Translational Inhibition in Vitro of Human Papillomavirus Type 16 L2 mRNA Mediated through Interaction with Heterogenous Ribonucleoprotein K and Poly(rC)-binding Proteins 1 and 2*

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Human papillomavirus (HPV) type 16 belongs to the group of “high risk” HPV types that are frequently detected in anogenital cancers. The expression of HPV-16 late genes encoding the virus capsid proteins L1 and L2 is restricted to terminally differentiated epithelial cells in the superficial layers of the squamous epithelium. We have previously identified negative elements in the 3’ end of L2 RNA that act in cis to reduce mRNA utilization without substantially affecting mRNA levels. The experiments reported here demonstrate the interaction of cellular proteins with an inhibitory sequence present in the coding region of the L2 mRNA. Using RNA gel shift assays and UV cross-linking, we have detected three cellular proteins interacting specifically with the sense strand of the L2 mRNA, two of which were identified as heterogeneous ribonucleoprotein K (hnRNP K) and the poly(rC)-binding protein (PCBP). Recombinant hnRNP K, PCBP-1, and PCBP-2 that were over expressed in bacteria and partially purified bound to the HPV-16 L2 mRNA in a sequence-specific manner. Interestingly, PCBP-1, PCBP-2, and hnRNP K specifically and efficiently inhibited translation of the HPV-16 L2 mRNA in vitro. Therefore, these proteins may play an important role in the regulation of HPV-16 late gene expression and virus production in vivo.

Human papillomaviruses (HPVs) are nonenveloped, epitheliotropic DNA tumor viruses with a circular double-stranded genome of approximately 8 kilobases (1). At present, more than 70 different types of HPVs have been identified that can be divided into mucosal or cutaneous types on the basis of their natural infection sites. All of the open reading frames are located on one strand of viral genomic DNA that consists of an early, a late, and a noncoding region (1). The late genes are expressed throughout the infected epithelium and are responsible for initiation of viral DNA replication, regulation of transcription, and transformation of cells (1, 5–9). The late gene code for the major and minor capsid proteins, L1 and L2, respectively, and their expression is thought to be regulated at both the transcriptional and the post-transcriptional level. Production of L1 and L2 protein is seen to be inhibited in dividing cells, and the L1 and L2 proteins are stabilized in the superficial layers of terminally differentiated squamous epithelial cells (1, 5–9). Sequences with inhibitory function have been identified on HPV-1 (10), HPV-16 (11), and BPV-1 (12) late mRNAs. Inhibitory RNA elements present in the HPV-16 L1 and L2 coding regions (13–15) reduce the levels of late mRNAs and proteins, and a negative element in the 3′-untranslated region (11) was seen to reduce RNA stability in vitro (11). The inhibitory RNA sequences on the late papillomavirus mRNAs are probably important determinants of virus late gene expression levels, presumably as a result of interactions with cellular RNA binding proteins (16–19). Nuclear proteins may affect splicing, stability, and export, whereas cytoplasmic proteins may affect the utilization of the mRNA by inhibiting translation, altering the subcytoplasmic localization or mRNA stability (20, 21).

