# The human papillomavirus type 16 negative regulatory RNA element interacts with three proteins that act at different posttranscriptional levels

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**In human papillomaviruses, expression of the late genes L1 and L2, encoding the capsid proteins, is restricted to the upper layers of the infected epithelium. A 79-nt GU-rich negative regulatory element (NRE) located at the 3′ untranslated region of the human papillomavirus 16 L1 gene was identified previously as key to the posttranscriptional control of late gene expression. Here, we demonstrate that in epithelial cells, the NRE can directly bind the U2 auxiliary splicing factor 65-kDa subunit, the cleavage stimulation factor 64-kDa subunit, and the Elav-like HuR protein. On induction of epithelial cell differentiation, levels of the U2 auxiliary splicing factor 65-kDa subunit decrease, levels of the cleavage stimulation factor 64-kDa subunit increase, and the levels of HuR remain unchanged, although redistribution of the HuR from the nucleus to the cytoplasm is observed. Late gene transcripts, which appear to be fully processed, are detected in undifferentiated W12 cells, but are confined in the nucleus. We propose that repression of late gene expression in basal epithelial cells may be caused by nuclear retention or cytoplasmic instability of NRE-containing late gene transcripts.**

Human papillomaviruses (HPVs), small double-stranded DNA viruses that infect squamous epithelia (1, 2), are divided into the "low risk" types and the "high risk" types, one of which, HPV16, is strongly implicated in the formation of genital neoplasms (3). The circular HPV genome comprises an early- and a late-coding region and some 1 kb of noncoding region. Early and late viral transcripts overlap and RNA 3′ ends are processed either at the 5′ proximal early polyadenylation [poly(A)] site, or at the late poly(A) site, respectively (4–6). Although the early genes are expressed throughout the epithelium, production of the L1 and L2 late structural proteins is restricted to terminally differentiated keratinocytes, in the upper layers of the epithelium (1, 7).

HPV L1 and L2 late gene expression is regulated at both the trancriptional (8) and posttranscriptional level: cis-acting negative regulatory RNA elements are found at the 3′ untranslated region (UTR) of HPV late mRNAs. In the bovine papillomavirus type 1 binding of the U1 small nuclear ribonucleoprotein to an unutilized 5′ splice site inhibits late poly(A) site usage (9, 10). In HPV1 binding of the hnRNPC1/C2 and HuR proteins to an AU-rich element regulates late mRNA stability and translation efficiency (11, 12). Inhibitory RNA elements were also found in the coding region of the HPV16 L1 and L2 (13, 14).

Our previous studies on HPV16 identified a negative regulatory element (NRE) present in the late 3′ UTR, which contains four putative 5′ splice sites and a GU-rich region. The NRE exerts a strong negative effect on the expression of a reporter gene (5), reduces mRNA stability *in vitro* (15), and binds a 65-kDa nuclear protein (16). On treatment of keratinocyte W12 cells (which harbor episomal HPV16 DNA and can be induced to differentiate, ref. 17) with phorbol-12-myristate-13-acetate (PMA), the negative effect on reporter gene activity is abrogated, NRE binding of the 65-kDa protein is reduced and NRE binding of a predominantly cytoplasmic 40-kDa protein is induced (16).

Here, we set out to determine which proteins interact specifically with the NRE. Apart from the previously suggested 65-kDa subunit of the auxiliary splicing factor U2AF (U2AF$^{65}$), normally required for recognition of the polypyrimidine tract upstream of 3′ splice sites (18, 19), other RNA-binding proteins involved in posttranscriptional mechanisms that interact with U-rich RNA sequences were considered as candidates for the 65- and 40-kDa proteins. A good candidate for the former was the 64-kDa subunit of the cleavage stimulation factor CstF (CstF-64), which binds GU-rich RNA motifs located downstream of poly(A) sites (20, 21), stabilizing formation of the cleavage and polyadenylation complex (20, 22), as well as recognizing elements located upstream of the poly(A) site (23). RNA-binding nucleocytoplasmic shuttling proteins were candidates for the 40-kDa protein, such as HuR (24, 25), that binds AU-rich elements to stabilize RNAs (26–28) and possibly transport them from the nucleus to the cytoplasm (29).

We show that U2AF$^{65}$, CstF-64, and HuR bind the HPV16 NRE. The levels of U2AF$^{65}$ and CstF-64 and the distribution of HuR are altered on epithelial differentiation, where the NRE inhibition is alleviated. The NRE-containing HPV16 late mRNAs are present in undifferentiated W12 cells and are apparently fully processed, but they are confined in the nucleus. Binding of these proteins to the NRE could regulate HPV16 late gene expression, through multiple steps involving polyadenylation, nucleocytoplasmic transport, and cytoplasmic instability.

## Materials and Methods

**Plasmids.** Plasmids CAT (chloramphenicol acetyltransferase) SE227 (containing 7,226–7,453 nt of the HPV16 DNA) and CAT PE445 (containing 7,008–7,453 nt) lacked or contained the NRE (16), respectively. Plasmid α-L1 contains a *Bam*HI/*Pst*I fragment of the L1 ORF (6,153–6,794 nt) ligated into *Bam*HI/*Pst*I cut pGEM-3Z vector (Promega). Plasmid α-U6 was a gift from I. W. Mattaj (30).

