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Generalized substitution of isoencoding codons shortens the duration of papillomavirus L1 protein expression in transiently gene-transfected keratinocytes due to cell differentiation

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ABSTRACT

Recently we reported that gene codon composition determines differentiation-dependent expression of the PV L1 genes in mouse primary keratinocytes (KCs) *in vitro* and *in vivo* (Zhao *et al*. 2005, Mol. Cell Biol. 25:8643–8655). Here, we investigated whether generalized substitution of isoencoding codons affects the duration of expression of PV L1 genes in mouse and human KCs in day 1 culture transiently transfected with native (*Nat*) and codon modified (*Mod*) L1 genes. Following transient transfection, KC continuously transcribed both *Nat* and *Mod* PV L1 genes for at least 12 days, with the levels of L1 mRNAs from the *Mod* L1 genes significantly higher than those from the *Nat* L1 genes. However, continuous L1 protein expression at day 9 post-transfection was observed for both mouse and human KCs transfected with the *Nat* L1 genes only. Further, aa-tRNAs prepared from D8 KC cultures enhanced translation of two PV *Nat* L1 DNAs in RRL lysate and PV *Nat* L1 mRNAs in D0 cell-free lysate, whereas aa-tRNAs from D0 KCs enhanced translation of PV *Mod* L1 mRNAs in D8 cell-free lysate. It appears that aa-tRNAs in less-differentiated and differentiated KCs differentially match the PV *Nat* and *Mod* L1 mRNAs to regulate their translations *in vitro*.

INTRODUCTION

The standard version of the genetic code includes 61 sense codons and 3 stop codons. The 61 sense codons in an mRNA molecular are translated to code 20 amino acids in the course of protein synthesis. Except for methionine and tryptophan, the 18 most common amino acids are coded by two to six codons called synonymous codons. Previous studies have clearly indicated that synonymous codons are used with unequal frequency in a protein-coding sequence and choice of the synonymous codons is far from random both within and between organisms (1–3). Consequently, the patterns of codon usage vary considerably among organisms, and also among genes from the same genome (4). Codon usage has been known to present a potential impediment to high-level gene expression in *Escherichia coli* and yeast (5,6). In humans, codon-mediated translational controls may play an important role in the differentiation and regulation of tissue-specific gene products (7).

Virus genomes frequently have their codon usage significantly different from their host species (8,9). We observed that papillomaviruses (PVs), like many mammalian DNA viruses, have a significantly greater usage of codons with third position of A/T, manifesting a A + T rich genome (10). Meanwhile, PV late (L1 and L2) genes frequently use the codons such as UUG, CGU, ACA and AUU that are rarely used in mammalian genes (10,11). Thus, when PV L1 and L2 genes are transfected into a wide range of eukaryotic cells, large amounts of the mRNAs can be transcribed, but no protein product is detected (12), which indicates that expression of the late genes is subject to post-transcriptional regulation (13). In HPVs, generalized substitution of isoencoding codons (mammalian preferred codons) with a higher G + C content allows expression of L1 and L2 proteins in different types of eukaryotic cells *in vitro* (10,14–17). The poor expression of several viral and other proteins has also been attributed to the unfavourable codon usage of their genes (18–20). The codon usage of the native GFP gene is not adapted to mammalian 'consensus' and

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unmodified GFP is poorly expressed in mammalian systems(18,21). Modification of the codon usage of HIV-1 genes to those used by highly expressed human genes has been found to significantly increase HIV-1 structural protein expression (22).

It is clear now that expression of hetero genes at translational levels can be significantly increased by synonymous codon substitution (18–20). However, we observed that the effects of synonymous codon substitution on translation of the PV L1 mRNAs in the KC cultures were dependent on the cell differentiation (23). In our study, the transiently transfection assay of the PV L1 gene expression in KC cultures was conducted at 48 h post-transfection as expression of a targeted gene has been generally overlooked in favour of early time points in other studies (24,25). In a recent study, we observed that hm *gfp* variants in which were introduced with different sets of six consecutive codons down stream the AUG codon show different mRNA translation efficiency and have different duration of the GFP expression in transiently GFP plasmids-transfected mammalian cells. (10,26). Thus, it is possible that generalized substitution of synonymous codons may improve not only the translation efficiency of the viral genes, but also change the duration of its expression in KCs post-transfection. In this article, we address the following issues: (i) how long expression of the PV L1 genes can be detected in primary mouse and human KC cultures following transient transfection of the *Nat* and *Mod* PV L1 gene expression constructs; (ii) whether generalized substitution of isoencoding codons affects the duration of the L1 gene expression due to cell differentiation; (iii) if cell-free lysates prepared from the mouse KC cultures can be used for translation of both *Nat* and *Mod* PV L1 mRNAs *in vitro* and (iv) whether supplementation of exogenous aa-tRNAs affects expression of *Nat* and *Mod* L1 DNAs and mRNAs in different cell-free *in vitro* translation systems.

MATERIALS AND METHODS

Plasmid construction and preparation

The four PV L1 gene expression plasmids: pCDNA3 *Nat* HPV6b L1, pCDNA3 *Mod* HPV6b L1, pCDNA3 *Nat* BPV1 L1 and pCDNA3 *Mod* BPV1 L1 used in the experiments have previously been described (10,15). Briefly, both BPV1 and HPV6b wt L1 ORFs are approximately 1.5 kb in length encoding 500 amino acids. The PV wt L1 genes show a strong codon usage bias, amongst degenerately encoded amino acids, toward 18 codons mainly with T at the third position that are otherwise rarely used by mammalian genes (10,11). We artificially modified BPV1 and HPV6b L1 genes such that the L1 ORFs were substituted with codons having G or C at the third position, which are preferentially used in the mammalian genome (10). All of the native and codon modified PV L1 ORFs were sequenced and cloned into the mammalian expression vector $pCDNA_3$, which contains the simian virus 40 (SV40) ori (Invitrogen, Australia), to give four expression plasmids $pCDNA_3$HPV6b *Nat* L1, $pCDNA_3$HPV6b *Mod* L1, $pCDNA_3$BPV1 *Nat* L1 and $pCDNA_3$BPV1 *Mod* L1. Correct orientation of the ORFs relative to the plasmids was confirmed by enzyme restriction analysis. All the PV L1 constructs were sequenced to confirm the desired sequences before use.

Primary mouse and human KC culture and DNA transfection

Mouse KCs were isolated from new born mouse skin as originally described (27) with some modifications. Briefly, isolated KCs were grown as adherent cultures at a density of 6.5×10^4 cells/cm^2 in freshly prepared 3:1 medium as previously described (23) for one day. The KC cultures were then transfected with the PV L1 gene expression constructs (pCDNA3 *Nat* HPV6b L1, pCDNA3 *Mod* HPV6b L1, pCDNA3 *Nat* BPV1 L1 and pCDNA3 *Mod* BPV1 L1) (10,28) using lipofectamine (Invitrogen, Australia) according to the manufacturer's protocol with some modifications. Following transfection, the DNA-transfected KCs continued to grow in KC-SFM medium with low calcium (0.09mM) and was collected for RNA and protein preparation at days 3 (D3), 6 (D6), 9 (D9) and 12 (D12) post-transfection.

Human KCs isolated from neonatal foreskins were cultured in KC serum-free medium (Life Technologies, Australia) up to confluence. The KCs were then passaged at a density of 6.5×10^4 cells/cm^2. After one day, the human KCs were transfected with the four PV L1 gene expression constructs as described above. Following transfection, the DNA-transfected KCs continued to grow in KC-SFM medium with low calcium (0.09mM) and collected for RNA and protein preparation at days 3 (D3), 6 (D6) and 9 (D9) post-transfection.

Quantitative reverse-transcriptase PCR (RT-PCR)

Here. 0.5 μg of RNA purified from cultured KCs transfected with the different PV L1 gene expression constructs was converted to complementary DNA using Oligo-dT primers and PowerScript reverse transcriptase (Promega, Australia) according to the manufacturer's protocol. We used 50 ng of each cDNA sample in 20 μl RT-PCR reactions using a Promega kit (Promega, Australia) supplemented with 3 mM $MgCl_2$ and Platinum Taq Polymerase (Invitrogen, Australia). RT-PCR was undertaken using the Taqman system (AB Applied Biosystems, Australia).