Expression of HPV-16 L2 from subgenomic expression plasmids is very inefficient and we were unable to detect L2 protein after transfection of eucaryotic expression plasmids designed to produce L2 in mammalian cells (14). The same vectors express high levels of various cellular and viral genes that we have tested previously. Due to this we speculated that the L2 coding region itself may contain sequences that repress L2 production. In an attempt to verify this idea, we found that sequences in the 5′ end of the L2 coding region were responsible for the relatively short half-life of the L2 mRNAs, whereas sequences in the 3′ end of L2 reduce protein levels approximately 10-fold without substantially affecting mRNA levels when inserted after a reporter gene (14). Here, we have identified cellular proteins that interact specifically with the 3′ end of the L2 coding region encompassing the inhibitory RNA sequences. These proteins were hnRNP K and PCBP-1 and -2. Translation of the L2 open reading frame in vitro was specifically inhibited by the presence of these proteins, suggesting that L2 production is regulated by binding of these factors to the L2 mRNA in the infected differentiating epithelial cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmid pKSH was constructed by first a polymerase chain reaction amplifying HPV-16 L2 sequences from nu-
The amount of pT7PCBP-1 used is indicated in the figure in the absence or presence of pT7PCBP-1, as described previously (13). Production of CAT protein was quantitated by a CAT assay 1:1000 dilution, as described elsewhere (13, 18, 27). HPV-16 L2 peptide antiserum (30) (provided by J. Dillner) was used at 1:100 in immunoblots and 1:100 in immunoprecipitations. The rabbit anti-DNase I sites were first introduced into the L2 sequence in pKSABCpl718, pKSABCpl718 was digested with Asp718 and NcoI, which resulted in a plasmid containing the last 228 nucleotides of HPV-16 L2. pT7PCBP-1 was generated by subcloning into an EcoRI-BamHI fragment encoding PCBP-1 from a GST-PCBP-1 plasmid (22) to pBluescript. The pT7CAT plasmid was produced by inserting a SalI-Asp718 CAT fragment from pNLCATB (13) into pBluescript. pT7CAT-L2 was generated by subcloning into pT7CAT of HPV-16 L2 sequences polymerase chain reaction amplified with oligonucleotides L2B and L2STOP. pT7lacz was constructed by cloning into pCR2.1 (Invitrogen) of the lacZ open reading frame polymerase chain reaction amplified from pCH110 (Amersham Pharmacia Biotech). L2 peptides and L2STOP peptides were expressed with the GST purification system (Amersham Pharmacia Biotech).

In Vitro Transcription Reaction, RNA Gel Shift and UV Cross-linking—In vitro transcription reactions were performed essentially as described previously (17). The resulting RNA probes are displayed in Fig. 1A. RNA mobility shift assays, RNAseT1 protection assay, UV cross-linking, and immunoprecipitations with UV cross-linked protein were performed as described previously (17, 18). 500 ng of recombinant GST fusion proteins were used in UV cross-linking assay.

In Vitro Translation—In vitro translation of presynthesized capped mRNA was performed in a rabbit reticulocyte lysate system (Promega) or in the transcription/translation-coupled rabbit reticulocyte lysate system (Promega) according to manufacturer's instructions in the absence or presence of recombinant GST fusion protein.

Transfections and Vaccinia Virus T7 RNA Polymerase Expression System—HeLa cells were infected with recombinant vaccinia virus vTF7-3 (28), producing bacteriophage T7 RNA polymerase, and transfected with 5 μg of pT7L2, pT7CAT, pT7CAT-L2, or pT7lacz in the absence or presence of pT7PCBP-1, as described previously (13). Briefly, the amount of pT7PCBP-1 used is indicated in the figure legend. The total amount of DNA in transfection mixes was adjusted to 15 μg per 0.5 ml of precipitate per 60-mm plate by using pUC18 carrier DNA. Each transfection experiment was performed at least four times.

CAT Enzyme-linked Immunosorbert Assay (ELISA) and Western Immunoblotting—Production of CAT protein was quantitated by a CAT antigen capture ELISA (Boehringer GmbH) according to the manufacturer's protocol. Western blot analysis was performed as described previously (13, 18). The rabbit anti-PCBP-2 serum (29) was generously provided by E. Ehrenfeld and was used at a dilution of 1:1000 in immunoblots and 1:100 in immunoprecipitations. The rabbit anti-HPV-16 L2 peptide antisera (30) (provided by J. Dillner) was used at a 1:1000 dilution, as described elsewhere (13, 18, 27).

