b in response to:			Stimulation index	
			No antigen ^c	Non-specific antigen ^d	Specific antigen ^e	Non-specific ^f	Specific ^g
1	VJmE1	Day 5	0.366 $\pm$ 0.027	0.441 $\pm$ 0.021	14.237 $\pm$ 1.566	1.2	38.9
2	VJmE1	Day 5	0.437 $\pm$ 0.012	0.640 $\pm$ 0.025	3.598 $\pm$ 0.293	1.4	8.2
3	VJmE1	Day 4	0.181 $\pm$ 0.040	0.234 $\pm$ 0.067	0.820 $\pm$ 0.125	1.3	4.5
4	VJmE1	Day 4	2.201 $\pm$ 0.312	2.145 $\pm$ 0.235	9.683 $\pm$ 0.702	0.9	4.3
5	VJmE2	Day 6	0.163 $\pm$ 0.014	0.355 $\pm$ 0.045	18.439 $\pm$ 2.104	2.1	114
6	VJmE2	Day 6	0.270 $\pm$ 0.040	0.585 $\pm$ 0.015	3.319 $\pm$ 0.404	2.1	19.7
7	VJmE2	Day 5	0.239 $\pm$ 0.022	0.324 $\pm$ 0.031	32.785 $\pm$ 2.639	1.3	137.1
8	VJmE2	Day 6	0.155 $\pm$ 0.013	0.402 $\pm$ 0.059	1.040 $\pm$ 2.500	2.5	6.7
9	VJmE6	Day 6	0.179 $\pm$ 0.019	0.567 $\pm$ 0.040	1.560 $\pm$ 0.078	3.1	7.5
10	VJmE6	Day 5	0.245 $\pm$ 0.047	0.448 $\pm$ 0.039	1.841 $\pm$ 0.000	1.8	7.5
11	VJmE6	Day 5	0.160 $\pm$ 0.017	0.239 $\pm$ 0.010	0.390 $\pm$ 0.050	1.4	2.4
12	VJmE6	Day 4	0.775 $\pm$ 0.089	1.092 $\pm$ 0.176	6.861 $\pm$ 0.218	1.4	8.8
13	VJmE7	Day 5	0.336 $\pm$ 0.037	0.600 $\pm$ 0.091	5.600 $\pm$ 0.000	1.7	16.6
14	VJmE7	Day 4	0.482 $\pm$ 0.057	1.939 $\pm$ 0.399	6.318 $\pm$ 1.380	4.0	13.1
15	VJmE7	Day 5	0.307 $\pm$ 0.013	0.352 $\pm$ 0.022	0.465 $\pm$ 0.135	1.1	1.5
16	VJmE7	Day 5	2.450 $\pm$ 0.310	5.136 $\pm$ 0.313	17.029 $\pm$ 3.159	2.0	6.9

^a Day indicated is day with highest value of response among day 4 to day 6.

^b Of triplicate wells.

^c Without addition of any antigen.

^d Addition of CRPV L2 protein.

^e Antigen corresponding to vaccinated antigen.

^f Ratio of counts per minute in response to non-specific antigen to counts per minute in response to no antigen.

^g Ratio of counts per minute in response to specific antigen to counts per minute in response to no antigen.

cure of papillomavirus-induced lesions can be successful only when the latter cells are eliminated by effector cells, such as cytotoxic T lymphocytes. A potential vaccine candidate gene/protein(s) therefore must be expressed in the infected, undifferentiated cells and, in addition, must be immunogenic. Experimental evidence for the viral proteins E1, E2, E6, and E7 in basal and suprabasal layers of infected epithelium has been indirectly established by RNA-RNA *in situ* hybridization studies (1, 23, 29). These four genes therefore are potential candidate vaccines. In the present study, E1 was the most effective of the four early genes for protection by vaccination of animals against viral challenge. These results are consistent with the findings of Jensen and coworkers (14). Immunization with the E6 gene only provided partial protection. A similar result was obtained by other investigators (27). Unexpectedly, T-cell-mediated immune responses for the E2 immunization group were the strongest of any of the vaccinated groups as determined by *in vitro* PBMC proliferation assays. However, none of the E2-vaccinated rabbits were completely protected from viral challenge, although viral tumor growth was significantly suppressed. E7 vaccination also induced cell-mediated immunity but neither induced protection of rabbits from viral challenge nor significantly suppressed viral tumor growth. In contrast, vaccination with the combination of the E1, E2, E6, and E7 genes provided complete protection of rabbits against subsequent viral challenge. It is possible that the expression levels of the E1, E2, E6, and E7 proteins are low in the basal and suprabasal layers of the infected epithelium. Low expression would result in a low density of virus-derived peptide/major histocompatibility complex (MHC) class I complexes on the cell surface. Efficient elimination of these virus-infected cells may be possible only if there is a pool of CD8⁺ T lymphocytes specific for E1, E2, E6, and E7 in certain MHC genetic backgrounds. In this study although the immunity induced by E7 vaccination alone did not affect viral infection, E7 vaccination may still play a role in protection and/or suppression of viral tumor growth in some rabbits with certain MHC genotypes which can efficiently process and present E7 antigen to T cells. E7 vaccination may also play a role in the prevention of cervical cancer.