Protein preparation and Western blot analysis

The L1-DNA-transfected KCs were lysed in cell lysis buffer containing 2 mM of PMSF and sonicated for 40 s. Forty microgram (40 μg) protein samples from whole-cell extracts were separated by SDS-PAGE and blotted onto PVDF membrane. The blots were first probed with monoclonal antibody (29) against PV L1 protein, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma, Australia). A chemiluminescence system (ECL kit; Amersham, Australia) was used to visualize the L1 signals. After probing for the L1 protein, the blots were stripped and relabeled with antibody against β-tubulin.

De novo Synthesis of L1 and cellular proteins

De novo synthesis of the PV L1 proteins in cultured mouse KCs was studied by labeling with [^{35}S]-methionine. KCs cultured in six-well plates for one day (D1) were transfected with the PV L1 gene expression constructs (pCDNA$_3$ *Nat* HPV6b L1, pCDNA$_3$ *Mod* HPV6b L1, pCDNA$_3$ *Nat* BPV1 L1 and pCDNA$_3$ *Mod* BPV1 L1) using lipofectamine (Invitrogen, Australia) as previously described (23). After transfection, L1 gene-transfected KCs continued to be grown in KC-SFM medium and at D2, D5 and D8 post-transfection were incubated in 2 ml of medium supplemented with 10 μCi of L-[^{35}S]Met (370 kBq) overnight. L1 gene-transfected KCs labelled with [^{35}S]-methionine at D3, D6 and D9 post-transfection were collected for protein analysis. Forty microgram (40 μg) protein samples, each in 1 ml of immunoprecipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethane sulphonyl fluoride (PMSF) (Sigma, Australia), 2 μg/ml benzamidine (Sigma, Australia), aprotinin 2 μg/ml and 1 μg/ml leupeptin (Auspep, Australia)) were immunoprecipitated using antibody against the L1 protein and then separated by SDS-PAGE. The SDS-PAGE gel was dried and autoradiographed.

Synthesis of two cellular proteins involucrin and tubulin in mouse KC cultures over time were also examined.

Immunofluorescence labeling

In parallel experiments, intracellular localization of the PV L1 proteins in mouse KC cultures were examined. KCs cultured for one day were transfected with each of the four PV L1 plasmids, and then fixed and permeabilized with 85% ethanol at 3 and 9 day post-transfection. The fixed KCs were then blocked with 5% skim milk-PBS, probed with monoclonal antibody against the PV L1 protein, and followed by Cy3-conjugated anti-mouse immunoglobulin G (IgG) (Sigma, Australia). The L1 labeled KCs were further blocked with 5% skim milk PBS and probed with polyclonal antibody against involucrin (Sigma, Australia), followed by FITC secondary antibody. Nuclei were countstained by 4′,6′-diamidino-2-phenylindole (DAPI). Antibody-labeled KCs were examined by immunofluorescence microscopy.

Cell-free *in vitro* translation of PV L1 plasmid DNAs in RRL system

One microgram of each plasmid DNA was incubated with 10 μCi of [^{35}S]methionine (Amersham, Australia) and 20 μl of T7 DNA-polymerase-coupled rabbit reticulocyte lysates (RRL) (Promega, Australia), with or without additional aa-tRNAs as indicated. Translation was performed at 30°C from 15 to 45 min and stopped by adding 2× SDS sample loading buffer. The L1 proteins were separated by SDS-PAGE on a 10% gel and blotted onto PVDF membrane. The blots were imaged using a phosphor screen and quantified by PhosphorImager analysis using the Imagequant program (Molecular Dynamics, USA).

Preparation of KC cell-free lysate for *in vitro* translation of the PV L1 mRNAs

Cell-free lysate was prepared from the mouse primary KCs cultured for one, three and eight days as described previously (30) with some modifications. Briefly, the cultured KCs were scraped into an extraction buffer containing 100 mM Hepes-KOH (pH 7.4), 120 mM potassium acetate (pH 7.4), 2.5 mM magnesium acetate, 1 mM dithiotreitol, 2.5 mM ATP, 1 mM GTP, 100 μM S-adenosyl-methionine, 1 mM spermidine, 20 mM creatine phosphate, 40 U of creatine phosphokinase (Sigma, Australia) per ml, and, importantly, an additional 100 mM sucrose. The collected KCs were then passed through a 25-gauge needle ten times, and the lysate was centrifuged at 4°C and 100 *g* for 2 min. The supernatant was collected to prepare the mRNA-dependent KC cell-free lysate. The endogenous mRNAs in the prepared KC cell-free lysate were hydrolysed by incubating the lysate at 20°C for 10 min in the presence of 10 U/per ml of micrococcal nuclease (31) and 1 mM $CaCl_2$. The enzyme was inhibited by adding 2.5 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, pH 7.0). The cell-free lysate was supplemented with 0.02 mM hemin before use.

In vitro translation of the PV L1 mRNAs in KC cell-free lysate system

In vitro transcription. The four PV L1 gene expression plasmids: pCDNA$_3$ HPV6b *Nat* L1, pCDNA$_3$ HPV6b *Mod* L1, pCDNA$_3$ BPV1 *Nat* L1 and pCDNA$_3$ BPV1 *Mod* L1 were linearized with EcoRI and transcribed by T7 RNA polymerase (Promega, Australia) at 37°C for 3 h. The transcription reaction contained 5 mM m7 GpppG to synthesize capped PV L1 mRNAs. Following transcription, 2 μl RNase-free DNase (2000 U/ml, Promega, Australia) was added into each reaction to digest the DNA template at 37°C for 1 h. The transcribed PV L1 mRNAs were then purified by phenol–chloroform extraction, followed by precipitation with 2 volumes of ethanol and washed twice with 70% ethanol. The purified PV L1 mRNAs resuspended in RNase-free water were used for *in vitro* translation.

In vitro translation. The *in vitro* translation mixture consisted of 17.5 μl KC cell-free lysate, 2 μl PV L1 mRNA (1 μg per reaction), 0.5 μl RNase inhibitor, 0.5 μl amino acid mixture (1 mM each amino acid) and 4.5 μl RNase-free H_2O. Translation reaction was carried out at 30°C for 1–3 h. The reactions were stopped with the addition of 2× SDS-sample buffer and then denatured at 80°C for 10 min. The translated PV L1 proteins were analysed by SDS-PAGE and blotted on PVDF membrane. The blots were examined for the PV L1 proteins by immunoblotting analysis using the monoclonal antibody against L1 proteins as mentioned above. The L1 signals were quantified by densitometric analysis using the Imagequant program (Molecular Dynamics, USA).

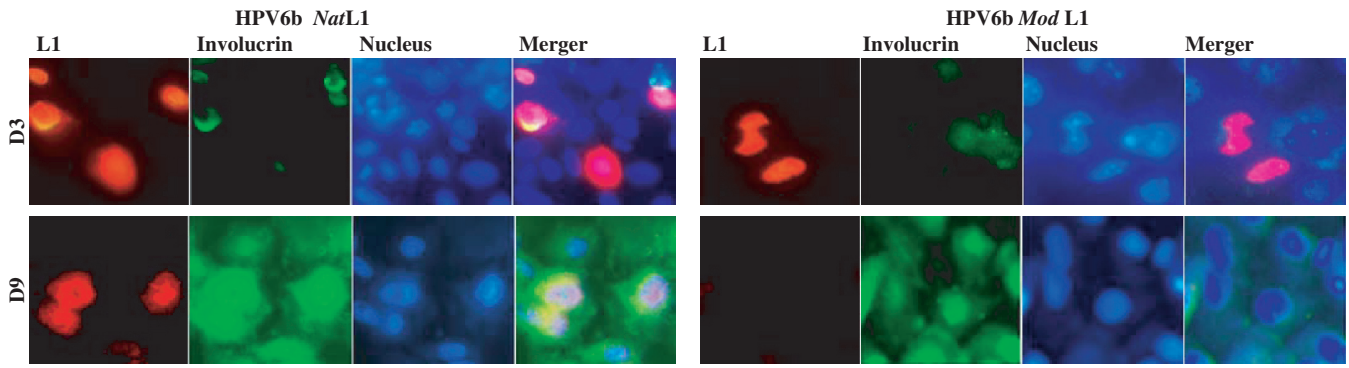

Figure 1. Expression of the HPV6b L1 protein in individual KCs. Mouse KCs grown in medium for 1 day were transfected with an expression construct for a HPV6b *Nat* or *Mod* L1 gene as indicated. At 3 and 9 days post-transfection, the KCs were stained by indirect immunofluorescence for HPV6b L1 protein (red) and for involucrin (green), and nuclei counterstained with DAPI (blue). Merger panes combine all three images. All pictures ×400.