RESULTS

Nuclear and Cytoplasmic Proteins Interact Specifically with an HPV-16 L2 RNA Sequence That Exhibits Inhibitory Activity—Expression of HPV-16 late genes L1 and L2 is restricted to terminally differentiated cells in squamous epithelium (1, 5–9). We have previously shown that the HPV-16 L2 mRNA binding region contains two sequences that inhibit expression of L2 in non-terminally differentiated cells (14). The 3' end of L2 contains cis-acting sequences that inhibit protein production without substantially affecting mRNA levels (14). To investigate whether cellular factors interact with the 3' end of the L2 mRNA, a series of RNA gel shift and UV cross-linking assays were performed, and the results are displayed in Fig. 1. The L2 RNA probes are depicted in Fig. 1A. As can be seen in Fig. 1B, L2 RNA H1 forms RNA-protein complexes when incubated with nuclear extract derived from a HeLa cell line. Complex formation was inhibited by an excess of cold L2 RNA but not by tRNA (Fig. 1B). Complex formation was less efficient using cytoplasmic extract, presumably because the cytoplasmic extract contains fewer L2 RNA-binding proteins (data not shown and see below). Since the results established the specific interaction of cellular proteins with the L2 inhibitory RNA sequence, it was of interest to perform UV cross-linking assays in order to visualize the individual RNA-binding proteins. Several proteins were found binding to the L2 RNA; however, only three cellular proteins A, B, and C with molecular masses of 66, 41, and 35 kDa, respectively, were shown to interact with sequence specificity since they bind to the sense L2 RNA strand and not to the antisense RNA, as shown in Fig. 1C (for protein A, see also Fig. 1D). Proteins A and C were found primarily in the nuclear fraction, while protein B was found primarily in the cytoplasmic fraction (Fig. 1C). UV cross-linking in the presence of 90 mM KCl instead of 120 mM resulted in more efficient detection of protein A (Fig. 1D). Analysis of L2 deletion mutants (Fig. 1A) revealed that proteins A and B interact with the 3' end of the L2 RNA probe while protein C interacts with the 5' end of the RNA probe (Fig. 1, C and E). A longer exposure of the gel was required to detect protein C (data not shown). Because proteins A, B, and C interact specifically with L2 RNA, they became of interest, and further investigation was encouraged since it was possible they may play functional roles in the regulation of HPV-16 late gene expression.

Characterization of Cellular Proteins Binding with Sequence Specificity to L2 mRNA—Since we had established that proteins bound to L2 RNA specifically it was of interest to characterize them in aid of their future identification. We first wished to establish whether the proteins interacting with the L2 RNA had affinities for specific homoribonucleotides. Competition experiments were therefore performed with poly(rA), poly(rC), poly(rU), and poly(rG), preincubated with cell extracts followed by the addition of radiolabeled L2 RNA and UV cross-linking. The results presented in Fig. 2A show the avid binding affinity of protein B for poly(rC) and also the binding affinity of protein C for poly(rU). Optimization of conditions for detection of protein A revealed that it had affinity for poly(rC) and that binding to L2 RNA was unaffected by poly(rA) and poly(rG) (Fig. 2B). Since many RNA-binding proteins have been characterized as having binding affinity for ssDNA (20, 21), it was of interest to examine the relative affinities of proteins A, B, and C for various ssDNAs. Two oligonucleotides with different pyrimidine content were selected. Oligo 1 with a pyrimidine content of 52% competed efficiently for proteins A and B (Fig. 2C, lane 1), whereas oligo 2 with a pyrimidine content of 40% failed to compete with the L2 RNA (Fig. 2C, lane 2), indicating that proteins A and B have affinity for pyrimidine-rich ssDNA.

