The 3′-untranslated regions (UTRs) of human papillomavirus 16 (HPV16) and bovine papillomavirus 1 (BPV1) contain a negative regulatory element (NRE) that inhibits viral late gene expression. The BPV1 NRE consists of a single 9-nucleotide (nt) U1 small nuclear ribonucleoprotein (snRNP) base pairing site (herein called a U1 binding site) that via U1 snRNP binding leads to inhibition of the late poly(A) site. The 79-nt HPV16 NRE is far more complicated, consisting of 4 overlapping very weak U1 binding sites followed by a poorly understood GU-rich element (GRE). We undertook a molecular dissection of the HPV16 GRE and identify via UV cross-linking, RNA affinity chromatography, and mass spectrometry that is bound by the CUG-binding protein 1 (CUGBP1). Reporter assays coupled with knocking down CUGBP1 levels by small interfering RNA and Dox-regulated shRNA, demonstrate CUGBP1 is inhibitory in vivo. CUGBP1 is the first GRE-binding protein to have RNA interfering knockdown evidence in support of its role in vivo. Several fine-scale GRE mutations that inactivate GRE activity in vivo and GRE binding to CUGBP1 in vitro are identified. The CUGBP1-GRE complex has no activity on its own but specifically synergizes with weak U1 binding sites to inhibit expression in vivo. No synergy is seen if the U1 binding sites are made weaker by a 1-nt down-mutation or made stronger by a 1-nt up-mutation, underscoring that the GRE operates only on weak sites. Interestingly, inhibition occurs at multiple levels, in particular, at the level of poly(A) site activity, nuclear-cytoplasmic shuttling, and transcription of the mRNA. Implications for understanding the HPV16 life cycle are discussed.

The human epidermis consists of layers of keratinocytes that broadly speaking represent separate stages of differentiation (1). The basal layer of cells are the rapidly dividing progeny of the basal layer that gives rise to the stratum corneum. The life cycle of papillomaviruses, small ~8000-bp DNA viruses, is intimately connected to the differentiation program of keratinocytes (2) such that they can readily infect almost any type of mammalian cultured cell but are unable to complete the viral life cycle. Human papillomaviruses (HPVs)2 infect squamous epithelia in the basal layer giving rise to papillomas that can lead to benign or malignant lesions (3). Of the more than 100 types of HPVs that are sequenced, only a handful account for nearly all cervical carcinomas and intraepithelial neoplasias, with HPV type 16 (HPV16) causing about 60% of the cases, making it the most clinically significant of the HPVs and one of the most studied (4, 5). In addition to HPV16, HPV1 that causes non-malignant lesions and bovine papillomavirus 1 (BPV1) have also been intensively studied and serve as prototypes for understanding HPVs (6).

The genome structure of papillomaviruses shares many common features. For example, all of the viral open reading frames are located on one strand of the viral genomic DNA with the early expressed genes being clustered and distinct from the late expressed genes (7–8). Downstream of the late L2 capsid gene open reading frame is the ~850-bp long control region that contains sequence elements that control DNA replication, transcription initiation, and post-transcriptional events (4). Viral early genes are expressed in undifferentiated and intermediately differentiated keratinocytes, whereas the products of the late genes, i.e. the L1 and L2 capsid proteins, are expressed only in the terminally differentiated epithelial layer. The viral P97 early promoter is active throughout all layers of the epithelial and governs transcription of the viral early genes (9). In contrast, the viral P670 (also called P742) late promoter is only active in partially as well as fully differentiated keratinocytes and this depends on cell differentiation factors as well as viral DNA amplification (10–12). Although P670 promoter activation is clearly necessary for late gene expression, many of the experimental approaches to establish this did not rule out changes in transcript stability as an alternative explanation. The fact that late transcripts are detected in partially differentiated keratinocytes is therefore addressed. Tel: 732-445-1016; Fax: 732-445-4213; E-mail: gunderson@biology.rutgers.edu.

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keratinocytes even though late proteins are restricted to terminally differentiated keratinocytes (13, 14) has led to increased focus on post-transcriptional control mechanisms that are now viewed as having an equal if not more important role than transcription activation in viral late gene expression (6). For example, the late transcripts partially overlap with early transcripts such that splicing of intron 2 of L1 removes the early poly(A) site. Cis-acting inhibitory elements, also referred to as negative regulatory elements (NREs), that repress late gene expression at the post-transcriptional level have been mapped to the L1 and L2 open reading frames of HPV16 (15–17), as well as the late 3′-UTRs of HPV16, BPV1, HPV1, and HPV31 (15–20). Among the NREs, BPV1 is the best understood mechanistically (Fig. 1A) and consists of a single autonomously acting 9-nt sequence that exactly matches the consensus 5′ splice site sequence that binds the U1 snRNP splicing factor (21). U1 snRNP is best known for its role in splicing where it nucleates early steps in spliceosome assembly by base pairing to the 5′ splice site. However, when a consensus 5′ splice site is located in the terminal exon, and so lacks a downstream 3′ splice site to catalyze splicing, it potently inhibits gene expression by binding to U1 snRNP leading to inhibition of poly(A) site activity (21–25). We designate as terminal-exon-located 5′ splice site sequences, like the one found in BPV1, U1 binding sites so as to clearly distinguish that they do not function in splicing. A battery of in vivo and in vitro assays has shown the complex formed between the BPV1 U1 binding site and U1 snRNP is sufficient to inhibit the BPV1 late poly(A) site as well as poly(A) site signals from other genes (21–24). The inhibitory mechanism involves an interaction between the 70-kDa subunit of U1 snRNP (U1–70K) and poly(A) polymerase (22). When the poly(A) site is inhibited, the primary transcript fails to mature as it lacks a poly(A) tail and so is degraded by the nuclear exosome. In its natural context the BPV1 U1 binding site is unusually close to the poly(A) signal (7 nt), however, work from many groups has shown that single U1 binding sites inhibit polyadenylation even when placed from 100s up to 1300 nt away from the poly(A) signal (21, 23–25). This has led to a proposal that inhibition involves disruption of the definition of the terminal exon by disrupting interactions between the poly(A) signal and the splicing out of the upstream 3′ terminal intron.

The identification of the highly inhibitory BPV1 NRE (i.e. U1 binding site) led to extensive mapping efforts to identify NREs in HPVs, including the 79-nt HPV16 NRE that spans from the last few nucleotides of the L1 coding region into the viral late 3′-UTR (Fig. 1B) (21, 26, 27). In reporter plasmids where the 3′-UTR and sequences past the poly(A) site are derived from the corresponding HPV16 sequences, expression of the reporter is low. Deletion of the NRE but not other parts of the 3′-UTR resulted in a large increase in expression ranging from 40- to 100-fold depending on the transfection conditions and cell types used (18, 21, 26, 27). As diagrammed in Fig. 1B, the HPV16 NRE can be divided into a 5′ segment and a 3′ GU-rich segment. The 5′ segment contains four weak, overlapping matches to the 5′ splice site consensus sequence that act as weak U1 binding sites (26). Unlike the case with BPV1, however, each of the four weak U1 binding sites of HPV16 has no activity on its own and there are many aspects of the inhibitory mechanism that remain unclear.