Preparation of aa-tRNAs for *in vitro* translation

Total tRNAs were extracted and purified from KC cultures using a Qiagen kit as instructed by the supplier (17). aa-tRNAs were produced for addition to either RRL system or KC lysate system as previously described (10). tRNAs (2.5×10^{-5} M) purified from D0 and D8 mouse and human KC cultures were added to a 50 µl reaction mixture containing 10 mM Tris-acetate (pH 7.8), 44 mM KCl, 12 mM $MgCl_2$, 9 mM β-mercaptoethanol, 38 mM ATP, 0.25 mM GTP and 15 µl of rabbit reticulocyte extract. The reaction was carried out at 25°C for 20 min and 30 µl of H_2O was then added to the reaction to dilute the tRNAs to 10^{-5} M. The aa-tRNAs were aliquoted and stored at −70°C for later use.

RESULTS

Codon modification alters continuous expression patterns of the PV L1 genes in both mouse and human KCs

Recently, we reported that expression of the PV L1 proteins from *Nat* PV L1 genes is only detected in differentiated KCs and that the codon composition of the *Nat* L1 genes determines the differentiation-dependent expression of the L1 proteins (23). Here, we have examined whether continuous expression of L1 protein, as observed *in vivo*, can be recapitulated *in vitro* and how the gene codon composition mediates the continuous expression of the L1 genes. We transiently transfected mouse KCs that had been cultured for one day (D1 cultured KCs) with the PV *Nat* and *Mod* L1 gene expression constructs. The L1-tranfected mouse KCs were collected for RNA and protein preparation at D3, D6, D9 and D12 post-transfection. Progressive changes of the cell morphology of the L1-transfected mouse KCs from D3 to D12 post-transfection were examined by light microscopy (data not shown). The morphological changes observed for the L1-transfected KCs over time reflected cell differentiation that was confirmed by the increased expression of involucrin that is a marker of keratinocyte terminal differentiation (32,33) using immunofluorescence microscopy (Figure 1) and [^{35}S]-methionine labeling experiments. We have also used immunofluorescence microscopy to investigate whether and how the L1 proteins encoded by the HPV6b *Nat* and *Mod* L1 genes were expressed in the L1-transfected KCs at D3 and D9 post-transfection (Figure 1). The L1 protein expressed from the *Nat* L1 gene was detected in the L1-transfected KCs at both D3 and D9, but from the *Mod* L1 genes only at D3 post-transfection (Figure 1). Meantime, expression of involucrin in L1-transfected KCs at both D3 and D9 post-transfection was examined (Figure 1). 20–30% of the L1-transfected KCs at D3 post-transfection exhibited weak involucrin signal, while all the L1-transfected KCs at D9 post-transfection had strong involucrin expression, suggesting that they proceed cell differentiation. (Figure 1).

The transcripts of both *Nat* and *Mod* PV L1 genes were then analysed by quantitative RT-PCR. Gel electrophoresis revealed that the L1 mRNAs can be detected in KCs up to 12 days post-transfection (Figure 2A). Given that the reported half life of the PV L1 mRNAs was approximately 5 h in mammalian cells (34) and mouse KCs (23), the results suggest that the cultured KCs can continuously transcribe the transiently transfected PV L1 genes over the time period examined. Quantitative RT-PCR analysis revealed that transcription of both *Nat* and *Mod* PV L1 genes was distinctly down-regulated in the L1-transfected KCs as the cell differentiated. Meantime, the levels of L1 mRNAs from the *Mod* L1 genes were significantly higher than those from the *Nat* L1 genes over the time course (Figure 2B), suggesting that replacement of the GC- ending codons in the PV L1 genes can improve their transcription efficiencies in KCs. In contrast, western blot analysis showed that the detected signals of the L1 proteins expressed from the two PV *Nat* L1 genes increased significantly over time, with the highest levels of the L1 proteins detected at D9 post-transfection (Figure 2B). Steady expression of the L1 proteins from the two PV *Mod* L1 genes was detected at D3 and D6 (Figure 1B) after which expression of the L1 proteins decreased dramatically and was not detectable in the HPV6b *Mod* L1 transfected KCs at D12 post-transfection (Figure 2B). The results suggest that the PV L1 proteins can be continuously detected in L1-transfected KCs for

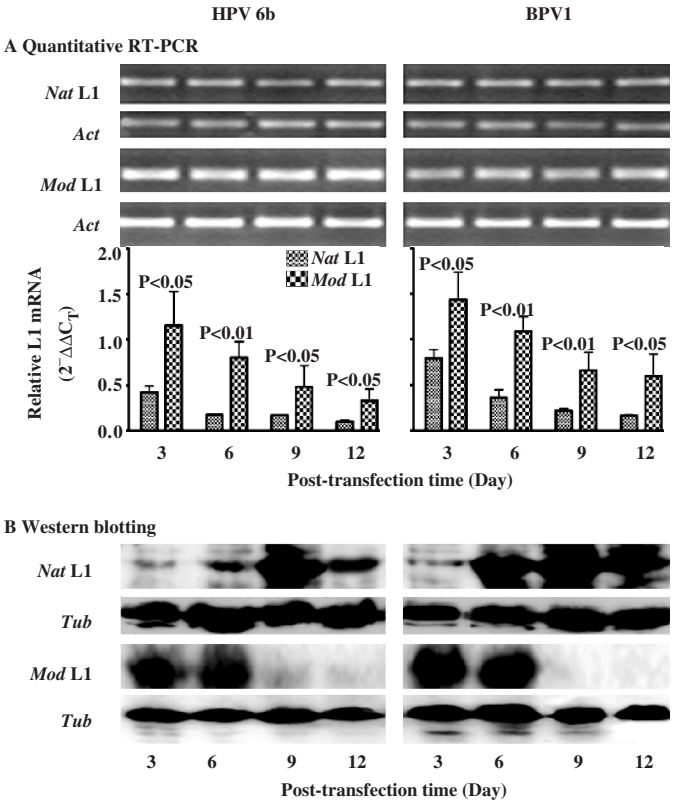

Figure 2. Time course assay for continuous expression of both *Nat* and *Mod* PV L1 genes in cultured mouse KCs. Mouse KCs grown in medium for 1 day were transiently transfected with the four L1 gene expression constructs for 16 h and then collected for analysis of the L1 gene expression for a 12-day period post-transfection. **(A)** Quantitative RT-PCR analysis of L1 mRNA in cultured KC cells. RNA samples were prepared at D3, D6, D9 and D12 post-transfection. 0.5 μg of each sample of DNase I-digested total RNA was used for RT-PCR. *Upper panel*: The RT-PCR products from L1 and actin mRNAs were electrophoresed on a 1% agarose gel. *Lower panel*: Relative L1 mRNAs are shown with the means (± S.E.M) of four separate assays from two independent experiments. Statistical analysis of the results was conducted. $P<0.01$ and $P<0.05$ represent the significant degree of the differences between *Nat* and *Mod* L1 mRNAs using *t* Test, respectively. **(B)** Western blotting analysis of L1 protein and β-tubulin in cultured mouse KCs. Monoclonal antibody against L1 protein was used to probe the blot. Upper panel shows the results of the L1 immunoblotting assay; lower panel shows the signals of β-tubulin showing comparable loading of the protein samples.