To further characterize the L2 RNA-binding proteins, UV cross-linking experiments were performed in the presence of increasing concentrations of KCl (90 or 500 mM) and MgCl₂ (3 or 8 mM), and the results are shown in Fig. 2D. As can be seen, the binding of proteins A and C is not detectable with increasing concentrations of KCl (Fig. 2D, lane 3), while in the presence of increasing concentrations of MgCl₂, binding affinity is shown to increase slightly in both cases (Fig. 2D, lane 4). Protein B also displays an increase in binding affinity with increasing concentrations of MgCl₂ but also interestingly displays a significant level of binding affinity in the presence of 500 mM KCl (Fig. 2D, lanes 3 and 4). A protein of 55 kDa was
FIG. 1. A, the late region of the HPV-16 genome is shown. Numbers refer to nucleotide positions in the HPV-16 genome (23). The various HPV-16 L2 RNA probes (H1–H6) are depicted. Drawing is not to scale. B, nuclear extract was incubated with in vitro synthesis radiolabeled HPV-16 L2 H1 RNA followed by RNaseT1 digestion. RNA-protein complexes were separated on non-denaturing polyacrylamide gels. Lane 1, L2 RNA and nuclear extract; lane 2, L2 RNA in the absence of cell extract; lane 3, L2 RNA incubated with proteinase K-treated nuclear extract; lane 4, L2 RNA and nuclear extract preincubated with a 50-fold excess of unlabeled L2 RNA probe; lane 5, L2 RNA and nuclear extract preincubated with a 50-fold excess of tRNA. C, UV cross-linking of nuclear and cytoplasmic proteins to HPV-16 L2 RNA. UV cross-linking of nuclear extract to the sense (S) (lane 1) or antisense (A) (lane 2) strand of HPV-16 L2 RNA probe H1 or to the sense (S) (lane 3) or antisense (A) (lane 4) strand of HPV-16 L2 RNA probe H2. UV cross-linking of cytoplasmic extract to the sense (S) (lane 5) or antisense (A) (lane 6) strand of HPV-16 L2 RNA probe H2. Proteins A, B, and C bind specifically to the sense strand of L2 RNA and not to the antisense strand. D, UV cross-linking of nuclear extract to the sense (S) or antisense (A) strand of HPV-16 L2 RNA probe H2 under conditions that result in efficient detection of protein A, in addition to proteins B and C. E, UV cross-linking of nuclear extract (NE) or cytoplasmic extract (CE) to HPV-16 L2 RNA probe H2 (lanes 1 and 4), H3 (lanes 2 and 5) and H4 (lanes 3 and 6). Proteins A, B, and C that bind specifically to the sense strand of L2 RNA and not to the antisense strand are indicated.
the formation of a doublet band at the expected molecular mass of 41 kDa. Normal rabbit serum did not precipitate UV cross-linked proteins (Fig. 3A). It is conclusive from this result that not only is PCBP-2 found interacting with the L2 RNA but that it interacts to a greater extent in the cytoplasmic fraction than in the nuclear fraction (Fig. 3A), reinforcing that it is predominantly active in the cytoplasm. A possible explanation for the precipitation of two proteins with the same serum may be that one is an isoform of PCBP-2 or perhaps it is PCBP-1, a closely related protein, since they both have similar properties and the serum toward PCBP-2 also reacts with PCBP-1 (31). This result was reinforced as two proteins were detected in the cytoplasmic extract of HeLa cells in a Western blot using the same serum (Fig. 3B). The appearance of two bands in the UV cross-linking immunoprecipitation assay could then be interpreted as the interaction of two related proteins with the L2 RNA.

To investigate whether the nuclear protein termed A interacting with the L2 RNA could be hnRNP K, nuclear extract was preincubated with hnRNP K monoclonal antibody (20) prior to analysis of the extract in RNA gels shift assay. This procedure was used since we were unable to immunoprecipitate hnRNP K with this monoclonal antibody. As can be seen in Fig. 3C, lanes 3 and 4, the incubation of L2 RNA with nuclear extract causes an RNA-protein complex to form, and if the monoclonal antibody against hnRNP K (20) is preincubated with nuclear extract, inhibition of complex formation is observed (Fig. 3C, lane 2). An unrelated monoclonal antibody to HPV-16 L1 protein has no effect on complex formation (Fig. 3C, lane 1) and thus establishes the specificity of the hnRNP K antibody. Therefore, the conclusion drawn from this experiment is that the monoclonal antibody against hnRNP K prevents interactions between hnRNP K and the L2 RNA.