**Cell Culture.** HeLa cells were grown in DMEM supplemented with 10% FBS. W12 cells (provided by M. Stanley, ref. 17) were

Abbreviations: HPV16, human papillomavirus type 16; NRE, negative regulatory element; UTR, untranslated region; U2AF$^{65}$, U2 auxiliary splicing factor 65-kDa subunit; CstF-64, cleavage stimulation factor 64-kDa subunit; poly(A), polyadenylation; PMA, phorbol-12-myristate-13-acetate; EMSA, electrophoretic mobility-shift assay; GST, glutathione *S*-transferase; RBD, RNA-binding domain; CAT, chloramphenicol acetyltransferase.

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maintained at subconfluence on mitomycin-C-treated (Sigma) 3T3 feeder cells in DMEM containing 10% FBS, 0.1 nM cholera toxin, 0.4 $\mu$g/ml hydrocortisone, and 10 ng/ml epidermal growth factor. Three methods were used to induce their differentiation: (*i*) 16 nM PMA was added to W12 cells for 5–10 days; (*ii*) W12 cells were grown in F medium (0.66 mM $Ca^{2+}$), supplemented with 5% FBS/0.1 nM cholera toxin/0.4 $\mu$g/ml hydrocortisone/10 ng/ml epidermal growth factor/5 $\mu$g/ml insulin/24 $\mu$g/ml adenine. When cells reached confluence, they were switched to F medium containing 1.2 mM $Ca^{2+}$ and 20% FBS; (*iii*) $10^7$ cells were suspended in 20 ml of F medium containing 20% FBS and 1.68% methylcellulose, for 1–8 days, as described (31).

**Cell Extract.** Nuclear and cytoplasmic extracts from HeLa cells were prepared as described (32). For W12 cells, feeder cells were removed first by treatment with 0.1% trypsin-0.5 mM EDTA and extracts from small quantities of cells were prepared (33). Nuclear and cytoplasmic extracts from the same batch of cells were dialyzed against buffer D (20 mM Hepes, pH 8.0/100 mM KCl/0.2 mM EDTA/1 mM DTT/0.5 mM PMSF/20% glycerol).

**Riboprobes, RNA Electrophoretic Mobility-Shift Assays (EMSA), and UV Cross-Linking.** Riboprobes: NRE, (spanning the 79-nt sequence 7,128–7,206 from HPV16); 5′, (the 5′ end 43 nt of the NRE); 3′, (the 3′ end 36 nt of the NRE); and B2P2 (19), were *in vitro*-transcribed as described (16) in the presence of [$\alpha$-$^{32}$P]rUTP. As control probes specific for CstF-64 binding, the adenovirus L3 poly(A) signal (L3) (34), and the *in vitro*-selected A-1 19-nt RNA ligand (35) were used.

For the EMSA assays, 50–100 fmol of radiolabeled RNA probe was incubated with 20 $\mu$g of nuclear or 50 $\mu$g of cytoplasmic extract in a total volume of 20 $\mu$l of binding buffer (10 mM Hepes, pH 7.6/60 mM KCl/3 mM $MgCl_2$/1 mM DTT/5% glycerol) in the presence of 2 $\mu$g of *Escherichia coli* tRNA for 15 min. The samples were then placed on ice, 100 $\mu$g of heparin was added, and they were incubated for an additional 10 min. In competition experiments, nuclear or cytoplasmic extracts were preincubated with antibodies or RNA competitors for 30 min on ice. The complexes were resolved on native 5% polyacrylamide gels (acrylamide/bisacrylamide, 60:1).

For the UV cross-linking, the RNA probe and the extract were incubated in binding buffer for 10 min at 30°C and placed on ice, and 2 $\mu$g of *E. coli* tRNA was added. UV cross-linking was performed in a Stratalinker (Stratagene) at a setting of 250 mJ. Unbound RNA was digested with RNase A (10 $\mu$g/ml) for 30 min. Samples were electrophoresed on 10% SDS/PAGE gels. For the binding experiments, 200 ng of recombinant histidine-tagged U2AF$^{65}$ (provided by A. MacMillan, University of Toronto, Canada), 70 $\mu$M of purified glutathione *S*-transferase (GST) 64 RNA-binding domain (GST-64RBD) (21), 1–10 $\mu$M of purified GST-HuR (provided by H. Furneaux, ref. 24), as well as purified CstF (36), were used.

**Western Blot Analysis.** Nuclear and cytoplasmic extracts were prepared as described above. mAb against cytokeratins 10 and 11 (Sigma) was used at a 1:1,000 dilution. The mAbs MC3 against U2AF$^{65}$ (provided by M. Carmo-Fonseca, ref. 37), $\alpha$64K against CstF-64 (36), and 19F12 against HuR (provided by H. Furneaux, Sloan Kettering Institute for Cancer Research, New York), were used at a 1:200, 1:100, and 1:2,500 dilution, respectively. mAbs against MEK-4 and ATF-2 (Santa Cruz Biotechnology) were used at a 1:1,000 dilution. Proteins were visualized by using enhanced chemiluminescence reagents (Amersham International), according to the manufacturer's instructions. Densitometric quantitation of the exposed film was done with the Phosphoimager QUANTITYONE program (Bio-Rad).