We have also immunized rabbits by (i) subcutaneous injection of recombinant baculovirus-expressed E1 and E2 proteins (unpublished observations), (ii) subcutaneous injection of recombinant retroviruses expressing E1 or E2 proteins (unpublished observations), and (iii) intramuscular injection of plasmid DNA encoding E1, E2, E6, or E7 respectively (12). Cell-mediated immunity was detectable in these vaccinated rabbits, but none of the above-listed vaccination strategies provided protection of rabbits against subsequent viral challenge. Based on our observations, gene gun-mediated intracutaneous genetic immunization is the most effective strategy for a papillomavirus early-gene-based vaccine. It has been demonstrated that gene gun-mediated intracutaneous inoculation of naked plasmid DNA directly delivered the DNA into skin-derived dendritic cells *in vivo* (7, 22). The proteins encoded by the plasmid DNA were expressed in the cytoplasm of dendritic cells in the draining lymph nodes. These procedures produced potent, antigen-specific cytotoxic T-lymphocyte-mediated protective tumor immunity (7).

In vivo delivery of an oncogene-expressing plasmid DNA always raises safety concerns. Papillomavirus E6 and E7 are multifunctional oncogenes based on *in vitro* assays. Gene gun-mediated delivery can directly introduce E6- and E7-expressing plasmid into cells. Whether they can transform the *in vivo* transfected cells, such as epithelial cells and Langerhans cells, is still an important issue to be studied. Genetic engineering of

E6 and E7 genes to inactivate their transforming functions may provide a solution to this potential problem.

Our results provide two important considerations for HPV vaccine development. First, vaccination with a combination of several early genes was an effective strategy for achieving complete protection in outbred populations. Second, gene gun-mediated intracutaneous genetic vaccination induced protective antipapillomavirus immunity. This strategy has great potential for therapeutic approaches to the treatment of HPV infections.

This study was supported by grant R01 CA47622 and the John G. Miller Memorial Golf Tournament. R. Han is the recipient of the 1994-1996 American Social Health Association/Marck Foundation Research Fellowship in sexually transmitted diseases.

REFERENCES

1. Bayn-Whitler, E., M. H. Stoler, F. Ghurfi, and E. P. Siller. 1990. Cell differentiation-related gene expression of human papillomavirus 33. *Mol. Microbiol. Immunol.* 17:185-192.
2. Bruchard, P., R. Kleinman, M. L. Hubbard, B. Naranjo, C. T. T. Chik-Dumoulin, G. Osh, J. T. Schiller, and D. R. Lowy. 1995. Immunization with virus-like particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J. Virol.* 69:3229-3233.
3. Campa, M. S. 1997. Vaccination against papillomavirus in cattle. *Clin. Dermatol.* 16:273-283.
4. Campa, M. S., G. J. Grindley, B. W. O'Neil, L. M. Choudhury, C. M. McGarvey, and W. F. R. Jervett. 1993. Prophylactic and therapeutic vaccination against a mucosal papillomavirus. *J. Gen. Virol.* 74:623-633.
5. Christensen, N. D., J. W. Kreider, N. C. Kim, and S. L. D'Aquila. 1991. The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inhibiting neutralizing epitopes. *Virology* 185:573-579.
6. Christensen, N. D., C. A. Reed, N. M. Chabot, R. Han, and J. W. Kreider. 1994. Immunization with virus-like particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomavirus. *J. Virol.* 70:889-894.
7. Cohen, C., B. C. Watkins, C. M. Collins, K. Thompson, and L. D. Fehn. 1994. DNA-based immunization by *in vivo* transfection of dendritic cells. *Nat. Med.* 2:1123-1128.
8. de Villiers, M.-E. 1997. Papillomavirus and HPV typing. *Clin. Dermatol.* 16:199-204.
9. Denny, J. J., D. Martinez, K. U. Jensen, R. W. Ellis, D. L. Montgomery, and M. A. Lin. 1994. Protection against papillomavirus with a polynucleotide vaccine. *J. Infect. Dis.* 170:314-320.
10. Gaudin, J., L. M. Choudhury, B. W. O'Neil, G. J. Grindley, G. Kramarz, and M. S. Campa. 1994. Vaccination of cattle with bovine papillomavirus type 4 L2 elicits the production of virus-neutralizing antibodies. *J. Gen. Virol.* 75:1577-1583.
11. Gherman, E. L., J. D. Walcott, K. E. de Vries, M. L. H. De Bruijn, R. Kleinman, R. B. Soden, D. R. Lowy, W. M. Kim, and J. T. Schiller. 1994. Cervical papillomavirus virus-like particles elicit antitumor immunity against the E7 oncoprotein in an HPV16 tumor model. *Proc. Natl. Acad. Sci. USA* 91:1820-1825.
12. Han, R., N. M. Chabot, C. Reed, and N. D. Christensen. 1999. Intramuscular injection of plasmid DNA encoding cottontail rabbit papillomavirus E1, E2, E6 and E7 induces cell-mediated but not humoral immune responses in rabbits. *Vaccine* 17:1328-1336.
13. Jensen, K. U., M. Naranjo, L. D. Schultz, H. E. Martins, J. C. Cook, J. J. Denny, D. Martinez, R. W. Ellis, and A. E. Shaw. 1993. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. *Vaccine* 11:1309-1314.
14. Jensen, E. B., R. Schvachman, E. Han, R. Ahmed, F. O. Wettstein, and J. F. Miller. 1997. Recombinant *Adenovirus* monovalent vaccination eliminates papillomavirus-induced tumors and prevents papilloma formation from viral DNA. *J. Virol.* 71:8467-8474.
15. Kleinman, R., L. M. Choudhury, B. W. O'Neil, E. R. Wagner, G. J. Grindley, A. Armstrong, G. M. McGarvey, J. T. Schiller, D. R. Lowy, and M. S. Campa. 1996. Virus-like particles of bovine papillomavirus type-4 in prophylactic and therapeutic immunization. *Virology* 219:37-44.
16. Kreider, J. W., and G. L. Bertlett. 1985. Shope rabbit papilloma-carcinoma complex—a model system of HPV infection. *Clin. Dermatol.* 3:20-34.
17. Kreider, J. W., N. M. Chabot, S. D. Patrick, P. A. White, S. L. D'Aquila, J. M. Seow, and N. D. Christensen. 1993. High efficiency infection of papillomas *in vivo* using recombinant cottontail rabbit papillomavirus DNA. *J. Virol. Methods* 55:333-344.
18. Lin, Y.-L., L. A. Saravanan, R. Ahmed, and F. O. Wettstein. 1993. Cottontail rabbit papillomavirus L2 protein-based vaccine: protection is achieved only with a full-length, nonrecombinant product. *J. Virol.* 67:4134-4142.