The 3′ segment of the HPV-16 NRE is GU-rich and hence is called the GU-rich element (GRE). Several reports have identified cellular factors that bind the GRE including U2AF65, HuR, ASF/SF2, and the CstF64 subunit of the cleavage stimulatory factor (26–29). Importantly, however, none of these GRE-binding proteins has been shown to function in vivo. For example, there are no reports that describe the effect on HPV16 NRE function of overexpression or RNA interference knockdown of these GRE-binding proteins. Although there is evidence in cell culture experiments that ASF/SF2 is up-regulated in HPV16-infected epithelial cells, the studies are correlative rather than causative (29).

Here we undertook a mechanistic analysis of the HPV16 NRE by focusing on the GRE and its interaction with the weak U1 binding sites. On its own the GRE has no inhibitory activity but in conjunction with the upstream weak U1 binding sites gives enhanced inhibition. No enhancement is seen when the upstream weak sites are made stronger by a 1-nt up-mutation or weaker by a 1-nt down-mutation underscoring that the GRE specifically enhances weak U1 binding sites. We identify that CUG-binding protein 1 (CUGBP1) binds the GRE and demonstrates by knockdown studies that CUGBP1 functions in vivo. The specificity of our results is demonstrated by the identification of a fine-scale GRE mutation that inactivates its activity in vivo and its binding to CUGBP1 in vitro. Interestingly, inhibition occurs at multiple levels, in particular at the level of poly(A) site activity, nuclear-cytoplasmic export, and translation of the mRNA. Thus, CUGBP1 is another member of the GRE-binding family of proteins and it is the first member to have direct in vivo knockdown data in support of its role in the function of the HPV16 GRE.

### EXPERIMENTAL PROCEDURES

**Plasmids—** The pSV40/RL reporter (Promega) was modified by insertion of double-stranded DNA oligonucleotides into the XbaI site in the 3′-UTR to make the plasmids in Figs. 1C and 2, A and B. The reporter plasmids shown in Figs. 1D, 2C, 3, and 5C that contain the entire 308-nt HPV16 3′-UTR and 150 nt of the HPV-16 sequence past the late poly(A) site were made by PCR amplification of genomic HPV16 (kindly provided by Dr. Craig Meyers, Penn State University, Hershey PA). The resulting PCR product was inserted into pSV40/RL thereby replacing the SV40 3′-UTR and poly(A) signal sequences with those from HPV16. Mutations of the U1 binding sites and GRE were made by site-directed mutagenesis. All plasmids were verified by sequencing.

**UV Cross-linking and Electrophoretic Mobility Shift Assays (EMSAs)—** Gel purified, 32P-radiolabeled RNA probe was incubated with HeLa nuclear extract (25–50 μg) in binding buffer (BB) (BB = 20 mm HEPES-KOH (pH 7.9), 100 mM KCl, 1.5 mM MgCl2, 5 mM dithiothreitol, 5% glycerol, 2 μg of tRNA) in total volume of 15 μl at room temperature for 15 min. The mixtures were placed as drops on a microowell plate and irradiated on ice with UV light at 254 nm for 10 min using a Stratalinker 2400 (Stratagene). Nonprotected RNA was digested for 20 min at 37 °C by addition of 1 μg of RNase A and then incubated for 10 min.
min in the presence of 0.05% SDS. The proteins were resolved on a 12.5% SDS-PAGE, and the gels were dried and subjected to autoradiography. EMSA was done as previously described (30).

Silencing of CUGBP1 with siRNA and Dox-regulatable shRNA—The RNA oligos for siRNA were synthesized by Dharmacon to target the following human CUGBP1 sequence: 5′-AAATTTGGCTGCACTAGCTGCT-3′. This same siRNA sequence has previously been shown to specifically silence CUGBP1 (31). The siRNA was transfected into HeLa cells using Lipofectamine 2000 plus (Invitrogen). The plasmid used to make the stable HeLa Trex cells that stably express a doxycycline-regulated anti-CUGBP1 shRNA off an H1 promoter was derived from pENTRM/H1/TO (Invitrogen). The shRNA sequence is 5′-TAGCAGCAGTAATTCTGTCAAC-3′ and corresponds to amino acids SSSNSVN of CUGBP1.

Expression and Purification of Recombinant (r) rCUGBP1—The plasmid encoding His-tagged CUGBP1 in a pET15 vector was kindly provided by Maurice Swanson (University of Florida). His-tagged rCUGBP1 was produced in BL21(DE3) cells and purified on Ni2+ -nitrilotriacetic acid (Qiagen). So as to facilitate removal of RNA that may remain bound to rCUGBP1, the purification included an RNase treatment step prior to the Ni2+-nitrilotriacetic acid step. The peak fraction was judged to be >98% pure when analyzed by Coomassie-stained SDS-PAGE (data not shown).

Affinity Chromatography with Biotinylated GRE RNA—Biotinylated GRE RNA was generated by in vitro transcription using a 3:2 ratio of biotin-rCTP to rCTP. 0.2 ml of streptavidin-agarose beads (Sigma) was washed 3 times in 1 ml of binding buffer 2 (BB2 = 200 mM KCl, 20 mM HEPES, pH 7.9, 0.05% Nonidet P-40, 1.5 mM MgCl2) and then preblocked in 1 ml of BB2, 0.5 mg of bovine serum albumin, 50 μg of tRNA, and 50 μg of glycogen by rotating for 30 min. After 2 more washes the beads were resuspended in 0.2 ml of BB2 including 1 mM dithiothreitol, 120 units of RNasin, and 25 μg of biotinylated GRE RNA probe. After 30 min of rotation the beads were washed 2 times and resuspended in 4 ml of BB2 including 1 mM dithiothreitol, 10% glycerol, 40 μg of tRNA, 200 units of RNasin, and combined with 1 ml of HeLa nuclear extract (7 mg of protein). After 30 min of rotation the beads were washed 3 times with 1 ml of BB2. Specifically bound proteins were eluted by addition of Laemmli buffer and separated by SDS-PAGE. Protein bands were cut from a Coomassie Blue-stained gel and submitted for Mass Spectrometry to the University of Medicine and Dentistry of New Jersey, Newark, proteomics facility.

Transfections—HeLa cells were passaged in standard conditions and transfected with Polyfect (Qiagen) as per the manufacturer’s instructions. For assays measuring just luciferase, 100 ng of each plasmid was transfected into 50,000 HeLa cells on a 24-well plate. 48 h after transfection luciferase activity was measured using the dual luciferase assay kit (Promega) as per the manufacturer’s instructions. Normal human epithelial keratinocytes were purchased from Cambrex, grown in keratinocyte growth medium, and transfected with Lipofectamine-2000 plus (Invitrogen).