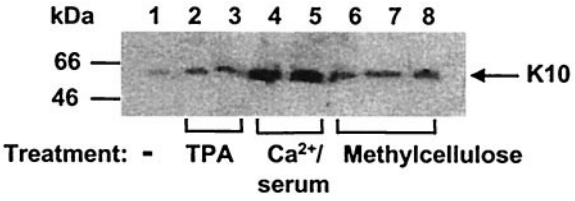


**Fig. 1.** Analysis of keratin 10 expression in W12 cells. Cells were untreated (lane 1), or treated with 16 nM PMA for 5 days (lane 2) and 10 days (lane 3), increased $Ca^{2+}$ and serum concentration for 5 days (lane 4) or 10 days (lane 5), or suspended in methylcellulose for 1 day (lane 6), 2 days (lane 7), or 8 days (lane 8). Total extracts (50 $\mu$g) were used and Western blot analysis was performed with the anti-K10 Ck 8.60 antibody.

**Transient Transfection.** Transient transfection was performed with the Lipofectamine reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Cells were harvested 48 h posttransfection.

**Northern Blot Analysis.** Total cytoplasmic RNA was prepared by lysis with Nonidet P-40 as described (38), whereas RNA from nuclei of the same cell preparation was purified by using Trizol (Life Technologies) according to the manufacturer's protocol. Poly(A)$^+$ RNA was selected with an Oligotex mRNA kit (Qiagen, Chatsworth, CA). L1, CAT, and U6 antisense probes were *in vitro*-transcribed from the corresponding plasmids with Sp6, T7, and T3 polymerases (Promega), respectively, in the presence of [$\alpha$-$^{32}$P]rUTP. [$\alpha$-$^{32}$P]dATP was used for random primer labeling of a DNA $\gamma$-actin probe (Amersham International).

## Results

**Differentiation of HPV16 Containing W12 Cells.** HeLa cells were used as a model for basal epithelial cells, but they cannot undergo differentiation *in vitro*. W12 cells, which are basal epithelial cells that contain episomal copies of the HPV16 genome (necessary for viral late gene transcription, ref. 39) and can be induced to differentiate (17, 31, 40), were also used.

To generate differentiating W12 cells and assess the degree of differentiation, we used the following approaches: (*i*) treatment with 16 nM PMA; (*ii*) increase the molarity of calcium (1.2 mM) and the percentage of serum (20%) in the F medium (31); and (*iii*) suspension in methylcellulose (31, 40). As a differentiation marker, the suprabasal layer-specific keratin K10 was used (41). Western blot analysis revealed that the levels of K10 were increased substantially in epithelial cells maintained in medium with increased $Ca^{2+}$ and serum (Fig. 1, lanes 4 and 5), whereas suspension in methylcellulose, or treatment with PMA induced levels of K10 to a lesser extent. Some K10 was present in W12 cultures maintained in the absence of inducers of differentiation, probably because of a small heterogeneity of the W12 cell population (4). Thus, for the experiments described, W12 cells were induced to differentiate by maintaining confluent epithelial cell monolayers in a high calcium and serum medium for 10 days.

**The HPV16 NRE Element Binds Cellular Proteins.** EMSA assays were performed by using the NRE RNA and nuclear or cytoplasmic extracts from HeLa and undifferentiated and differentiating W12 cells (Fig. 2). Three RNA–protein complexes formed after incubation of the NRE with nuclear extracts, with no significant differences between HeLa, undifferentiated, and differentiating W12 cells, apart from a small increase in the mobility of the upper band in the differentiating W12 extract (Fig. 2, lane 3). When cytoplasmic extracts were used, the NRE formed a single complex with the HeLa cell extract (Fig. 2, lane 4), whereas two complexes were formed after incubation with W12 cell extracts (Fig. 2, lanes 5 and 6). The larger complex was more abundant in the differen-

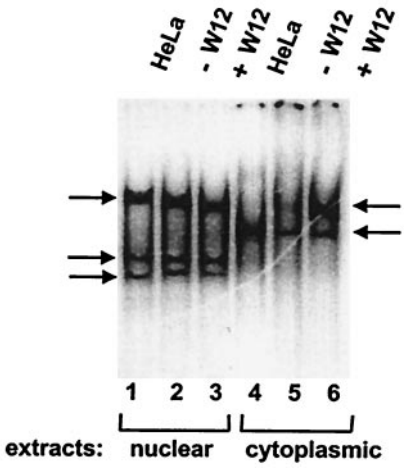


**Fig. 2.** The NRE RNA binds cellular proteins. EMSA with HeLa (lanes 1 and 4), undifferentiated (−, lanes 2 and 5) and differentiating (+, lanes 3 and 6) W12 cell nuclear (lanes 1–3) and cytoplasmic (lanes 4–6) extracts. A $^{32}$P-labeled *in vitro*-transcribed sense probe, homologous to the NRE (NRE RNA) was used. Arrows indicate the complexes formed.