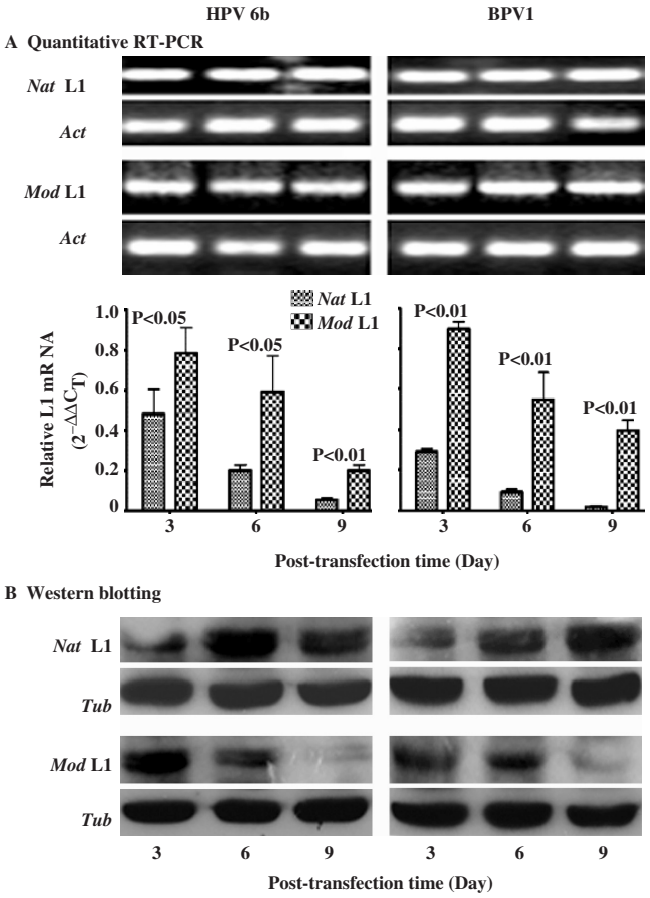

Figure 3. Time course assay for continuous expression of both *Nat* and *Mod* PV L1 genes in cultured human KCs. KCs at a density of 6.5×10^4 cells/cm^2 in medium grown for 1 day were transiently transfected with the four L1 gene expression constructs for 16 h and then collected for analysis of the L1 gene expression for a 9-day period post-transfection. **(A)** Quantitative RT-PCR analysis of L1 mRNA in cultured KC cells. RNA samples were prepared at D3, D6 and D9 post-transfection. 0.5 μg of each sample of DNase I-digested total RNA was used for RT-PCR. *Upper panel*: The RT-PCR products from L1 and actin mRNAs were electrophoresed on a 1% agarose gel. *Lower panel*: Relative L1 mRNAs are shown with the means (± S.E.M) of four separate assays from two separate transfections. Statistical analysis of the results was conducted. $P<0.01$ and $P<0.05$ represent the significant degree of the differences between *Nat* and *Mod* L1 mRNAs using *t* Test, respectively. **(B)** Western blotting analysis of L1 protein and β-tubulin in cultured human KCs. Monoclonal antibody against L1 protein was used to probe the blot. Upper panel shows the results of the L1 immunoblotting assay; lower panel shows the signals of β-tubulin showing comparable loading of the protein samples.

a certain period, and that codon composition of the L1 mRNA plays a key role in regulation of the continuous expression of the L1 proteins.

Considering that human KCs are the host cells of HPV infection, we also investigated the expression of the four PV L1 gene expression constructs in primary human KC culture. Both L1 mRNA transcription examined by quantitative RT-PCR and protein expression analysed by western blot were investigated. Similar results were obtained (Figure 3A and B). Thus, the data produced from both mouse and human primary KCs suggest that the continuous expression of the PV L1 proteins is also post-transcriptionally regulated and associated with cell differentiation.

Synthesis of the PV L1 proteins from the PV *Mod* L1 genes diminishes with time in cultured KCs

To investigate whether the PV L1 proteins continuously detected from both *Nat* and *Mod* L1 genes in L1-transfected KCs over the time course are due to continuous synthesis of the L1 proteins, rather than their intrinsic stability in cultured KCs. The L1-transfected mouse KCs maintained in normal KC medium were labeled overnight with [^{35}S]-methionine at D2, D5 and D8 post-transfection, respectively. The extended labeling time is required to incorporate sufficient radioactivity into the

newly synthesized proteins to be detected. Following [^{35}S]-methionine labeling, the L1-transfected KCs were collected at D3, D6 and D9 post-transfection and analysed for the synthesis of involucrin, β-tubulin and L1 protein (Figure 4A and B). Immunoprecipitation of the methionine-labeled involucrin and β-tubulin showed that the level of methionine-labeled involucrin steadily increased in cultured KCs over time accompanied by a slight decrease of the β-tubulin synthesis observed in the D9 KC culture (Figure 4A). The increasing expression of the involucrin over time reflects the L1-transfected KC cultures' proceeding cell differentiation.

Immunoprecipitation of the methionine-labeled L1 proteins showed levels of the newly synthesized L1 proteins expressed from the two PV *Nat* L1 genes were low at D3 (Figure 4B), but were significantly up-regulated at D9. In contrast, the L1 protein synthesis in the KCs transfected with the two PV *Mod* L1 expression constructs was active at D3 post-transfection (Figure 4B). The synthesis of the L1 protein by the HPV6b *Mod* L1 expression construct was active in the L1-transfected KCs at D6, but not detectable at D9 post-transfection (Figure 4B). The L1 protein synthesis in the KCs transfected with the BPV1 *Mod* L1 expression constructs was significantly down-regulated from D3 to D9 post-transfection. To compare the relative levels of the newly synthesized L1 proteins from the PV *Nat* L1 gene expression constructs to those from the PV *Mod* L1 gene expression constructs over the time course, we examined the ratio of the newly synthesized L1 protein to the tubulin (L1/tubulin) by densitometric analysis of L1 protein and β-tubulin signals (Figure 4C). The ratios of the L1/tubulin produced from the PV L *Nat* 1-transfected KCs were 0.66 ± 0.15 for HPV6b and 0.66 ± 0.04 for BPV1 at D3 post-transfection and 0.92 ± 0.25 for HPV6b and 0.76 ± 0.01 for BPV1 at D6 post-transfection, but were significantly increased up to 1.65 ± 0.16 for HPV 6b and 1.75 ± 0.55 for BPV1 at D9 post-transfection. In contrast, the ratios of the L1/tubulin produced from the *Mod* PV L1-transfected KCs were 1.70 ± 0.02 for HPV 6b and 1.34 ± 0.03 for BPV1 at D3 post-transfection and 1.87 ± 0.39 for HPV 6b and 0.82 ± 0.05 for BPV1 at D6 post-transfection, but were significantly decreased to 0.09 ± 0.01 for HPV 6b and 0.29 ± 0.02 for BPV1 at D9 post-transfection. The ratios between PV *Nat* L1/tubulin and PV *Mod* L1/tubulin were significantly different over the time course, except for BPV1 L1 at D6 post-transfection (Figure 4C). The data suggest that the detected continuous expression of the L1 proteins from the PV L1 genes (Figure 4C) was due to the continuous synthesis of the L1 proteins and that codon composition of the L1 mRNA sequences was a major determinant of the continuous synthesis of the L1 proteins.