RNA Preparation and Analysis by Ribonuclease Protection Assay (RPA)—Uniformly 32P-labeled RNA probes were made by in vitro transcription with T7 or SP6 RNA polymerase in the presence of [32P]UTP as previously described (30). For assays measuring mRNA levels, the transfection was scaled up to 4 million cells and 5 μg of plasmid. 48 h after transfection the cells were harvested and split into two parts. 5% of the cells were used to measure luciferase so as to confirm the inhibition of Renilla luciferase activity, whereas the remaining 95% were used either to make total RNA or to make RNA from nuclear and cytoplasmic fractions as below. In all cases RNA was isolated using an RNAeasy kit (Qiagen). Preparation of nuclear and cytoplasmic fractions was done on ice by resuspending the cell pellet in 10 mM Tris (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.05% Triton X-100. After a brief centrifugation at 8000 × g for 1 min, the supernatant was collected as the cytoplasmic fraction. The nuclear pellet was resuspended in 20 mM Tris (pH 7.9), 1.5 mM MgCl2, 420 mM KCl, 25% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. After a brief centrifugation at 14,000 × g for 1 min, the supernatant was collected as the nuclear fraction. Proportional amounts of RNA from the cytoplasmic and nuclear fractions were analyzed by RPA. RPAs were done using gel-purified 32P-radiolabeled RNA probes and protected products were quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software with the final values being corrected for the U content of the probe.

Western Blotting—Samples were separated onto a 12% SDS-PAGE gel, then transferred to an Immobilon-P membrane (Millipore) in transfer buffer (192 mM glycine, 25 mM Tris, 20% (v/v) methanol) with 300 mA, 25 volts, and 10 watts for 12 h. The membrane was then blocked with 1 × PBS, 0.1% Triton, 0.7% (v/v) nonfat milk for 30 min followed by probing with the primary antibody in new blocking solution. The anti-CUGBP1 antibody clone 3B1 (Upstate Biotechnologies, New York) was diluted 1:2000-fold and the anti-GAPDH antibody (Chemicon) was diluted 1:10,000. After 1–2 h of rocking, the membrane was washed twice with 1 × PBS + 0.1% Triton, 5 min for each wash. Then the membrane was incubated for 1 h with horseradish peroxidase antibody (Amersham Biosciences) that recognizes the primary antibody. After being washed twice with 1 × PBS + 0.1% Triton + 1% milk powder, twice with 1 × PBS + 0.1% Triton, and once with 1 × PBS, 5 min each time, the membrane was soaked in ECL reagent (PerkinElmer Life Sciences) for 60 s and exposed to x-ray film.

Northern Blotting—RNA samples were separated on an 8% (24:1) urea-acrylamide denaturing gel, then transferred to an Hybond N+ membrane (Amersham Biosciences) in TBE buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3) at 300 mamps, 10 volts, 5 watts for 3 h. After one wash in ddH2O, the wet membrane was UV cross-linked twice in a Stratalinker, 30 s each time. The membrane was then air-dried and prehybridized at 42 °C by rocking for 30 min in prehyb buffer consisting of 50% (v/v) formamide, 5 × SSC buffer (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 5 × Denhardt’s buffer (1 mg/ml bovine serum albumin, 1 mg/ml polyvinylpyrrolidone, 1 mg/ml Ficoll-400), 1% SDS, and 0.1 mg/ml sonicated salmon sperm DNA.
The HPV16 3′-UTR Cis-element Inhibits by Binding CUGBP1

FIGURE 1. Analysis of the HPV16 NRE: cis-acting elements. A, shown is the well characterized BPV1 NRE that acts as an autonomous U1 snRNP base pairing site herein called a U1 binding site. The U1 snRNP-U1 binding site complex inhibits the BPV1 late gene poly(A) site (PAS) leading to reduced mRNA biosynthesis and hence reduced mRNA levels. B, shown is the structure and sequence of the 79-nt HPV16 NRE that is divided into two parts: one having four weak U1 binding sites and the other being a GRE. Also shown are mutations that inactivate these elements that are used in this work. The three UGUUU repeats are underlined. C, determination of the inhibitory activities of the wild type (wt) or mutated (mt) GRE and U1 binding sites when inserted alone or together into the 3′-UTR of a Renilla reporter construct. Each plasmid was transiently transfected into HeLa cells along with a control firefly reporter to normalize for transfection efficiency. The inhibitory activity of each inserted sequence was calculated by comparing its normalized Renilla luciferase activity to that of the parental pSV40/RL plasmid that was set to 1.0. For example, the normalized Renilla luciferase activity of pSV/wtU1 is 9.1-fold lower than that of pSV40/RL, therefore the 4 weak HPV16 U1 binding sites have an inhibitory activity of 9.1-fold. The inhibitory activity of the 9-nt BPV1 NRE is shown for comparison. 80 past PAS, 80 nt as measured from the poly(A) site to downstream. D, inhibitory activities of the GRE and U1 binding sites were determined as in panel C but in the context of the full-length 308-nt HPV16 3′-UTR and 150-nt of HPV16 sequence past the poly(A) site. Experiments were done as in panel C except that mtU1/GREnt was used as the reference plasmid that had its inhibitory activity set to 1.0. 150 past PAS, 150 nt as measured from the poly(A) site to downstream. For panels C and D, the results are from >5 independent transfection assays.

RESULTS

Analysis of the HPV16 NRE: Cis-acting Elements—To determine the inhibitory activities of the HPV16 U1 binding sites and GRE, we inserted them alone or together into the 3′-UTR of pSV40/RL, a standard Renilla luciferase reporter plasmid as shown in Fig. 1C. pSV40/RL has its 3′-UTR and poly(A) signal sequences derived from the SV40 late poly(A) signal region. As a specificity control, matching plasmids with mutated insertions were also made with the mutations indicated in Fig. 1B. The mutated HPV16 U1 binding sites are identical to a previously published mutation (21, 27) that inactivates this element because it inactivates base pairing to U1 snRNP. In contrast there is no published fine-scale substitution mutant of the GRE. Given that it has three UGUUU repeats (Fig. 1B, underlined) we chose to mutate all 3 repeats to acaau to make the GREnt mutant as shown. For the sake of comparison we also tested the inhibitory activity of the well characterized 9-nt single BPV1 U1 binding site by inserting it into the same position of pSV40/RL. Each plasmid was transfected into HeLa cells and the resulting Renilla luciferase activity (after 48 h) normalized to a co-transfected firefly reporter that was present in all transfections. Consistent with previous results, insertion of the single BPV1 U1 binding site resulted in strong (17-fold) inhibition of normalized Renilla luciferase activity as compared with the original pSV40/RL plasmid. The inhibition is specific as insertion of a mutated BPV1 U1 binding site gave no inhibitory activity. Insertion of the weak HPV16 U1 binding sites resulted in a 9.1-fold inhibition, consistent with their poor base pairing potential to U1 snRNP and in agreement with prior work (21, 27). The specificity of this inhibition was demonstrated by the fact that mutating each site, so that its base pairing potential to U1 snRNP was weakened by 1 base pair, caused complete loss of inhibitory activity. In contrast to the U1 binding sites, neither the wild type nor the mutated HPV16

million disintegrations/min of [32P]RNA probes, either anti-U1 snRNA alone or with anti-U6 snRNA were then added to the prehyb buffer and incubated with the membrane at 42 °C by rocking for 12 h or overnight. After hybridization, the membrane was washed twice with 2× SSC buffer (0.3 M NaCl, 30 mM sodium citrate), 5 min for each wash at room temperature; twice with 2× SSC buffer, 0.1% SDS, 30 min for each wash at 55–60 °C. The final wash was twice with 2× SSC, 1 min each time at room temperature and then the membrane was exposed to x-ray film.
The HPV16 3′-UTR Cis-element Inhibits by Binding CUGBP1