tiating W12 extract (Fig. 2, lane 6). Poly(rU) competed efficiently in the formation of nuclear and cytoplasmic RNA–protein complexes, whereas poly(rA), poly(rG), and poly(rC) did not, indicating that the proteins detected bind U-rich RNA (data not shown). To identify the NRE-binding proteins, we used antibodies against

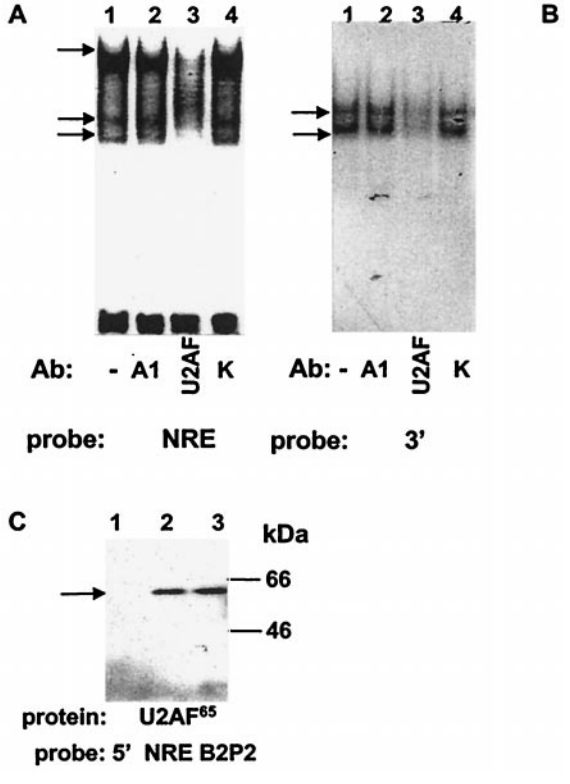


**Fig. 3.** U2AF$^{65}$ interacts with the HPV16 NRE RNA. EMSA with (*A*) the NRE RNA probe or (*B*) a probe homologous to the 3′ half of the NRE (3′ probe), incubated with HeLa nuclear extract with no Ab (lane 1) or in the presence of an Ab against hnRNPA1 (lane 2), U2AF$^{65}$ (lane 3), and hnRNPK (lane 4). (*C*) His-tagged U2AF$^{65}$ was UV cross-linked to the B2P2 probe (lane 3), the NRE probe (lane 2), and the 5′ half of the NRE (5′ probe) (lane 1). Arrows indicate the complexes formed.

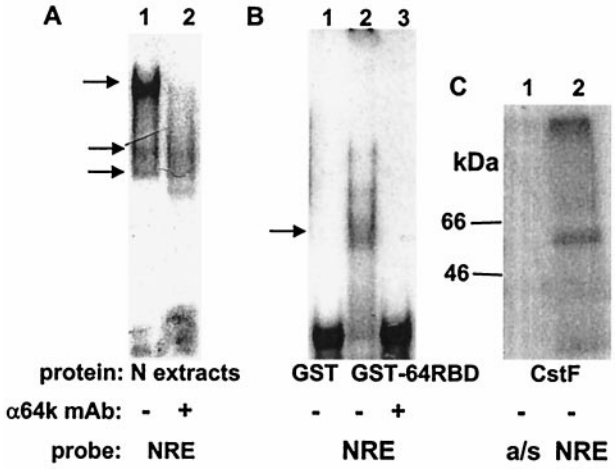


**Fig. 4.** CstF-64 interacts with the HPV16 NRE RNA. (*A*) EMSA with the NRE RNA probe incubated with HeLa cell nuclear extract in the absence (lane 1) or presence (lane 2) of a mAb against CstF-64 (α64k). (*B*) EMSA with the NRE probe incubated with 70 μM of GST-64RBD in the absence (lane 2) or presence of the α64k mAb (lane 3). As a control, 1 μg of GST protein was incubated with the NRE probe (lane 1). Arrows indicate the complexes formed. (*C*) Purified CstF from HeLa cells was UV cross-linked to an antisense (a/s) NRE probe (lane 1) and to the NRE (lane 2).

various proteins involved in RNA processing, including hnRNPA1, hnRNPK, U2AF$^{65}$, CstF-64, and HuR. Only the latter three bound the NRE as described below.

**The U2AF$^{65}$ Protein Binds the HPV16 NRE RNA.** EMSA assays were performed after preincubation of nuclear extracts with a mAb against U2AF$^{65}$ (MC3). In the presence of the MC3 mAb, the upper complex formed with the NRE is disrupted whereas the two lower complexes are possibly supershifted (Fig. 3*A*, lane 3), demonstrating the presence of U2AF$^{65}$ in all these complexes. When the 3′ half of the NRE was used as a probe, addition of the mAb also disrupted complex formation (Fig. 3*B*, lane 3), indicating that the GU-rich sequence present in this probe is sufficient for binding. Because the mapped epitope of the MC3 mAb overlaps with the beginning of the U2AF$^{65}$ RNA-binding domain (37), preincubation with the mAb results mainly in disruption of the complexes formed. Preincubation of nuclear extract with Abs against hnRNPA1 and hnRNPK did not affect the complex formation on the NRE (Fig. 3*A*, lanes 2 and 4) or the 3′ probe (Fig. 3*B*, lanes 2 and 4).