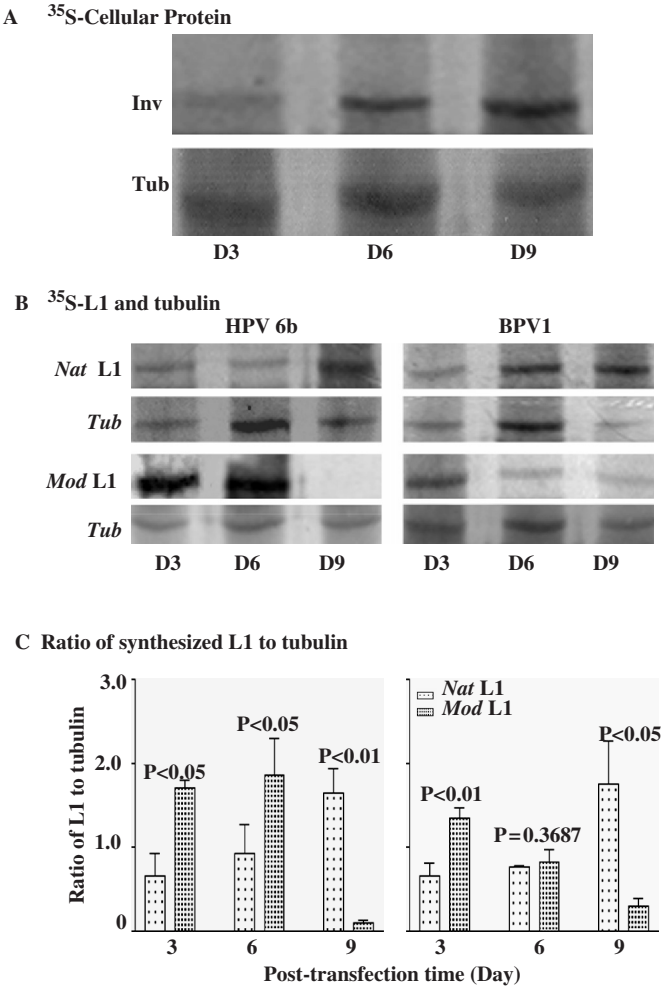

Figure 4. Continuous synthesis of L1 proteins studied by labeling with [^{35}S]-methionine. Cultured mouse KCs at D1 were transiently transfected with 2 μg of the PV L1 gene constructs for 16 h. The PV L1 gene-transfected KCs were then incubated in 2 ml normal KC culture medium labeled with 10 μCi of L-[^{35}S]Met (370 kBq) at D2, D5 and D8 post-transfection. Protein samples were collected for analysis of L1 protein and β-tubulin incorporated with [^{35}S]-methionine at D3, D6 and D9 post-transfection. Forty μg protein samples, in 1 ml of immunoprecipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with protease inhibitors and phosphatase inhibitors), were immunoprecipitated by monoclonal antibodies against either L1 protein or β-tubulin or by polyclonal antibody against involucrin, separated by SDS-PAGE and autoradiographed for 48 h. **(A)** The results of both involucrin and β-tubulin synthesis are representative of two independent experiments. **(B)** The results of both L1 and β-tubulin synthesis are representative of two independent experiments. **(C)**. Ratio of synthesized L1 protein to β-tubulin (L1/tubulin) was examined by densitometric analysis of L1 protein and β-tubulin signals from two independent experiments. Statistical analysis between *Nat* L1/tubulin and *Mod* L1/tubulin at individual time points was conducted using paired *t* test. $P < 0.05$ or $P < 0.01$ was compared between *Nat* L1/tubulin and *Mod* L1/tubulin as shown.

aa-tRNAs from D8 mouse KC cultures enhance translation of the PV *Nat* L1 genes in RRL translation system

Recently, we showed that translation of L1 from PV *Nat* L1 genes in a RRL cell-free translation system was preferentially enhanced by aa-tRNAs from differentiated KCs *in vivo* (23). Here, we have examined whether and how aa-tRNAs prepared from the mouse KCs cultured for one (D0) or eight (D8) days affect translation of L1 proteins from *Nat* L1 genes in the RRL cell-free translation system. We showed that translation of the L1 proteins from PV *Nat* L1 genes was much slower than

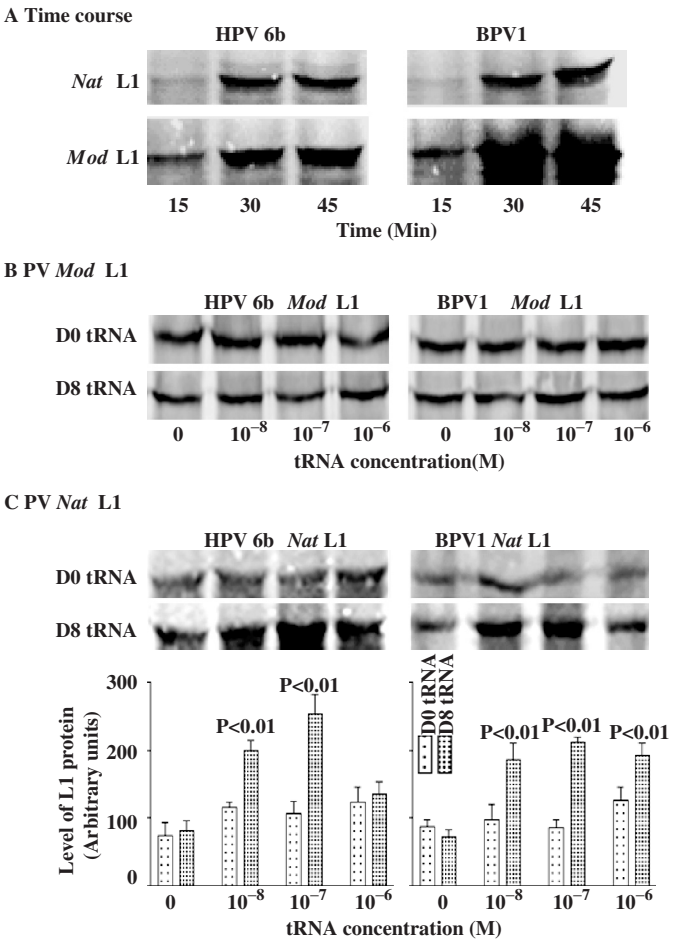

Figure 5. Translation of PV *Nat* L1 genes is preferentially enhanced by aa-tRNA from D8 cultured mouse KCs in a rabbit reticulocyte lysate cell-free *in vitro* translation system. **(A)** Both *Nat* and *Mod* HPV6b and BPV1 L1 genes from were translated in a cell-free system using rabbit reticulocyte lysate (RRL) in the presence of [^{35}S]methionine over a time course from 15 to 45 min. Production over time of L1 protein from *Nat* L1 genes (HPV6b or BPV1) is much slower than from *Mod* L1 genes. **(B)** Supplementation of the *in vitro* translation reaction with aa-tRNA from D0 or D8 cultured KC did not affect L1 production from *Mod* PV L1 genes at 15 min. **(C)** Supplementation of the *in vitro* translation reaction with aa-tRNA from D0 or D8 cultured KC enhances L1 production from native L1 genes in a dose-dependent manner from 10^{-8} to 10^{-6} M at 15 min: inhibition of translation is evident at the highest concentrations of aa-tRNA. *Upper panel:* representative western blots of three independent experiments. *Lower panel:* histograms showing means (± S.E.M.) of L1 protein production from six individual blotting assays from three independent experiments quantified by use of a PhosphorImager. Statistical analysis of the results from individual tRNA concentrations from these experiments was conducted using paired *t* test. $P < 0.01$ was compared between D0 and D8 aa-tRNA treatments as shown.

that from the PV *Mod* L1 genes over a time course of 15–45 min (Figure 5A). Supplementation of the aa-tRNAs from D0 or D8 KCs had no distinct effects on the translation of the *Mod* L1 genes (Figure 5B). In contrast, introduction of exogenous aa-tRNAs from D0 KCs did not enhance translation of PV *Nat* L1 genes, but addition of exogenous aa-tRNAs from D8 KCs significantly enhanced translation of *Nat* PV L1 genes in a dose-dependent manner with optimum efficiency at 10^{-7}M (Figure 5C). Addition of 10^{-7}M aa-tRNA from D8 cultured KCs produced strong L1 bands from both PV L1 genes at 15 min, while the corresponding L1 bands from the control reactions without added aa-tRNAs were weak (Figure 5C). It was observed that 10^{-7}M aa-tRNAs from D8 cultured KCs gave enhanced translation of L1, 1.59 ± 0.6-fold for HPV6b and 2.09 ± 0.8 for BPV1 (Figure 5C, lower panel). The results suggest that the improved translation of native L1 mRNA and inhibition of the translation of codon modified L1 mRNA in differentiated epithelial cells is probably due to an association of gene codons with the cellular aa-tRNAs as the tRNA gel profiles were different between D0 and D8 cultured KCs (unpublished data).