To determine the inhibitory activity of these elements in the more natural context of the HPV16 3′-UTR, the experiments in Fig. 1C were repeated using reporter plasmids where the entire 3′-UTR and sequences 150 nt past the poly(A) site were derived from HPV16. So as to not alter spacing between the various elements, we limited the analysis to testing substitution rather than deletion or insertion mutants. As shown in Fig. 1D, inhibitory activities were comparable with that observed in Fig. 1C indicating the U1 binding sites and the GRE function in the context of the natural 3′-UTR. Thus we conclude: 1) the GRE has no activity on its own; 2) the weak HPV16 U1 binding sites have modest activity on their own as compared with the BPV1 U1 binding sites; and 3) when the GRE and weak HPV16 U1 binding sites are placed together, a much stronger synergistic inhibition is observed. HeLa cells are derived from basal cervical carcinoma cells and so are a good mimic of the natural host cell. That said, they are immortalized and contain HPV16-derived genes and so only approximately match the natural host cell. Given these considerations, we repeated the Fig. 1D transfections but replaced HeLa cells with normal human epithelial keratinocytes, the natural host for HPV16, and found the same inhibitory pattern indicating the results with normal human epithelial keratinocytes are the same as with HeLa (data not shown).

The HPV16 GRE Specifically Synergizes with Weak, but Not Strong, U1 Binding Sites—It is striking that the GRE and the HPV16 U1 binding sites synergize. Given the complex, overlapping configuration of the four weak HPV16 U1 binding sites, it seemed plausible that this synergy would not be observed with other U1 binding sites. To test this, a series of reporter plasmids, having a simple, single U1 binding site that ranged from very weak to strong, were made (Fig. 2A). U1ε has the weakest U1 binding site as it can only make a 6-bp duplex with U1 snRNP. U1δ is incrementally stronger as it can make a 7-bp duplex and U1γ is the strongest as it can make an 8-bp duplex. Therefore, a finer scale GRE mutation, where the three weak U1 sites are not accessible to U1 snRNP. To examine this possibility, a finer scale GRE mutation, where the three UGUUU repeats were mutated to UGaaU, was tested and we found the same loss of inhibitory activity as the acaaU mutation (data not shown). Thus our data support the view that these loss of function mutations in the GRE specifically affect GRE function and are not due to a gross rearrangement of the folding pattern of the RNA.

GRE had significant activity on its own. When, however, the wild type GRE was placed with the weak HPV16 U1 binding sites, the inhibitory activity was significantly boosted from 9.1- to 52.2-fold. Thus placement of both elements together gave a synergistic level of inhibition. The specificity of this synergy is supported by the fact that the mutated GRE only slightly raised inhibition from 9.1- to 13-fold. One concern was the Gre mutation changes 12 nt and so it may have additional undesired effects on the activity of the mRNA. For example, having so many changes may induce a misfolding of the RNA such that the weak U1 sites are not accessible to U1 snRNP. To examine this possibility, a finer scale GRE mutation, where the three UGUUU repeats were mutated to UGaaU, was tested and we found the same loss of inhibitory activity as the acaaU mutation (data not shown). Thus our data support the view that these loss of function mutations in the GRE specifically affect GRE function and are not due to a gross rearrangement of the folding pattern of the RNA.

FIGURE 2. The GRE enhances activity of weak, but not strong, U1 binding sites. A, as was done in Fig. 1C, three reporter plasmids were made from pSV40/RL by insertion of a single U1 binding site that had incrementally increased base pairing potential with U1 snRNP. As indicated shading, the U1ε plasmid has a U1ε binding site that potentially makes a 6-bp duplex with U1 snRNP, the U1δ plasmid can potentially make a 7-bp duplex, and U1γ can potentially make an 8-bp duplex. Three matching plasmids were made by inserting the GRE immediately downstream of the single U1 binding site keeping the spacing as found in HPV16. As was done in Fig. 1C, the inhibitory activity of each of the six plasmids was determined by transfection into HeLa cells. As in Fig. 1C, the experiments in panel A were repeated with the only difference being the plasmids contained two tandem U1 binding sites in place of the single U1 binding site. This was done to more closely mimic the HPV16 case. C, the ability of the GRE to enhance only weak U1 binding sites was tested in the context of the full-length 308-nt HPV16 3′-UTR sequence past the poly(A) site. U1-up4 is where the fourth U1 binding site is up-mutated to make a 10-bp duplex with U1 snRNP. Transfections were done as in panel B and the inhibitory values calculated using the reference plasmid mtU1/GREmt that had its inhibitory value set to 1.0. The results are from 4 independent transfection assays.
synergizes only with the U1₁₆ site. In contrast, the very weak U₁₆ site is unable to synergize with the wtGRE. As expected, the U₁₆ site has no activity on its own, consistent with our previous work that 6-nt U1 sites have no activity because they cannot base pair to U1 snRNP (22, 24). Equally striking is the observation that no synergy is observed with the intrinsically strong U₁₆ binding site.

The configuration of the overlapping U1 binding sites in HPV16 is such that it is likely only two U1 snRNPs can bind simultaneously. To more closely mimic the HPV16 configuration, the experiments in Fig. 2A were repeated but with two tandem U1 binding sites in place of the single site (Fig. 2B). The results as shown in Fig. 2B indicate the inhibitory pattern seen with the two tandem U1 binding sites matches what we observe with the single U1 binding site in that synergy with the GRE was only observed with the U₁₆ sites. Thus the GRE can synergize with both single or multiple upstream U1 binding sites as long as they are neither too strong nor too weak. We point out that the high level of inhibition (188-fold) seen with the U₁₆ sites has been observed by us and others in prior work were two tandem U1 sites inserted into a reporter plasmid give inhibitory levels in the 100–700-fold range (21, 24). To determine whether the specificity of this synergy could be observed in the context of the natural HPV16 sequences, the fourth U1 binding site in the wtU1/ wtGRE plasmid in Fig. 1D was up-mutated so that it matched the consensus U1 snRNP binding site. This up-mutation, designated U1-up4, was placed with either a wtGRE or a mutated GRE and the inhibitory activities determined by transfection into HeLa cells. The results as given in Fig. 2C indicate the U1-up4 mutation has strong activity that was not GRE-dependent, meaning its inhibitory activity could not be further increased when the wild type GRE was present. Thus we conclude that HPV16 GRE specifically enhances weak, but not strong or very weak U1 binding sites.