To show a direct binding, we tested the ability of bacterially expressed his-tagged U2AF$^{65}$ protein to UV cross-link to the NRE. Whole NRE RNA bound the protein (Fig. 3*C*, lane 2), whereas the 5′ half of the NRE did not (Fig. 3*C*, lane 1). U2AF$^{65}$ also bound to the control B2P2 probe (Fig. 3*C*, lane 3), a known U2AF$^{65}$ binding site (19), with high sequence similarity to the GU-rich region at the 3′ half of the NRE (16).

**The CstF 64-kDa Subunit Binds the HPV16 NRE RNA.** To further characterize the RNA–protein interactions observed in Fig. 2, EMSA assays were performed after preincubation of nuclear extract with a mAb against CstF-64 (α64K). The presence of the α64K mAb disrupted the upper complex formed between the extract and the NRE probe but not the two lower complexes (Fig. 4*A*, lane 2). Interestingly, the same complex was also disrupted by incubation with the MC3 mAb, indicating that the upper complex contains both U2AF$^{65}$ and CstF-64 proteins. EMSA assays were also performed by incubating the NRE with a GST fusion protein of the RBD of CstF-64. GST-64RBD bound the NRE probe (Fig. 4*B*, lane 2) and the α64K mAb

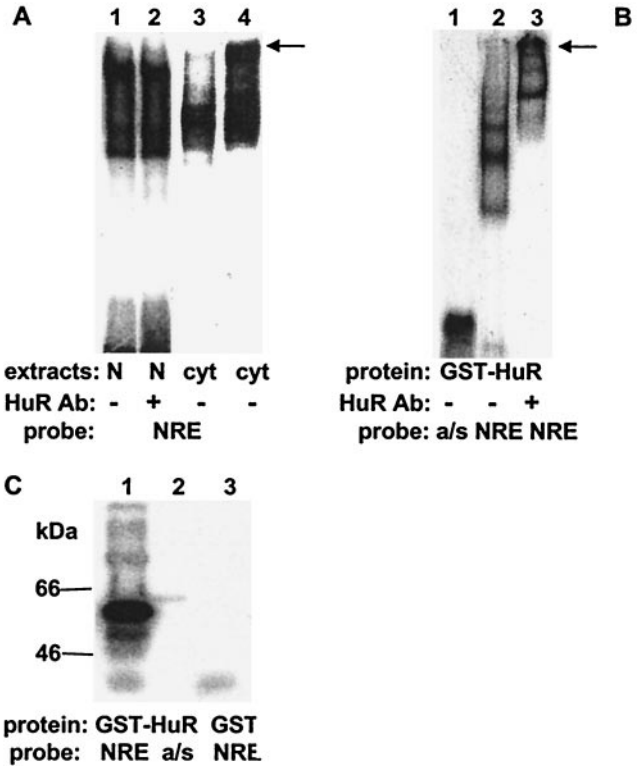


**Fig. 5.** HuR binds the HPV16 NRE RNA. (*A*) EMSA with the NRE RNA probe incubated with HeLa cell nuclear (lanes 1 and 2) or cytoplasmic extract (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a mAb against HuR (19F12). The arrow indicates the supershift of the bound RNA induced by the mAb. (*B*) EMSA with 10 $\mu$M GST-HuR incubated with the NRE probe in the absence (lane 2) or presence (lane 3) of the HuR mAb, or with an antisense (a/s) NRE probe (lane 1). (*C*) GST-HuR protein (10 $\mu$M) was UV cross-linked to the NRE RNA (lane 1) and an antisense (a/s) NRE probe (lane 2). As a control, GST protein was UV cross-linked to the NRE (lane 3).

prevented the RNA–protein complex formation (Fig. 4*B*, lane 3). However, the binding of GST-64RBD protein to the NRE seems to be 3-fold weaker compared with previously identified RNA elements of similar size, determined by the protein concentration required for detectable shifted complexes (42).

To show a direct binding, UV cross-linking experiments were performed by incubating NRE RNA with highly purified CstF. The results revealed that CstF-64 binds the NRE (Fig. 4*C*, lane 2), but not an antisense probe (Fig. 4*C*, lane 1).

**The Elav-Like HuR Protein Interacts with the HPV16 NRE RNA.** EMSA were performed by incubating the NRE RNA with nuclear or cytoplasmic extracts in the presence of a mAb against HuR (19F12), a candidate for the 40-kDa NRE-binding protein. The mAb induced a supershift only when preincubated with cytoplasmic extract (Fig. 5*A*, lane 4), but not with nuclear extract (Fig. 5*A*, lane 2). Purified GST-HuR also formed a complex with the NRE probe (Fig. 5*B*, lane 2), but not with an antisense probe (Fig. 5*B*, lane 1) which was supershifted by the HuR mAb (Fig. 5*B*, lane 3). For direct binding, we tested UV cross-linking of GST-HuR to the NRE, showing that GST-HuR cross-linked efficiently to the NRE RNA (Fig. 5*C*, lane 1), but not to an antisense probe (Fig. 5*C*, lane 2), whereas GST alone did not cross-link to the NRE (Fig. 5*C*, lane 3).