aa-tRNAs from D0 and D8 human KC cultures differentially enhance translation of the PV *Mod* and *Nat* L1 genes in RRL translation system

To prove that aa-tRNAs from the human KCs would have similar effects, we have examined further how the tRNAs (10^{-7} M) prepared from both D0 and D8 human KC cultures affected translation of the L1 proteins from the PV *Nat* and *Mod* L1 genes in the RRL cell-free translation system. Again, introduction of exogenous 10^{-7} M aa-tRNAs from D8 human KCs dramatically increased the production of L1 protein from the two PV *Nat* L1 gene constructs while they had few effects on expression of the two PV *Mod* L1 genes (Figure 6B). Surprisingly, supplementation of 10^{-7} M aa-tRNAs from D0 human KCs could significantly enhance translation of the two PV *Mod* L1 genes, but not of the two PV *Nat* L1 genes (Figure 6A). The results indicate that aa-tRNAs from D0 and D8 human KC cultures differentially enhance translation of the PV *Mod* and *Nat* L1 genes in RRL translation system.

In vitro translation of the PV *Nat* and *Mod* L1 mRNAs differs in cell-free lysates prepared from the mouse KC cultures

Next, we developed a cell-free lysate translation system from the primary mouse KC cultures to investigate whether and how the PV L1 mRNAs that were *in vitro* transcribed could be translated *in vitro*. We observed that the PV L1 mRNAs, whether they were transcribed *in vitro* from the *Nat* or *Mod* PV L1 genes, could be *in vitro* translated in the KC cell-free lysates when the L1 mRNAs were capped with m7 GpppG and the endogenous mRNAs in the cell-free lysates were completely hydrolysed (Figure 7). We observed further that the PV *Nat* L1 mRNAs could be *in vitro* translated in the D0 lysate, but the translational efficiencies were distinctly lower than those in the D3 and D8 lysates (Figure 7). In contrast, although the PV *Mod* L1 mRNAs could be translated *in vitro* both in the D0 and D8 lysates (Figure 7), the translational efficiencies of the L1 proteins from the PV *Mod* L1 mRNAs in the D8 lysate were dramatically decreased (Figure 7). The data suggest that the PV *Nat* L1 mRNAs have the translational preference to the cell-free lysates prepared from the differentiated mouse KC

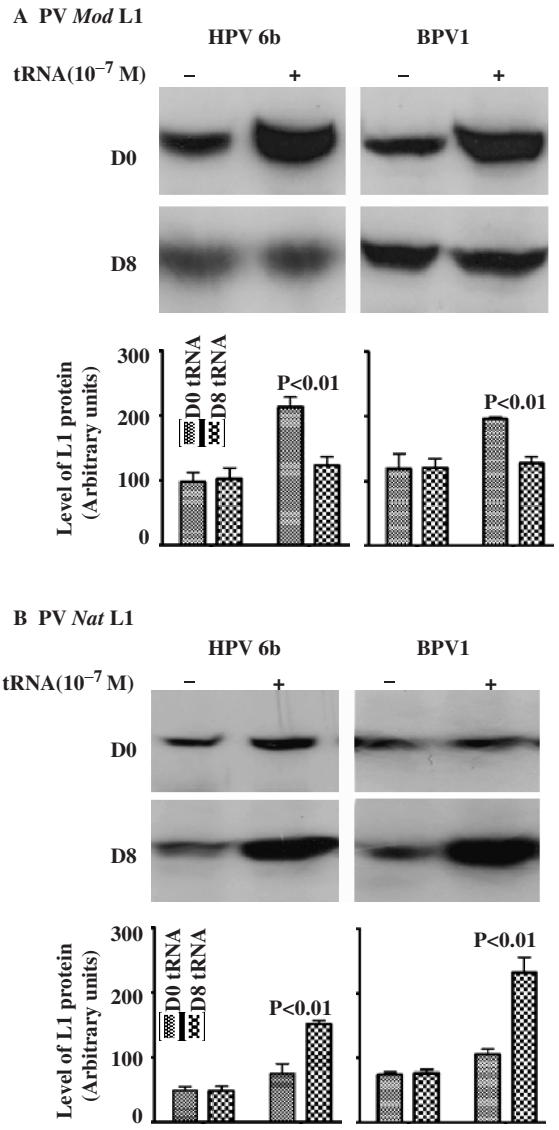

Figure 6. Translation of PV *Nat* and *Mod* L1 genes is differentially enhanced by aa-tRNA from D0 and D8 human KC cultures in a rabbit reticulocyte lysate cell-free *in vitro* translation system. **(A)** Supplementation of the *in vitro* translation reaction with aa-tRNA (10^{-7} M) from D0 cultured human KCs increased L1 production from *Mod* PV L1 genes at 20 min. **(B)** Supplementation of the *in vitro* translation reaction with aa-tRNA (10^{-7} M) from D0 or D8 cultured KC enhances L1 production from PV *Nat* L1 genes at 20 min. *Upper panel:* representative immunoblots of two independent experiments. *Lower panel:* histograms showing means (± S.E.M.) of L1 protein production from three individual immunoblotting assays from two independent experiments by use of a monoclonal antibody against the L1 protein. Statistical analysis of the results from these experiments with or without supplementation of aa-tRNAs was conducted using paired *t* test. $P<0.01$ was compared between D0 and D8 aa-tRNA treatments as shown.

cultures, while the PV *Mod* L1 mRNAs prefer to translate the L1 proteins in the cell-free lysate prepared from less-differentiated mouse KC cultures. The results are consistent with the western blot analysis of continuous expression of the PV L1 proteins from both *Mod* and *Nat* L1 genes in L1-transfected KCs (Figure 2B).

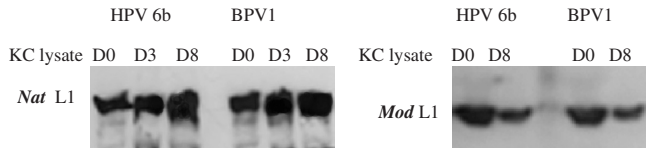

Figure 7. Translation of PV *Nat* and *Mod* L1 mRNAs is different among the KC lysates from the primary mouse KCs cultured for different period (D0, D3 and D8). 1 μg of each of the PV L1 mRNAs was used for the *in vitro* translation reaction in KC lysates. Translation reaction was carried out at 30°C for 3 h.

aa-tRNAs from D0 and D8 mouse KC cultures differentially enhance translation of the PV *Mod* and *Nat* L1 mRNAs in KC cell-free lysate translation system

We have also examined whether additional supplementation of the aa-tRNAs (10^{-7} M) prepared from D0 and D8 mouse KC cultures could enhance translation of the PV *Nat* and *Mod* L1 mRNAs in the D0 and D8 lysates *in vitro* (Figure 8). Introduction of exogenous aa-tRNAs from D0 KCs only enhanced significantly the translation of the BPV1 *Nat* L1 mRNAs in the D0 KC lysate at the 2 h time point (Figure 8A and B). In contrast, addition of exogenous aa-tRNAs from D8 KC culture significantly enhanced the translation of both HPV6b and BPV1 *Nat* PV L1 mRNAs in the D0 lysate at the two time points (1 or 2 h) (Figure 8A and B). The aa-tRNAs from D0 KC culture significantly enhanced translation of the *Mod* L1 mRNAs from both HPV6b and BPV1 in D8 lysate, while the aa-tRNAs from D8 KC culture only enhanced translation of the BPV1 *Mod* L1 mRNA in D8 lysate at the 1 h time point (Figure 8C and D). The results support the findings for the translation of the PV L1 genes *in vitro* in RRL translation system (Figures 5 and 6).