The HPV16 NRE Affects mRNA Biosynthesis and Nuclear Export—Prior work from a number of groups has shown that a single strong U1 binding site, having a ≥8 of 10 match to the consensus sequence, inhibits poly(A) site activity leading to reduced mRNA levels (Refs. 23 and 24 and Introduction). Although we expected the HPV16 NRE to do likewise, the unusual synergy with the GRE indicated a more thorough analysis was in order. Furthermore, the NREs of other well-characterized HPVs affect disparate steps in gene expression (see Introduction). As a rigorous method to quantitate mRNA levels produced from the HPV16-containing Renilla reporters, we employed a RPA where the Renilla RPA probe includes 100 nt of unrelated vector sequence and 295 nt that span the Renilla coding region, thus giving a 295-nt protected fragment. As a control, we also performed RPA to measure endogenous GAPDH mRNA levels using a probe derived from a commercially available plasmid that gives a 307-nt protected product.

In Fig. 3A, RPA was used to measure transcript levels produced from the Renilla reporter and from endogenous GAPDH from total RNA isolated from transfected and untransfected HeLa cells. The transcript levels produced by the mtU1/GREmt and mtU1/wtGRE plasmids were similar to the original pSV40/RL Renilla reporter plasmid, whereas the wtU1/GREmt plasmid produced about 7-fold less transcript. The RPA data for these four plasmids closely correlates with the Renilla luciferase activity seen in Figs. 1, C and D, indicating the reduced Renilla level for the wtU1/GREmt plasmid is due to a reduced transcript level. Such a correlation was not observed, however, for the wtU1/wtGRE plasmid, as it produced about the same amount of transcript as the wtU1/GREmt plasmid but far less (~5-fold) Renilla luciferase activity. When lower amounts of these plasmids (down to 0.25 μg) were transfected a similar pattern of Renilla luciferase activity and mRNA levels was observed arguing that factors that regulate these HPV16 elements are not limiting in vivo (data not shown). As shown in supplementary Fig. S1A, the amounts of total RNA were varied so as to facilitate the quantitation and to confirm that we were in the linear range of the assay.

Although we expected the reduced mRNA levels produced from the wtU1/GREmt plasmid would be due to inhibition of poly(A) site activity, it was also possible that they could arise from changes in mRNA stability, especially as the contribution of GRE to the inhibitory mechanism is unknown. Thus, we determined the mRNA half-lives of transcripts produced from each plasmid by measuring mRNA decay rates after arresting transcription with actinomycin D. As shown in supplementary Fig. S1B, the mRNA half-lives of the four HPV16 reporter plasmids were similar (~2.5 h) indicating that the different levels of mRNA are not due to differential transcript stability. Therefore, we considered a model where the combination of the GRE and weak HPV16 U1 binding sites promotes nuclear retention of the mRNA. Such a nuclear retention would be in addition to the reduced mRNA levels seen with wtU1/GREmt plasmid. To test this, nuclear and cytoplasmic fractionation of the transfected cells was done, total RNA was extracted from each fraction and analyzed by RPA. Measurement of U1 snRNA by Northern blotting (Fig. 3B, lanes 11–23) and U6 snRNA and GAPDH (supplementary Fig. S2) indicated the fractionation was successful in that there was only limited cross-contamination of the two compartments. Fig. 3B, lanes 1–10, is a representative RPA and the right side of Fig. 3C gives the quantitation of three independent experiments. Of the four HPV16 reporters only wtU1/wtGRE had an unusual nuclear retention pattern in that a smaller proportion of its mRNA was found in the cytoplasm. This is most apparent when comparing the wtU1/wtGRE with the wtU1/GREmt plasmids in that the latter produced 2.2-fold less cytoplasmic mRNA even though both plasmids had a similar level of nuclear transcripts. Given that these two plasmids had a 5-fold difference in Renilla luciferase activity, the nuclear retention activity only accounts for part of the synergistic inhibition. Thus it is likely that the remaining inhibition occurs in the cytoplasm, presumably at the level of translation. Nevertheless, the data clearly show the combination of the GRE and U1 binding sites leads to increased nuclear retention. Finally, we repeated the actinomycin D experiments to test the half-lives of the nuclear and cytoplasmic fractionated transcripts and found no change in half-life in the different cellular compartments (data not shown). Thus the different levels of cytoplasmic mRNA are not due to differential mRNA stability.

Identification That CUGBP1 Binds the GRE—To determine what factor(s) bind the HPV16 GRE we performed UV cross-linking with [32P]-uniformly labeled RNA containing the 79-nt
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FIGURE 3. Affect of the GRE and U1 binding sites on mRNA. A, the upper autoradiogram is an RPA with an antisense RNA probe corresponding to the coding region of the Renilla reporter plasmid. Lane 2 contained 20 μg of total RNA from untransfected cells. Lanes 3–7 contained 20 μg of total RNA from 4 million HeLa cells transfected with 5 μg of each plasmid as indicated. Lane 1 contained 20 μg of yeast RNA in place of the total RNA. The protected Renilla reporter-specific band is predicted to be 295 nt. The lower autoradiogram matches the 7 lanes of the upper panel except the probe is designed to detect endogenous GAPDH mRNA and only 5 μg of total RNA or tRNA were used. On the right are indicated the sizes in nucleotides of 32P-labeled Msp markers (see panel B). B, RPA was used to analyze equal amounts of RNA isolated from cytoplasmic (C) (lanes 1–5) and nuclear (N) (lanes 6–10) fractions of transfected and untransfected cells. To assess the quality of the nuclear and cytoplasmic fractionation, Northern blotting to detect U1 snRNA (lanes 14–23) was done with 2 μg of RNA loaded per lane. Lanes 14–23 are the same RNA samples as lanes 1–10. Lanes 11–13 were done to assess the dose response of the Northern blot to detect U1 snRNA. C, quantitation of the RPAs and comparison with Renilla luciferase activities. The four bar graphs on the left are from quantitation of the RPA signals from total RNA samples in panel A and supplemental Fig. S1A and from 2 other independent transfection/RPA experiments. As the reference point, the Renilla mRNA signal from the transfected mtU1/GREmt plasmid was set to 100%. The eight bar graphs in the middle are RPA signals of total RNA derived from the cytoplasmic (C) and nuclear fractions (N) with the values and error bars derived from 3 independent transfection assays. The cytoplasmic RNA from the transfected mtU1/GREmt plasmid serves as the reference point for the cytoplasmic RNA samples and is set to 100%. Likewise the nuclear RNA from the transfected mtU1/GREmt plasmid serves as the reference point for the nuclear RNA samples and is set to 100%. All of the RPAs were quantitated by phosphoimagery using ImageQuant software. The four bar graphs on the right are Renilla activities of these plasmids taken from Fig. 1D and are shown here to facilitate a side-by-side comparison of mRNA levels with Renilla luciferase activities.