Because three proteins (U2AF$^{65}$, CstF-64, and HuR) bound the NRE, competition experiments were performed to examine their relative binding affinities. EMSA incubating the NRE probe with

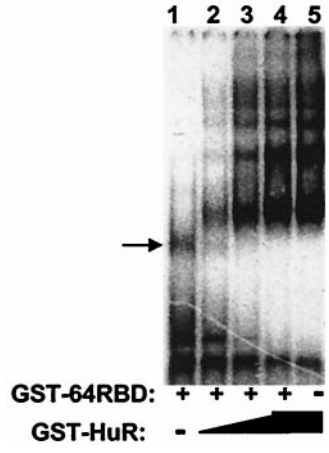


**Fig. 6.** Competition between CstF-64 and HuR for NRE RNA binding. EMSA with the NRE RNA probe incubated with 70 $\mu$M of GST-64RBD in the absence (lane 1) or presence of increased concentrations of GST-HuR: 1 $\mu$M (lane 2), 5 $\mu$M (lane 3), and 10 $\mu$M (lane 4). In lane 5, the NRE was incubated with 10 $\mu$M GST-HuR, alone. The arrow indicates the complex formed between GST-64RBD and NRE.

70 $\mu$M GST-64RBD revealed that addition of as little as 1 $\mu$M GST-HuR is sufficient to disturb complex formation (Fig. 6, lane 2). Although GST-HuR seems to bind the NRE much stronger than the GST-64RBD, purified CstF protein binds RNA elements of the NRE size 2–10 times stronger than the GST-64RBD protein (21, 42), thus the actual difference between HuR and CstF-64 NRE binding *in vivo* could be a lot less than the 70-fold observed *in vitro*.

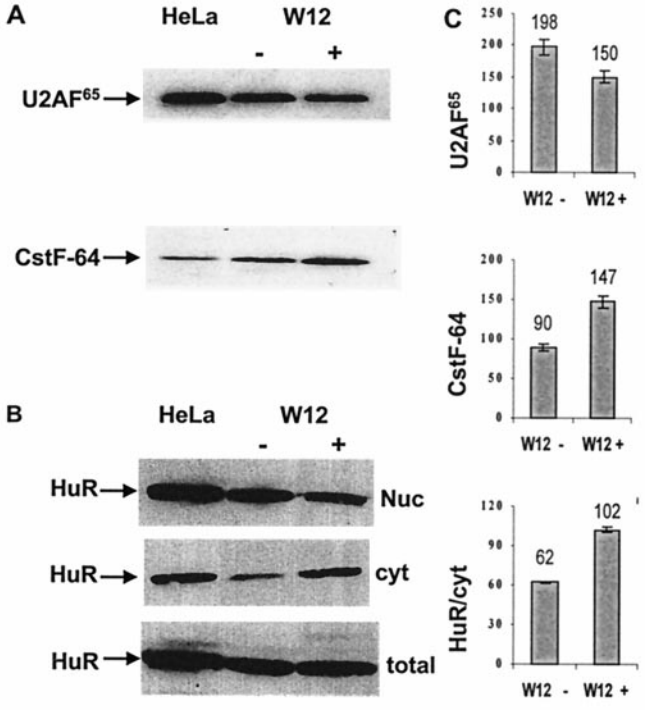


**Fig. 7.** Western blot analysis of U2AF$^{65}$, CstF-64, and HuR expression in HeLa cells, and undifferentiated (−) and differentiating (+) W12 cells. (*A*) Nuclear extract (10 $\mu$g) was used for the detection of U2AF$^{65}$ with the MC3 mAb, and CstF with the $\alpha$64k mAb. (*B*) Nuclear and cytoplasmic extracts equivalent to $10^5$ cells, or 50 $\mu$g of total extracts were used for the detection of HuR with the 19F12 mAb. (*C*) Densitometric quantification of three independent experiments was performed and the differences found between undifferentiated and differentiated W12 cells are shown with standard deviations from the means.

U2AF$^{65}$ binding to the NRE could not be compared because the his-tagged U2AF$^{65}$ bound the NRE-RNA in UV cross-linking experiments but not in EMSA.

**Levels of NRE-Binding Proteins in Differentiating W12 Cells.** We investigated the levels of expression of the three NRE-binding proteins on induction of W12 cell differentiation. Various amounts (10–90 $\mu$g) of cell extracts were analyzed to ensure linear detection. Densitometric quantification of data from three experiments showed a 20–30% decrease in U2AF$^{65}$ levels and a 30–40% increase in CstF-64 levels in differentiating W12 cells (Fig. 7 *A* and *C*). Because there was no significant change in the intensity of the complexes formed between the NRE and undifferentiated or differentiating W12 nuclear extracts, but only a shift in mobility of the uppermost complex (Fig. 2, compare lanes 2 and 3), this could indicate a change in the representation of the two proteins in the complex. During differentiation where U2AF$^{65}$ levels are reduced, more CstF molecules could bind the NRE. Because HuR is a shuttling protein (29), we analyzed its expression in the nucleus and cytoplasm of undifferentiated and differentiating W12 cells. The Western blots were also probed with Abs against the transcription factor ATF-2 (for nuclear extract), and against MEK kinase 4 (for cytoplasmic extract), as controls for protein loading and efficient fractionation (data not shown). Interestingly, the levels of HuR in the cytoplasm increased about 40%, on induction of differentiation, whereas the total levels of HuR remained the same (Fig. 7 *B* and *C*). The change in distribution of HuR is paralleled by a concomitant increase in NRE–protein binding in the cytoplasm of differentiating W12 cells (Fig. 2, lane 6), where the NRE no longer exerts its inhibitory effect on gene expression (16).