DISCUSSION

The PV life cycle is tightly linked to epithelial cell differentiation. Translation of the virus capsid proteins (L1 and L2) is restricted to the differentiated KCs although a range of polycistronic mRNAs including late open reading frames (L1 and L2 genes) can be detected in undifferentiated PV-infected KCs (35–39). Thus, different posttranscriptional mechanisms that likely prevent capsid protein translation have been proposed to explain why measurable late gene mRNAs are not associated with production of late proteins in less differentiated cells. First, an AU-rich element in the 3' untranslated region of the late gene polycistronic mRNA of several HPVs has been reported to inhibit translation of the late genes in undifferentiated epithelial cells (13,40,41). However, a range of strong constitutive promoters including retroviral LTRs, CMV and SV40 have been used to construct many authentic sequence L1 gene expression constructs from different HPV types in various laboratories (42,43). These L1 expression constructs that do not contain the inhibitory elements can easily produce the L1 transcripts when they are transiently or stably transfected to different mammalian cells. But efficient translation of these authentic sequence L1 gene constructs has not

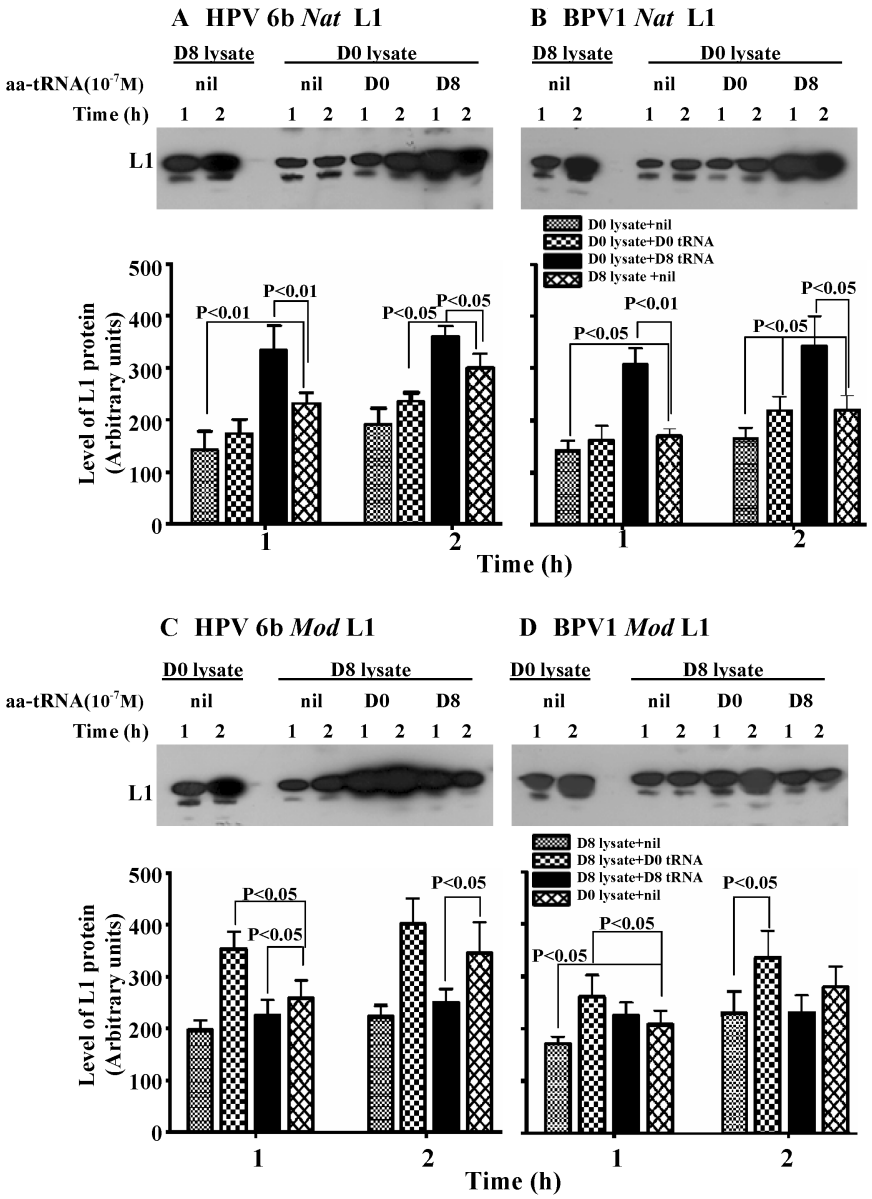

Figure 8. Translation of PV *Nat* and *Mod* L1 mRNAs is differentially enhanced by aa-tRNAs from D0 and D8 mouse KC cultures in KC lysate system. Supplementation with 10^{-7} M aa-tRNA from D8 cultured KCs significantly enhanced L1 protein production from native L1 mRNAs in D0 KC lysate *in vitro* (**A** and **B**), while the L1 protein production from modified L1 mRNAs in D8 KC lysate *in vitro* was significantly enhanced by supplementation with 10^{-7} M aa-tRNA from D0 cultured KCs (**C** and **D**). *Upper panel:* representative western blot analysis of four blots from two independent experiments. *Lower panel:* histograms showing means (± S.E.M.) of L1 signals from the four blots quantified by densitometric analysis using the Imagequant program (Molecular Dynamics, USA). Statistical analysis of the results from these experiments at the two time points was conducted. $P<0.01$ and $P<0.05$ represent the significant degree of the differences between two treatments using *t* Test, respectively.

been reported. Second, a splicing enhancer or silencer in HPV genome is involved in the expression of the late genes. A splicing enhancer in the E4 coding region of HPV16 is required for inhibition of premature late gene expression (44). Zhao *et al*. (2005) (45) reported further that an hnRNP A1-dependent splicing silencer in the HPV16 L1 coding region prevents premature expression of the L1 gene. Unfortunately, whether the reported splicing enhancer or silencer can induce protein expression of the late genes has not been reported. Third, blockage to translation of L1 mRNA has been attributed to sequences within the L1 ORF (46,47). Tan *et al*. reported first that most of the sequence of L1 ORF is inhibitory for L1 translation (46) A major inhibitory element located within the first 129 nucleotides of the L1 gene was then reported (47). Again, expression of L1 and L2 proteins from HPV native L1 and L2 gene expression constructs has not been observed in transiently transfected mammalian cells (47). Evidently, the mechanism of the tight control of PV late gene expression is not understood well.

Recently, by establishing mouse primary KC culture, we were successful, for the first time, to express the L1 proteins of two PV types (HPV6b and BPV1) by transient transfection of authentic or codon modified L1 gene

expression plasmids (23). KC cultures are heterogeneous, which contain two cell populations: replicating and terminally differentiating cells (48). The terminally differentiating KCs that do not divide in culture progressively proceed differentiation and increase their cell size (49). Considering the specific characteristics of the KC cultures, we used lipofectamine to transfect the L1 gene plasmid DNAs to the cultured KCs at 80–90% confluence instead of 50–70% confluence, which is recommended by the manufacturer. We then used flow cytometry analysis to examine the transfection efficiency of the KC cultures transfected with a GFP expression construct and observed that 30–40% of the cultured KCs expressed GFP (unpublished data). The L1 gene expression constructs used in our experiments were driven by a strong constitutive CMV promoter. Therefore, it is possible that the levels of expression of L1 mRNA and protein in the present experimental systems would be much higher than those ever achieved during a real infection. It is also clear that the claimed inhibitory elements in the L1 ORFs are not the active inhibitors of translation of L1 mRNAs in our previous and present studies.