wild type HPV16 NRE incubated in HeLa nuclear extract (Fig. 4A). After UV irradiation, excess RNA was removed by treatment with RNase A under semidenaturing conditions and the samples analyzed by SDS-PAGE. Fig. 4A is a representative autoradiograph where the most dominant polypeptide band as bind to and effect the stability of the interleukin-2 mRNA (32). As shown in the supplemental Fig. S3, the fourth polypeptide was CUGBP1 that has a reported mobility of 54 kDa clearly marking it as the likely candidate to be the 54-kDa UV cross-linked band.
CUGBP1 is a member of the embryonic lethal abnormal vision-type RNA-binding protein family and has 3 conserved RNA recognition motifs (31, 33–35). CUGBP1 is ubiquitously expressed in many tissues, is found both in the nucleus and cytoplasm, and has one known phosphorylated form. CUGBP1 is best known for its role in CUG trinucleotide repeat diseases such as myotonic dystrophy where it binds tightly to nuclear RNAs with >200 CUG repeats (for a review, see Ref. 35). However, CUGBP1 can also regulate alternative pre-mRNA splicing by the HPV16 GRE.

Knockdown of CUGBP1 Levels Alleviates GRE-mediated Inhibition—Having established that the GRE is specifically bound by CUGBP1, we wanted to determine whether CUGBP1 functions in vivo. To this end we performed two types of CUGBP1 knockdown experiments, one being a transient knockdown with a published anti-CUGBP1 siRNA (31) that specifically knocks down CUGBP1 levels, and the second being a stable but regulatable knockdown with shRNA. Although both approaches gave similar results, the siRNA approach was more problematic due to variable levels of knockdown (data not shown) and so we focused our efforts on the stable shRNA approach. HeLa Trex cells (Invitrogen) are HeLa cells engineered to stably express the tetracycline transactivator protein so that it in turn can activate transcription as part of the “Tet-On” system where addition of doxycycline (Dox), a more stable analog of tetracycline, activates tetracycline transactivator so that it in turn can activate transcription of genes with Tet operons. Individual clones of stable HeLa Trex cells were produced that stably express an anti-CUGBP1 shRNA driven off an H1 promoter containing Tet operons. As shown in Fig. 5A, the addition of Dox to this stable clone results in a 3-fold reduction in CUGBP1 levels as compared with unin-
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**FIGURE 5.** Silencing of CUGBP1 specifically affects expression of an HPV16-derived reporter. A, shown are Western blots to demonstrate specific silencing of CUGBP1. HeLa Trex cells (Invitrogen) were used to make a stable cell line (called anti-CUGBP1 shRNA HeLa) that stably expresses a doxycycline-regulatable shRNA designed to target endogenous CUGBP1. Lane 1 is from uninduced and lane 2 from doxycycline-induced anti-CUGBP1 shRNA HeLa Trex cells. Lane 3 is from untreated and lane 4 from doxycycline-treated HeLa Trex cells that do not contain an shRNA plasmid. 0.03 μg/ml Doxycycline was used and 20 μg of protein were loaded on each lane. The upper panel was probed with anti-CUGBP1 antibody and the lower panel with anti-GAPDH antibody. B, 50 μg of whole cell lysate made from the same cells in lanes 1 and 2 of panel A were used in a UV cross-linking assay. The probe is the same as in Fig. 4A, lane 1. C, the wild type and mutated reporter plasmids from Fig. 1D were transfected into uninduced or doxycycline-induced anti-CUGBP1 shRNA HeLa Trex cells and inhibitory activities were calculated as described in the legend to Fig. 1. The hatched bar graphs are from untreated and doxycycline-treated HeLa Trex cells that do not contain an shRNA plasmid. Results are from three independent transfections.

produced cells (Fig. 5A, compare lanes 1 and 2). Note the upper band of the doublet is likely phosphorylated CUGBP1 because the position of the band and intensity relative to the major band matches what has been reported for the phosphorylated form of this protein in HeLa cells (35). As a control, GAPDH levels were not affected by Dox treatment. Re-probing the blot demonstrated that the level of other proteins, such as NFAT, β-actin, and the U1 snRNP-associated proteins U1A and U1−70K were not affected by Dox (data not shown). Additionally no knockdown was observed when the original HeLa Trex cell line was treated with Dox (Fig. 5A, lanes 3 and 4). GAPDH was also probed by Western blotting to demonstrate the specificity of the knockdown.

Having a stable knockdown of CUGBP1 allowed us to directly test whether the 54-kDa UV cross-link in Fig. 4 was indeed CUGBP1. To this end we made total cell lysates from the cells shown in Fig. 5A, lanes 1 and 2, and performed the UV cross-linking assay as was done in Fig. 4. As can be seen the Dox treatment specifically reduced the levels of the 54-kDa cross-link relative to the nonspecific cross-linked bands. Note that the overall cross-link pattern in Fig. 5B has a higher level of nonspecific bands as compared with Fig. 4A. This is because total cell extract was used rather than nuclear extract. As was done in Fig. 4A, the specificity of the CUGBP1 cross-link was confirmed by parallel experiments with radiolabeled RNA having the mutated GRE (data not shown). Thus, these experiments demonstrate that CUGBP1 is the polypeptide in HeLa cell extracts that cross-links to the GRE-containing RNA.

To determine whether reduced CUGBP1 levels would affect GRE activity in vivo, we transfected the HPV16-derived reporter plasmids in Fig. 1D into the anti-CUGBP1 shRNA stable cells grown either with or without Dox. In the absence of Dox the inhibitory activity is GRE-dependent, with an inhibitory pattern similar to that of ordinary HeLa cells in Fig. 1 that do not express the tetracycline transactivator protein. In the presence of Dox, CUGBP1 levels are reduced and this specifically affected the inhibitory activity of wtU1/wtGRE but not when the GRE was mutated. Other anti-CUGBP1 shRNA stable clones gave similar results indicating the GRE-dependent effect seen with the CUGBP1 knockdown is not specific to a particular clone. Thus we conclude the CUGBP1-GRE complex specifically synergizes with the U1 snRNP-U1 binding site complex to give enhanced inhibition of the expression of the viral late 3′-UTR and poly(A) signal sequences.