UV Cross-linking of PCBP-1, PCBP-2, and hnRNP K GST Fusion Proteins to the L2 mRNA—To verify the results obtained by immunoprecipitation and gel shift assays, GST fusion proteins of hnRNP K, PCBP-1, and PCBP-2 were synthesized in a bacterial expression system and were partially purified. It was due to the similar properties of PCBP-1 and PCBP-2 and the fact that two UV cross-linked proteins were immunoprecipitated with the PCBP-2 antisera that we decided to investigate whether PCBP-1 also interacted with L2 RNA. The results presented in Fig. 3D (lanes 2 and 4) show the specific interaction of both PCBP-1 and PCBP-2 to L2 sense RNA and show that these proteins have very slight binding affinity to the L2 antisense RNA (Fig. 3D, lanes 3 and 5). The binding to the antisense L2 RNA presumably occurs because the recombinant proteins are present in higher concentrations than those in the HeLa cell extracts. Fig. 3D, lanes 6 and 7, show the positive interaction of GST-hnRNP K to L2 mRNA; once again it is clear that hnRNP K displays a higher affinity toward L2 sense mRNA than to the L2 antisense mRNA. Purified GST did not UV cross-link to L2 RNA (Fig. 3D, lane 1). As described above for the cytoplasmic proteins A and B, PCBP-1, PCBP-2, and hnRNP K interacted primarily with the 3' end of the L2 RNA sequence (Fig. 3E and data not shown). RNA gel shift assays revealed that cold RNA H5 competed efficiently with the probe for binding of the proteins, whereas cold RNA H3 did not (Fig. 3F), further demonstrating the specificity of the interactions. Taken together, these results confirmed our previous results that both PCBP-2 and hnRNP K do indeed interact in a sequence specific manner with the L2 mRNA. We also extended these results to include the binding of PCBP-1 to the HPV-16 L2 mRNA.

We have previously shown that the insertion of the complete HPV-16 L1 or L2 coding regions downstream of the CAT gene resulted in 50–200-fold inhibition of CAT production (13–15).

also observed binding to the L2 RNA in the presence of 500 mM KCl and 8 mM MgCl₂ (Fig. 2D), but this protein was not observed to interact specifically since it appeared to bind also to the antisense L2 RNA strand. However, it may be of interest because it appears to have strong affinity for the L2 RNA.