In the present study, we have investigated continuous expression patterns of PV *Nat* and *Mod* L1 genes in cultured mouse and human KCs following a single transfection of the L1 plasmid DNAs. Regarding expression of the PV genes in different mammalian cells, previous studies have reported that synonymous codon substitution can overcome the translation blockage of the viral capsid gene mRNAs (10) and increase levels of E7 protein up to 100-fold due to highly efficient translation of the codon-replaced viral mRNAs (50). A deeper understanding of the quantitative relationship between codon usage and protein levels must include studies on the relationship between codon usage and mRNA levels, and subsequently also the relationship between mRNA levels and protein productivity. Previous studies have reported that the mRNAs of *myc* and *fos* genes can be continuously expressed in primary cultured KCs, irrespective of their proliferative or differentiated state (51). However, many small vectors with heterologous promoters are prone to vector loss and transcriptional silencing although continuous expression of a transgene at therapeutic levels is required for successful gene therapy (52). Only the viral vectors containing U1 snRNA promoters may be an attractive alternative to vectors containing viral promoters for continuous high-level expression of therapeutic genes or proteins (53). Therefore, we examined first whether and how mRNAs of the PV L1 genes, whether or not they are codon modified, were continuously transcribed in primary KC cultures over twelve days following a single transfection of the L1 gene expression constructs in D1 mouse and human KC culture. Both the *Nat* and *Mod* PV L1 genes cloned into the pCDNA3 vector were driven to express by the CMV promoter, they exhibited continuous mRNA transcription in mouse and human KC culture. But the levels of the *Mod* PV L1 mRNAs were significantly higher than those of the *Nat* PV L1 mRNAs in the cultured KCs over the time course investigated, irrespective of their differentiation status, indicating that codon usage can improve transcription efficiency of the viral genes examined in the cultured KCs. The *Mod* PV L1 genes were substantially substituted with the codons ending with G or C, exhibiting GC manifest of their mRNA sequences because the *Nat* PV L1 genes have a strong bias of using codons ending with A or T (11). Thus, from the gene transcription perspectives, the PV L1 genes favour the codons ending with G or C in their gene sequences.

An interesting observation in the present study is that the expression of the PV L1 proteins from the two *Mod* PV L1 genes was scarcely detected at D9 post-transfection. This expression pattern is distinctly different from that of the two *Nat* PV L1 genes, which showed continuously up-regulated expression of the L1 proteins and increased the duration of translation of their mRNAs in both mouse and human KC cultures. It has been reported that genes known to be expressed during latency display codon usage strikingly different from the genes that are expressed during lytic growth in the Epstein-Barr virus (54). In particular, the percentage of codons ending with G or C is persistently lower (about 20%) in all latent genes than in non-latent genes (54). Also, the latent genes have codon usage substantially different from that of host cell genes to minimize deleterious consequences to the host of viral gene expression during latency (54). Thus, the principal explanation to account for the disparity of continuous expression of the L1 proteins from *Nat* and *Mod* PV L1 genes in the present study appears to be their synonymous codon usage. The strong A/T codon usage bias of the *Nat* PV L1 genes due to natural selection may be a main determinant for the increased duration of translation of their mRNAs in differentiated KCs.

Codon usage bias as the main determinant of efficient mRNA translation has been observed in many genes of *Saccharomyces cerevisiae* (55). In the present study, we showed that up- and down- regulated translations of either *Nat* or *Mod* PV L1 mRNAs in cultured mouse and human KCs were dependent on cell differentiation. Generally, differentiation-dependent translation of genes is determined by interaction of regulatory mRNA sequences with translational regulators (56). Two proteins, hnRNPK and hnRNP E1/E2 from rabbit reticulocytes, mediate translational silencing of cellular and viral mRNAs in a differentiation-dependent way by binding to specific regulatory sequences (57). Expression of 15-lipoxygenase (LOX) gene is typically regulated by a translational regulatory process. LOX mRNA accumulates early during differentiation, a differentiation control element in its 3′ untranslated regions confers translational silencing until late stage erythropoiesis (58). The human cytomegalovirus (HCMV) major immediate-early (MIE) genes, encoding IE1 p72 and IE2 p86, are activated by a complex enhancer region that operates in a cell type- and differentiation-dependent manner (59). Enzymes that are responsible for the translational control of gene expression have also been observed (57,60). A novel mechanism of translational gene regulation by which silenced mRNAs can be translationally activated by the c-Src kinase was reported (57). In HPV, raft cultures treated with activators of protein kinase C can induce post-transcriptional

changes in the late gene expression, which may occur through inactivation or down-regulation of splicing factors that inhibit use of the late region polyadenylation site, resulting in increased instability of late region transcripts (60). As discussed above, different translational inhibitory mechanisms have been proposed to explain the block to PV L1 and L2 protein expression (47,61). However, neither the 'block' nor the inhibitory element is incomplete as how else would new infections/particles arise. Based on the systematical analysis in the present study, it is evident that the primary determinant mediating differentiation-dependent translation of the PV L1 mRNAs in KCs is the synonymous codon usage of the PV L1 genes, supporting that codon-mediated translational controls may play an important role in the differentiation and regulation of tissue-specific gene products in humans (7).

A question arising from the present study is why the gene codon usage can differentially turn translation of the PV L1 genes on and off by a post-transcriptional regulatory process. In the bacterium *E. coli* and in yeast, the use of synonymous codons is strongly biased, comprising both bias between codons recognized by the same transfer RNA (tRNA) and bias between groups of codons recognized by different synonymous tRNAs. Therefore, highly expressed genes use a subset of optimal codons in accordance with their respective major iso-acceptor tRNA levels (5,6). A model was developed for the co-evolution of codon usage and tRNA abundance explaining why there are unequal abundances of synonymous tRNAs leading to biased usage between groups of codons recognized by them in unicellular organisms (62). Recently, a significant correlation was found between tRNA relative abundances and codon composition of Buchnera genes (63). Evidently, the match between codon usage and tRNA that mediates translational regulation is a widely used mechanism of translational control. However, in multicellular eukaryotes, there is limited experimental data on tRNA abundance (64) and its function in regulating gene expression. Although a relationship between tRNA abundance and codon usage in *Drosophila* has been reported (65), no experimental evidence of how codon usage parallels tRNA content to regulate gene translation has been provided. Therefore, it is not clear how tRNA abundance in cells reflects the corresponding codons in mRNAs of the target genes to mediate mRNA translation. Recently, we have reasoned that if the PV L1 protein production by the native L1 genes, which is blocked in less-differentiated epithelial cells, is due to the restricted availability of the appropriate tRNAs, the tRNA pools should be different between proliferative and differentiated KCs. Consequently, tRNA profiles of the differentiated KCs *in vivo*, obtained by high pressure liquid chromatography (HPLC) separation, were distinct from those of undifferentiated cells *in vivo* in mouse and bovine (23). Meanwhile, aa-tRNAs from differentiated mouse KCs significantly enhanced translation of native PV L1 genes in a rabbit reticulocyte lysate cell-free *in vitro* translation system (23). In the present study, we observed that the tRNA profile of D0 KCs differs from that of D8-cultured KCs using tRNA gel electrophoresis (unpublished data). Functionally, aa-tRNAs from D8 cultured KCs significantly enhanced translation of the *Nat* PV L1 genes or mRNAs in both RRL and D0 lysate *in vitro* translation systems, but only aa-tRNAs from D0 human KC cultures significantly enhanced translation of the *Mod* PV L1 DNAs in RRL *in vitro* translation system and from D0 mouse KC cultures significantly enhanced translation of the *Mod* PV L1 mRNAs in D8 cell-free lysate *in vitro* translation system. Previous studies have shown that tRNAs from uninfected cells can rescue the translation of global proteins in *Autographa californica* nucleopolyhedrovirus-infected Ld652Y cells (66). Stimulating expression of GTase with $tRNA^{AGA}$ and $tRNA^{AGG}$ resulted in a 5-fold increase in GTase production in *E. coli* in which codon usage is highly biased and the tRNAs specific to codons AGA^{arg} and AGG^{arg} are extremely rare (67). A correlation between tRNA content constraints and gene expression levels has been clearly demonstrated using the concept of optimal codons (68). Here, the inability to express PV L1 protein from the native L1 gene sequence in undifferentiated KCs and from modified L1 gene sequence in differentiated KCs appears to be due to availability of the cellular tRNAs. Native PV L1 mRNAs may translate in undifferentiated KC lysate in which endogenous mRNAs (host mRNAs) were completely hydrolysed, which may have evolved to exploit the tRNA resources available for their efficient translation in differentiated KCs. The argument is strongly supported by our early work that showed that the problem of translation blockage of PV L1 genes in non-differentiated mammalian cells can be alleviated by gene codon modification (10) or by cellular tRNA population alteration (17).

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