**DISCUSSION**

We show CUGBP1 binds to the HPV16 GRE and synergizes with the upstream weak U1 binding sites to give enhanced inhibition of the viral late poly(A) site. Multiple lines of evidence support this conclusion. Both a 12-nt and a finer scale 6-nt mutation of the GRE leads to loss of this inhibitory activity in vivo whereas, RNAs with these same mutations exhibit loss of CUGBP1 binding in vitro. Knockdown of CUGBP1 levels in vivo also reduces inhibitory activity and importantly this reduction is only seen with the wild type, but not the mutated, GRE. Although the relevance of the CUGBP1-mediated inhibition to the viral life cycle still needs to be established in cells that produce infectious virions (e.g. in raft culture), this is the first in vivo data directly showing GRE-binding protein functions in vivo and as such identifies it as a target for future work. We also examined which steps in the biosynthesis and activity of mRNA are affected by the GRE and U1 binding sites. The GRE alone has no inhibitory activity whereas, the U1 binding sites alone inhibit mRNA biosynthesis in the nucleus, most likely at the poly(A) site, rather than transcription initiation, as this is how U1 binding sites function in other contexts (21–25). When both elements are together in their natural context, additional levels of inhibition occur, namely at the level of nuclear retention and cytoplasmic function of the mRNA, the latter presumably at the level of translation. In contrast, the U1 binding sites and the GRE had no effect on mRNA stability. Interestingly, the GRE specifically synergizes with weak but not strong U1 binding sites even when a single simple U1 binding site is used. Finally we caution that although our data indicate an important role for CUGBP1, it is nearly certain that additional factors are involved due to the inherent complex nature of the HPV16 NRE and that its inhibitory activity changes during keratinocyte develop-
Role of CUGBP1—CUGBP1 was originally characterized through its involvement with trinucleotide repeat diseases such as myotonic dystrophy (DM) where it binds to CUG repeats (typically 100s up to 1000s of repeats are found) located in the 3'-UTR of the DM1 gene (31, 33–39). DM pathology arises not by effecting the function of the DM1 gene but rather from sequestration of CUGBP1 thereby preventing it from performing other functions. Despite much work, however, the mechanistic role that CUGBP1 has in the DM disease remains unclear. Interestingly, CUG repeats are also bound by the muscle-blind like 1 (MBNL1) protein that is a major antagonist of CUGBP1 function both in DM1-related diseases as well as during normal development. The best characterized example of this is where both proteins function as regulators of alternative splicing during development of skeletal tissue and heart muscle (35). During postnatal heart development, CUGBP1 is down-regulated and its function is replaced by that of MBNL1. CUGBP1 also stimulates translation of the p21 gene although in this case it seems unlikely that MBNL1 plays a role (34). In general CUGBP1 is ubiquitously expressed in many tissues, is found in the nucleus and cytoplasm, and has one known phosphorylated form. The fact that CUGBP1 is down-regulated in a variety of differentiation systems suggests a model that it is down-regulated during keratinocyte differentiation thereby leading to up-regulation of the HPV16 late poly(A) site. Given that other models are also plausible, such as binding of antagonistic factors (e.g. MBNL1), future work will be needed to establish how the CUGBP1-mediated repression is alleviated. Whatever the mechanism may be, our CUGBP1 knockdown data directly demonstrates that the consequences of lowering CUGBP1 are to increase expression from reporters containing the full-length HPV16 3'-UTR and that this increase depends on the GRE.

CUGBP1 Phosphorylation—An extensive literature has shown that phosphorylated CUGBP1 levels increase in a variety of cell types and growth conditions and that this is associated with increased translation of CUGBP1-targeted mRNAs (39). Interestingly, the GRE-RNA affinity purified material gave a doublet in the 54-kDa size range with the upper band being less abundant than the lower band. Analysis by mass spectrometry of both bands identified the lower as CUGBP1 (see “Results” and supplemental Fig. S3) and the upper band as phosphorylated CUGBP1 (data not shown). Notably, we did not visualize the phosphorylated form in the UV cross-linking assay. We suspect this is because it is far less abundant than the main unphosphorylated CUGBP1 species and UV cross-linking causes band broadening due to trace amounts of cross-linked RNA remaining attached to the polypeptide due to incomplete RNase digestion. At present we cannot determine how well the phosphorylated CUGBP1 binds the GRE as compared with the unphosphorylated form, thus the functional significance of our detection of phosphorylated CUGBP1 remains unclear.

Regulation of U1 Binding Site Activity—Our original intention in initiating these experiments was to gain a mechanistic understanding of the function of the weak U1 HPV16s binding sites and whether their activity would be influenced by flanking sequences. Up to now, U1 binding sites had only been found in papillomaviruses and it was of particular interest to determine whether cellular genes utilize U1 binding sites within terminal exons to regulate gene expression. We just recently identified the first example of a mammalian gene having a natural U1 binding site (40). Although this site matches the consensus sequence, and so should be constitutively active, its activity is influenced by two types of flanking sequences, one that represses via an RNA secondary structure and the other that stimulates via binding a trans-acting factor. Thus, like the weak HPV16 U1 binding sites, the inhibitory activity of strong U1 binding sites can be readily regulatable and so represent an additional mechanism for the cell to regulate the biosynthesis and activity of mRNA. Currently we have used bioinformatic approaches to identify genes having consensus or near consensus U1 binding sites in their terminal exon as these would be predicted to repress expression by poly(A) site inhibition. Such bioinformatic approaches will now have to include the possible influence of flanking sequences on U1 binding site activity thereby significantly broadening and compounding the scope of such an analysis.

The Role of Other GRE-binding Proteins—Several reports have shown U2AF65, ASF/SF2, HuR, and CstF64 to be part of a complex that binds to the HPV16 GRE (26–29) where U2AF65 directly cross-links to the GRE. We do not understand why our approach presented here failed to detect specific binding of these factors, especially U2AF65, but we suspect differences in extract preparation and conditions used for UV cross-linking and RNA affinity purification play a major role. We would argue that the most rigorous method to demonstrate specific binding to the GRE is to use a mutated control GRE RNA sub-state that differs from the wild type in as few positions as possible. These prior reports used control RNAs having deletions or even unrelated RNAs as specificity controls and this fact significantly reduces the rigor of these assays and compounds a reliable interpretation of the data. Also it is generally recognized that UV cross-linking is highly idiosyncratic and provides an unreliable measure of the affinity of a protein for its cognate RNA. Indeed, one of the highest affinity, sequence-specific RNA-binding proteins known, the U1A protein, is unable to give detectable cross-links to its target RNA unless a photocross-link group is built into the RNA target (41). Furthermore, unlike CUGBP1, none of these GRE-binding proteins has been subjected to in vivo testing by overexpression or RNA interference-mediated knockdown.

With all that said, we wish to state that our data does not rule out the involvement of U2AF65, or these other GRE-binding proteins, in the HPV16 late gene system. Perhaps our selection conditions are too stringent resulting in loss of these factors from the complex which, incidentally, would be consistent with their having a regulatory role. Nevertheless, our analysis has progressed much further in that we have fine-scale mutations that inactivate the GRE and we observe a tight correlation of CUGBP1 binding activity with inhibitory activity seen in vivo. Furthermore, the role of CUGBP1 in vivo is supported by two types of silencing experiments involving siRNA and Dox-regulated shRNA, something not yet done with ASF/SF2, U2AF65, CstF64, or HuR.
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An important question for further work will be to determine the mechanism of synergy between the GRE and U1 binding sites. Of particular interest is the exquisite specificity in that the GRE only synergizes with weak, but not strong, U1 sites. It will be important to determine whether the GRE-CUGBP1 complex forms in the absence of the U1 binding sites as that would indicate whether the inhibitory mechanism is based on synergistic binding or synergistic inhibition, the latter a specific property of the larger ternary complex. Ultimately, the relevance of any work done on HPV16 will need to be tested in a system that supports the full virus life cycle. To this end the effect of GRE mutations in the context of the full-length virus on viral activity in raft culture, the only cell culture system known to produce infectious virions, will need to be tested. Our current efforts are being directed to answer these questions.