Identification of L2 RNA-binding Proteins as hnRNP K and PCBP-2—In retrospect of the results obtained, we postulated the identities of proteins A and B as being hnRNP K and PCBP-2, respectively. hnRNP K is a 67-kDa, nuclear RNA-binding protein with shuttling function and affinity for poly(rC) and ssDNA (20), while PCBP-2 is a 41-kDa cytoplasmic RNA-binding protein that also binds poly(rC) and ssDNA (22). We received a monoclonal antibody to hnRNP K (20) and a polyclonal rabbit antiserum against PCBP-2 (29) which were used in a series of RNA gel shift assays and immunoprecipitation assays. As can be seen in Fig. 3A, L2 mRNA UV cross-linked in the presence of nuclear and cytoplasmic extract and immunoprecipitated with a polyclonal antiserum to PCBP-2 (29) caused
Fig. 3. A, immunoprecipitation of UV cross-linked nuclear (N) or cytoplasmic (C) proteins with normal rabbit serum (P) or anti-PCBP-2 rabbit antiserum (I) (29). B, Western immunoblot of cytoplasmic extract (lane 1) or anion exchange column high salt eluate of partially purified nuclear extract (lane 2). The doublet band representing PCBP-2 is indicated. C, HPV-16 L2 RNA probe H5 was incubated with or without nuclear extract (lanes 3 and 4) or nuclear extract preincubated with monoclonal antibody against hnRNP K (20) (lane 2) or monoclonal antibody against HPV-16 L1 (lane 1). D, the sense strand (S) or antisense strand (A) of HPV-16 L2 RNA probe H2 was UV cross-linked to partially purified GST (lanes 1), GST-PCBP-1 (lanes 2 and 3), GST-PCBP-2 (lanes 4 and 5), or GST-hnRNP K (lanes 6 and 7). E, partially purified GST-PCBP-2 was UV cross-linked to HPV-16 L2 RNA probe H2 (lane 1), H3 (lane 2), H4 (lane 3), H5 (lane 4), and H6 (lane 5). Probes are depicted in Fig. 1A. F, RNA gel shift with HPV-16 L2 RNA probe H2 and PCBP-1. 50-, 10-, or 2-fold molar excess of competitor H3 or H5 RNAs were used. The positions of the RNA-protein complexes (Co) and the radiolabeled free probe (P) are indicated on the left. G, RNA gel shift with HPV-16 L2 RNA probe H2 and PCBP-1. Lane 1, probe H2 and PCBP-1; lane 2, probe H2; lane 3, probe H2 and PCBP-1 incubated in the presence of a 5-fold molar excess of HPV-1 L2 unlabeled competitor RNA; lane 4, probe H2 and PCBP-1 incubated in the presence of a 5-fold molar excess of HPV-16 L1 unlabeled competitor RNA. The positions of the RNA-protein complexes (Co) and the radiolabeled free probe (P) are indicated on the left. H, RNA gel shift with HPV-16 L2 RNA probe H2 and PCBP-1 in the absence (lane 2) or presence of a 125-, 25- or 5-fold molar excess of HPV-1 L2 unlabeled competitor RNA (lanes 3–5) or a 125-, 25- or 5-fold molar excess of HPV-16 L1 unlabeled competitor RNA (lanes 6–8). Unbound and bound RNA were quantitated in a PhosphorImager and percent bound RNA is shown.
The complete L1 and L2 coding sequences from HPV-1-inhibited CAT production 3–10-fold (14). The negative element mapped to the 3’-end of the HPV-16 L2 coding sequence inhibited gene expression 12–13-fold (14), the negative element in the 3’-end of HPV-16 L1 inhibited gene expression 33-fold (13) and the corresponding region from HPV-1-inhibited gene expression less than 2-fold in transfected cells. These results suggested that the poly(C)-binding proteins detected with the HPV-16 L2 RNA probe used here may bind strongly also to HPV-16 L1 while they may interact with lower affinity with the HPV-1 L2 sequence that contains weak inhibitory sequences. To test this idea, the last 337 nucleotides of the HPV-16 L1 open reading frame and the last 532 nucleotides of the HPV-1 L2 open reading frame were used as competitors in an RNA gel shift assay with the HPV-16 L2 RNA probe H2 and PCBP-1. The results revealed that HPV-16 L1 RNA competed efficiently with the HPV-16 L2 probe, suggesting that PCBP-1 also may affect HPV-16 L1 production (Fig. 3). In contrast, the HPV-1 L2 RNA did not compete efficiently with the HPV-16 L2 RNA probe (Fig. 3). The results obtained with serially diluted competitors were quantitated and are shown in Fig. 3H. In conclusion, the inhibitory activity of the various HPV sequences in cells correlates with their affinities for the poly(C)-binding proteins.