181
203

19. Lin, Y. L., L. A. Baranetska, R. Salzman, R. Ahmed, and F. O. Wettstein. 1992. Effective vaccination against papilloma development by immunization with L1 or L2 structural proteins of cottontail rabbit papillomavirus. *Virology* 189:612-619.
20. Montgomery, D. L., J. W. Stober, K. E. Lander, E. C. Perry, A. Friedman, B. Martin, J. B. Ulmer, J. J. Dannehy, and M. A. Lin. 1993. Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol.* 12:777-783.
21. Orth, G. 1987. *Epidemiology and prevention*, p. 199-243. In N. P. Salzman and P. M. Hawley (ed.), *The Papovaviridae*, vol. 2. The papillomaviruses. Plenum Press, New York, NY.
22. Pargader, A., K. E. Irvine, A. Ismail, E. H. Barlow, N. P. Restick, and E. H. Gorman. 1992. Proinflammatory role for directly transfected dendritic cells in antigen presentation to CD8⁺ cells after gene gun immunization. *J. Exp. Med.* 175:1575-1582.
23. Schmitt, J., M. Hanna, P. Cassinelli, G. Orth, and F. Breitburg. 1997. A cottontail rabbit papillomavirus strain (CRPV6) with strikingly divergent E5 and E7 open reading frames: an insight in the evolution of papillomaviruses. *Virology* 228:228-234.
24. Salzman, R., L. A. Baranetska, Y.-L. Lin, R. Ahmed, and F. O. Wettstein. 1993. Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. *J. Virol.* 67:602-605.
25. Stoler, M. H., C. E. Mouton, A. Woldrich, S. M. Wolford, L. T. Chow, and T. R. Broker. 1992. Human papillomavirus 16 and 18 gene expression in cervical neoplasia. *Hum. Pathol.* 23:117-128.
26. Sander, P., R. E. Tigelaar, and J. L. Brumson. 1997. Intranasal vaccination of rabbits with the cottontail rabbit papillomavirus (CRPV) L1 gene protects against virus challenge. *Vaccine* 15:664-671.
27. Sander, P., R. E. Tigelaar, W. Xing, and J. L. Brumson. 1998. Intranasal vaccination of rabbits with the E5 gene of cottontail rabbit papillomavirus provides partial protection against virus challenge. *Vaccine* 16:611-623.
28. Smith, J. A., S.-J. Ghim, F. J. Palmer-Hill, W. J. White, J. E. Towner, J. A. Hill, J. A. Newman, A. E. Jones, and R. Salzman. 1993. Systemic immunization with papillomavirus L1 proteins completely prevents the development of viral induced papillomas. *Proc. Natl. Acad. Sci. USA* 90:11553-11557.
29. Zehner, R., L. A. Baranetska, F. O. Wettstein, and T. Ulmer. 1994. Changes in RNA expression pattern during the malignant progression of cottontail rabbit papillomavirus-induced tumors in rabbits. *J. Virol.* 68:3620-3630.
30. van Haanen, H., and E.-M. de Villiers. 1994. Human papillomaviruses. *Annu. Rev. Microbiol.* 48:437-447.