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REFERENCES

1. Fuchs, E., and Raghavan, S. (2002) *Nat. Rev. Genet.* 3, 199–209
2. zur Hausen, H. (1996) *Biochim. Biophys. Acta* 1288, F55–F78
3. Howley, P. M. (1996) in Fields *Virology* (Fields, B. N., Knipe, D. M., and Howley, P. M., eds) 3rd Ed., Vol. 2, pp. 3074–3083, Lippincott-Raven, Philadelphia
4. zur Hausen, H., and Schneider, A. (1987) in *The Papovaviridae* (Salzman, N., and Howley, P. M., eds) pp. 245–263, Plenum Press, New York
5. Bosch, F. X., Manos, M. M., Munoz, N., Sherman, M., Jansen, A. M., Peto, J., Schiffman, M. H., Moreno, V., Kurman, R., and Shah, K. V. (1995) *J. Natl. Cancer Inst.* 87, 796–802
6. Zheng Z. M., and Baker, C. C. (2006) *Front. Biosci.* 11, 2286–2302
7. Chan, S.-Y., Delius, H., Halpern, A. L., and Bernard, H.-U. (1995) *J. Virol.* 69, 3074–3083
8. Baker, C. C. (1990) in *Papillomavirus and Human Cancer* (Psister, H., ed) pp. 91–112, CRC Press Inc., Boca Raton, FL
9. Smotkin, D., and Wettstein, F. O. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 4680–4684
10. Grassmann, K., Wilczynski, S. P., Cook, N., Rapp, B., and Iftner, T. (1996) *Virology* 223, 185–197
11. Longworth, M. S., and Laimins, L. A. (2004) *J. Virol.* 78, 3533–3541
12. Bodily, J. M., and Meyers, C. (2005) *J. Virol.* 79, 3309–3321
13. Stoler, M. H., Wolinsky, S. M., Whitbeck, A., Broker, T. R., and Chow, L. T. (1989) *Virology* 172, 331–340
14. Barksdale, S. K., and Baker, C. C. (1993) *J. Virol.* 67, 5605–5616
15. Collier, B., Gooblar-Larsson, L., Sokolowski, M., and Schwartz, S. (1998) *J. Biol. Chem.* 273, 22648–22656
16. Tan, W., Felber, B. K., Zoлотухин, А. S., Pavlakis, G. N., and Schwartz, S. (1995) *J. Virol.* 69, 5607–5620
17. Sokolowski, M., Tan, W., Jelline, M., and Schwartz, S. (1998) *J. Virol.* 72, 1504–1515
18. Kennedy, I. M., Haddow, J. K., and Clements, J. B. (1990) *J. Virol.* 64, 1825–1829
19. Furth, P. A., and Baker, C. C. (1991) *J. Virol.* 65, 5806–5812
20. Cumming, S. A., Repellin, C. E., McPhilips, M., Radford, J. C., Clements, J. B., and Graham, S. V. (2002) *J. Virol.* 76, 5993–6003
21. Furth, P. A., Choe, W., Rex, J. H., Byrne, J. C., and Baker, C. C. (1994) *Mol. Cell. Biol.* 14, 5278–5289
22. Gunderson, S. I., Polycarpou-Schwarz, M., and Mattaj, I. W. (1998) *Mol. Cell* 1, 255–264
23. Beckley, S. A., Liu, P., Stover, M. L., Gunderson, S. I., Lichtler, A. C., and Rowe, D. W. (2001) *Mol. Cell. Biol.* 21, 2815–2825
24. Fortes, P., Cuevas, T., Guan, F., Liu, P., Pentlicky, S., Jung, S. P., Chantar, M. L., Prieto, J., Rowe, D., and Gunderson, S. I. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 8264–8269
25. Wassarman, K. M., and Steinz, A. J. (1993) *Genes Dev.* 7, 647–659
26. Koffa, M. D., Graham, S. V., Takagaki, Y., Manley, J. L., and Clements, J. B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 4677–4682
27. Cumming, S. A., McPhilips, M. G., Veerapradsit, T., Milligan, S. G., and Graham, S. V. (2003) *J. Virol.* 77, 5167–5177
28. Dietrich-Goetz, W., Kennedy, I. M., Levens, B., Stanley, M. A., and Clements, J. B. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 163–168
29. McPhilips, M. G., Veerapradsit, T., Cumming, S. A., Karali, D., Milligan, S. G., Boner, W., Morgan, I. M., and Graham, S. V. (2004) *J. Virol.* 78, 10598–10605
30. Gunderson, S. I., Vagner, S., Polycarpou-Schwarz, M., and Mattaj, I. W. (1997) *Genes Dev.* 11, 761–773
31. Timchenko, N. A., Patel, R., Jakova, P., Cai, Z. J., Quan, L., and Timchenko, L. T. (2004) *J. Biol. Chem.* 279, 13129–13139
32. Kao, P. N., Chen, L., Brock, G., Ng, J., Kenny, J., Smith, A. J., and Cortes, B. (1994) *J. Biol. Chem.* 269, 20691–20699
33. Timchenko, N. A., Cai, Z. J., Welm, A. L., Reddy, S., Ashizawa, T., and Timchenko, L. T. (2001) *J. Biol. Chem.* 276, 7820–7826
34. Phillips, A. V., Timchenko, L. T., and Cooper, T. A. (1998) *Science* 280, 737–741
35. Ramun, L. P., and Cooper, T. A. (2006) *Annu. Rev. Neurosci.* 29, 259–277
36. Takahashi, N., Sasagawa, N., Suzuki, K., and Ishiiura, S. (2000) *Biochem. Biophys. Res. Commun.* 277, 516–523
37. Faustino, N. A., and Cooper, T. A. (2005) *Mol. Cell. Biol.* 25, 879–887
38. Marquis, J., Paillard, L., Audic, Y., Cosson, B., Danos, O., Le Bec, C., and Osborne, H. B. (2006) *Biochem. J.* 400, 291–301
39. Timchenko, L. T., Salisbury, E., Weng, G. L., Nguyen, H., Albrecht, J. H., Hersh, J. W., and Timchenko, N. A. (2006) *J. Biol. Chem.* 281, 32806–32819
40. Guan, F., Caratozzolo, R. M., Goracznia, R., Ho, E. S., and Gunderson, S. I. (2007) *RNA (Cold Spring Harbor)*, in press
41. Stump, W. T., and Hall, K. B. (1995) *RNA (Cold Spring Harbor)* 1, 55–63