PCBP-1, PCBP-2, and hnRNP K Inhibit the Translation of the L2 mRNA in Vitro—Since we had established that PCBP-1, PCBP-2, and hnRNP K were binding specifically to the L2 mRNA, it was obvious to examine the functional role of the proteins. A combination of these proteins has recently been shown to inhibit in vitro translation of the erythroid 15-lipoxygenase RNA (36). Therefore, we observed the effect of PCBP-1, PCBP-2, and hnRNP K on the translation of the full-capped L2 mRNA and on the capped CAT reporter mRNA, used here as an internal control. The experiment was performed in duplicate using two different systems, the coupled transcription/translation system (Promega) and the uncoupled system programmed with presynthesized L2 and CAT-capped RNA. As all three proteins are produced in HeLa cells (20, 31), we first investigated if a mix of the three proteins affected in vitro translation of HPV-16 L2. As shown in Fig. 4A, a mixture of PCBP-1, PCBP-2, and hnRNP K (lane 2) combined inhibits translation of the L2 mRNA. A 3-fold serial dilution of the GST fusion proteins (lanes 3 and 4) shows an alleviation of translational inhibition. No translational inhibition is observed on the internal control CAT mRNA (Fig. 4A). Results using the coupled system are shown to the left in Fig. 4A, and the results from the uncoupled to the right. Quantitation of the protein levels by densitometry revealed that addition of the three proteins reduced the translation of L2 mRNA 3.5- and 4.7-fold, respectively, in the two translation systems (Fig. 4B, lanes 2). The mean fold inhibition value of L2 in the absence or presence of PCBP-1, PCBP-2, and hnRNP K in three independent experiments was 3.9-fold. The CAT protein levels were quantified with densitometry and in CAT ELISA. The CAT protein acting as the internal control displays no significant fold difference when PCBP-1, PCBP-2, and hnRNP K are present or absent (Fig. 4, A and B), indicating specific translational inhibition toward L2 mRNA and not CAT mRNA. Interestingly, each protein alone inhibited L2 translation to a similar extent as the mixture of the three proteins (Fig. 5, A and B), while CAT was unaffected (Fig. 5, A and B). The presence of the L2 sequence downstream of the CAT gene, as in pT7CAT-L2, inhibited CAT production in the presence of PCBP-1 (data not shown), suggesting that the L2 sequence acts as a landing pad for the inhibitory proteins, while in a parallel experiment, translation of CAT from pT7CAT-derived mRNAs was unaffected by

PCBP-1.

PCBP-1 Inhibits the Production of L2 Protein in Transfected Cells—To investigate whether the PCBP proteins inhibit production of HPV-16 L2 also in intact cells, we used the vaccinia virus T7 expression system (28), which allows production of detectable levels of HPV-16 L2 despite the presence of inhibitory sequences (14), presumably a result of the high expression levels in this system. The HPV-16 L2 expression plasmid pT7L2 and the CAT plasmid pT7CAT were transfected into vaccinia virus vTF7-3 (28)-infected HeLa cells in the absence or presence of the serially diluted PCBP-1 expression plasmid pT7PCBP-1. Each transfection experiment was performed at least four times. Production of L2 was assayed by immunoblotting, the L2 levels were quantitated by densitometry, and the CAT levels were quantitated in a CAT capture ELISA. The results show that PCBP-1 inhibits production of HPV-16 L2 also in intact human epithelial cells (Fig. 6A), while the levels of CAT produced from the internal control plasmid pT7CAT are
unaffected by PCBP-1 overexpression (Fig. 6B). One of the quantitated L2 immunoblots is shown in Fig. 6C. Cotransfection of pT7L2 and pT7CAT with various amounts of the pT7 vector, the empty vector lacking PCBP-1 coding sequences, did not affect production of HPV-16 L2 (Fig. 6C). We conclude that PCBP-1 inhibits L2 production also in human cells.