**HPV16 L1 mRNA Is Predominantly Nuclear.** Although production of the HPV16 late proteins is confined to the most superficial differentiated cells of the epithelium (17, 43), we could readily detect late gene transcripts in undifferentiated W12 cells. The subcellular localization of the late gene mRNAs in undifferentiated W12 cells was examined by Northern blot analysis of poly(A)$^+$-selected RNA by using a L1 riboprobe. Two size classes of polyadenylated late messages were detected (1.9 and 4.9 kb), both present mainly in the nuclear fraction (Fig. 8*A*, compare lanes 1 and 2). The 1.9-kb transcript is the L1 mRNA (4), whereas the 4.9-kb band most likely represents an L2-L1 bicistronic transcript, similar to those described for HPV31 and HPV18 (44, 45).

To investigate whether the NRE affected the distribution between the nucleus and cytoplasm of a reporter gene mRNA, HeLa cells were transiently transfected with plasmids pCAT445 and pCAT227 containing or lacking the NRE, respectively. Northern blot analysis of fractionated HeLa cells revealed that the presence of the NRE resulted in the localization of the CAT transcripts predominantly in the nucleus (Fig. 8*B*, lane 1), leading to a substantial reduction in CAT activity (16). With the construct lacking the NRE, an equal distribution of CAT transcripts was observed between the nucleus and the cytoplasm (Fig. 8*B*, lanes 3 and 4). Northern blots were rehybridized with probes for $\gamma$-actin and U6 RNAs (the former was located mainly in the cytoplasm, whereas the latter was largely retained in the nucleus) to examine the efficiency of nuclear and cytoplasmic fractionation.

## Discussion

We have shown that the HPV16 NRE binds three cellular proteins involved in RNA-processing events: U2AF$^{65}$, CstF-64, and HuR. U2AF$^{65}$ requires only the NRE 3′ portion, which is GU-rich, and CstF requires the whole NRE sequence (data not shown). CstF-64 binds the NRE relatively weakly (it is readily displaced by HuR binding), although *in vivo* the downstream poly(A) site may stabilize further its binding via interaction with cleavage polyadenylation specificity factor (22, 46). Both U2AF$^{65}$ and CstF-64 can bind the NRE in the nucleus, indicating either competitive or cooperative binding of the two proteins. In contrast, although HuR is present both in the nucleus and cytoplasm of HeLa and W12 cells, it is capable of binding the NRE only in the cytoplasm. On differentiation of W12 cells, the levels of U2AF$^{65}$ moderately decrease and the levels of CstF-64 increase, whereas the levels of HuR remain unchanged.

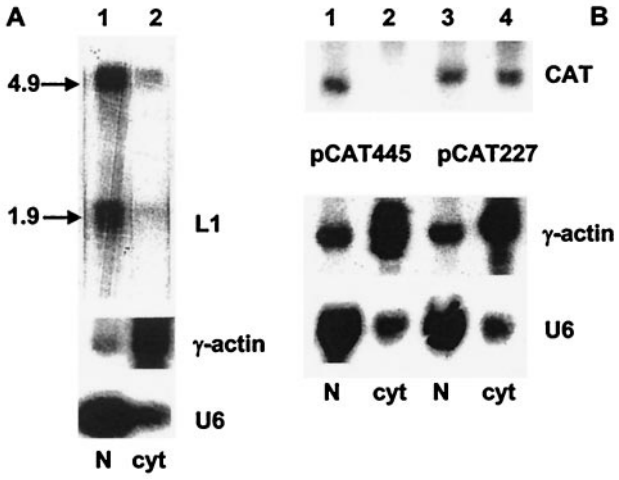


**Fig. 8.** The NRE-containing transcripts are confined in the nucleus. Northern blot analysis of nuclear (N) and cytoplasmic (cyt) poly(A)$^+$ RNA extracted from (*A*) undifferentiated W12 cells with an L1-specific riboprobe; the arrows indicate the presence of two L1-containing transcripts, or (*B*) from HeLa cells transfected with pCAT445 (lanes 1 and 2), or pCAT227 (lanes 3 and 4) by using a CAT-specific riboprobe. Blots were stripped and rehybridized with probes for $\gamma$-actin and U6 RNA.

Although the auxiliary splicing factor U2AF$^{65}$ is not known to bind 3′ UTR sequences, other splicing-associated factors bind such elements. Binding of U1 small nuclear ribonucleoprotein to a bovine papillomavirus type 1 element down-regulates late gene expression (9) by direct contact of the U1 70-kDa protein with the poly(A) polymerase (10). Most recently, in *Caenorhabditis elegans*, U2AF$^{65}$ binding to its own mRNA caused its nuclear retention. U2AF$^{65}$ binding at the 3′ UTR of a reporter gene dramatically suppressed its expression by inhibiting RNA export to the cytoplasm, without affecting RNA accumulation or processing in the nucleus (47). Consequently, splicing factors can act to retain pre-mRNA in the nucleus, by preventing association of mRNA with proteins required for nuclear export (48, 49). Whether binding of U2AF$^{65}$ to NRE-containing RNAs may directly cause their retention in the nucleus, and conversely, whether epithelial cell differentiation-induced reduction of U2AF$^{65}$ levels facilitates their export, remains to be tested.

CstF-64 is known to bind 3′ UTR sequence elements. Although this factor normally regulates specificity and efficiency of polyadenylation by binding GU-, or U-rich sequences located downstream of poly(A) sites (20, 21), it can also bind GU-rich elements upstream of the poly(A) site. Binding of polypyrimidine tract-binding protein and CstF-64 upstream of the C2 complement poly(A) site stimulates cleavage and poly(A) addition (23). Furthermore, levels of CstF-64 are involved in regulating poly(A) site selection. CstF-64 is up-regulated during B-cell differentiation and overexpression of the protein is sufficient to switch IgM heavy chain expression from membrane bound, where polyadenylation occurs at a strong downstream poly(A) site, to the secreted form, by using the upstream weaker 5′ proximal poly(A) site (42, 50). The HPV16 NRE is located

upstream of three potential late polyadenylation sites, LP1, LP2, and LP3. LP2 is functional in HeLa cells *in vitro* (5) and undifferentiated W12 cells *in vivo* (K. McGuire & S.V.G., unpublished results). LP1 is most likely a weak site caused by a poorly functional downstream GU-rich element (5). The up-regulation of CstF-64 levels on epithelial cell differentiation could result in increased recruitment of this polyadenylation factor to the NRE, increasing the overall efficiency of the LP2 site utilization, or activating the NRE-proximal weak LP1 site.

Although we found elevated levels of CstF-64 on differentiation of W12 cells, Terhune *et al.* (51) showed a reduction of CstF-64 levels in differentiating foreskin epithelial cells (LKP-31) transfected with HPV31b DNA. This difference may be attributed either to the different cell line we have used, or to the different methods used to induce differentiation. Suspension in semisolid medium used for the LKP-31 cells provides differentiation signals hypothesized to mimic cell detachment from the basement membrane via integrin signaling pathways (40), whereas for W12 cells, the increase in calcium concentration results in direct activation of protein kinase C (52).

Only cytoplasmic HuR was found to bind the NRE. It is possible that in the nucleus, U2AF$^{65}$ and CstF-64-bound NRE-containing transcripts are less accessible to HuR and its nuclear binding is not detectable. In undifferentiated epithelial cells, sequestration of late gene transcripts, perhaps accomplished by U2AF$^{65}$ and CstF-64 binding of NRE-containing mRNAs may prevent interaction with the nucleocytoplasmic transport machinery or assembly of export-competent hnRNPs (53, 54). On W12 cell differentiation, when the inhibitory activity of the NRE is relieved (16), we observed a change in cellular distribution of HuR paralleled by increased HuR-NRE binding in the cytoplasm. The differentiation signal that induces redistribution of HuR to the cytoplasm may also be responsible for alleviating the inhibitory activity of the NRE. HuR may facilitate export of NRE-containing late mRNAs via its shuttling sequence (29), or alternatively, because the NRE may act as a mRNA instability element *in vitro* (15), redistribution of HuR could stabilize NRE-containing mRNAs in the cytoplasm (28, 55). Usually HuR increases the stability of AU-rich element containing mRNAs (26–28). Although the HPV16 NRE lacks the characteristic AUUUA tandem repeat found in AU-rich element containing transcripts, it has two GUUUG motifs repeated in tandem which resemble the binding sites of HuB, a tissue-specific member of the human ELAV (embryonic lethal abnormal visual system) family (56).

Although HPV16 late proteins are expressed only in terminally differentiated W12 cells, we detected HPV16 late mRNAs in undifferentiated W12 cells, perhaps attributable to rare cells which underwent spontaneous differentiation (4). However, we favor the hypothesis that the presence of mature late gene transcripts confined to the nucleus reflects their nuclear retention or cytoplasmic instability. On differentiation, efficient export and stabilization of the NRE-containing late mRNAs, plus elevated efficiency of the late poly(A) site and increased transcription driven by the differentiation-dependent promoter (8) could allow expression of late L1 and L2 genes. This model is in agreement with the detection of late L1 and L2 HPV16 transcripts in the nuclei of less differentiated cells in the lower layers of squamous intraepithelial lesions, where expression of the late proteins is down-regulated (6). These data provide further evidence that the HPV16 NRE exerts its activity via different mechanisms, with closely spaced or overlapping sequences potentially contributing to regulation at the level of polyadenylation, RNA transport from the nucleus, and cytoplasmic RNA instability.

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