In addition, transfection of cells with pT7CAT-L2, a plasmid that contains L2 sequences downstream of CAT, in the presence of increasing concentrations of pT7PCBP-1 resulted in a gradual decrease in CAT levels (Fig. 7A), further indicating that the presence of the L2 sequence on the mRNA inhibits expression of a heterologous mRNA. The β-galactosidase levels produced from the pT7lacZ internal control plasmid were unaffected by PCBP-1 overexpression (Fig. 7B). As a further control, we cotransfected pT7CAT-L2 and pT7lacZ with various amounts of the pT7 vector, the empty vector that lacked PCBP-1 coding sequences. Cotransfection with pT7 vector did not affect CAT production from pT7CAT-L2 (Fig. 7C) or β-galactosidase from pT7lacZ (Fig. 7D). In conclusion, overexpression of PCBP-1 in HeLa cells specifically inhibited protein production from mRNAs containing HPV-16 L2 sequences.

**DISCUSSION**

From HeLa extracts, three proteins were observed to interact specifically with the L2 sense RNA strand. A 35-kDa poly(rU)-binding, nuclear protein and two poly(rC)-binding proteins were identified as PCBP-2 and hnRNP K. Although positive identification of the 35-kDa protein was not achieved, a number of proteins with similar properties have been described.
may contain optimal binding sites for PCBP and hnRNP K proteins, perhaps due to RNA secondary structure.

Interaction between RNA-binding proteins and mRNA is achieved through RNA binding domains present in the proteins. It is conceivable that the interaction between hnRNP K and the L2 inhibitory element is facilitated by the presence of three copies of a motif termed the KH domain as well as two RGG boxes (34). The structural similarity between PCBP-2 and PCBP-1 led us to believe that PCBP-1 could also interact with L2, and in vitro experiments performed verified this prediction. The PCBP-1 and -2 do not contain RGG boxes but contain three KH domains each (34). Therefore, it is most likely that the KH domains of both PCBP-1 and -2 and hnRNP K are responsible for the interaction with the L2 mRNA. This interpretation is reinforced by the fact that KH domains have been shown to bind poly(rC) (35) and that poly(rC) competes with L2 RNA for binding to these proteins.

To analyze a functional role for these RNA-binding proteins, one must associate their binding with the progressive life cycle of the papillomavirus, which is strictly linked to the differentiation of stratified epithelium (5–9). The ability of hnRNP K and PCBP-1 and -2 to inhibit translation of the L2 mRNA appears to suggest a mechanism of decreasing the levels of L2 protein in the lower layers of the epithelium upon infection of the virus. It is therefore suggestive that HPV utilizes not only host factors to selectively repress its viral capsid production but also the level of differentiation present in the epithelium. In a stratified epithelium, L2 protein is only present from the granular layer to the stratum corneum, while the mRNA can be found in lower layers. This would suggest that, through the interaction of hnRNP K and PCBP-1 and -2 with L2 mRNA coupled with environmental changes experienced in the epithelium as differentiation occurs, L2 mRNA is inefficiently translated, but upon progression to epithelium differentiation, translational inhibition is alleviated.

Growing evidence now suggests that the controls determining the functional integrity of cytoplasmic mRNAs may be susceptible to the level of differentiation of the cell. It has been shown previously that hnRNP K and PCBP-1 inhibit the in vitro translation of 15-lipoxygenase mRNA (36). The levels of 15-lipoxygenase mRNA are high in immature red blood cells, but 15-lipoxygenase protein is undetectable. In the course of differentiation, the translational repression is released. The silencing of translation is seen to be conferred by a differentiation control element in the 5'-untranslated region and through the binding of hnRNP K and PCBP-1 to this region. The mechanism used by these proteins involves the specific inhibition of 80 S ribosome assembly on 15-lipoxygenase mRNA and control of cap-dependent and internal ribosome entry site-mediated translation by binding to differentiation control elements (36). The fact that the inhibitory L2 sequence is active, when located downstream of the translated open reading frame, suggests that translation initiation is affected as in 15-lipoxygenase mRNA. It is possible that hnRNP K and PCBP-1 and -2 regulate translation of cellular and virus mRNAs in response to fluctuating differentiation signals in different cell types.

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