SF2/ASF Binds the Human Papillomavirus Type 16 Late RNA Control Element and Is Regulated during Differentiation of Virus-Infected Epithelial Cells

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Received 2 February 2004/Accepted 13 May 2004

Pre-mRNA splicing occurs in the spliceosome, which is composed of small ribonucleoprotein particles (snRNPs) and many non-snRNP components. SR proteins, so called because of their C-terminal arginine- and serine-rich domains (RS domains), are essential members of this class. Recruitment of snRNPs to 5' and 3' splice sites is mediated and promoted by SR proteins. SR proteins also bridge splicing factors across exons to help to define these units and have a central role in alternative and enhancer-dependent splicing. Here, we show that the SR protein SF2/ASF is part of a complex that forms upon the 79-nucleotide negative regulatory element (NRE) that is thought to be pivotal in posttranscriptional regulation of late gene expression in human papillomavirus type 16 (HPV-16). However, the NRE does not contain any active splice sites, is located in the viral late 3' untranslated region, and regulates RNA-processing events other than splicing. The level of expression and extent of phosphorylation of SF2/ASF are upregulated with epithelial differentiation, as is subcellular distribution, specifically in HPV-16-infected epithelial cells, and expression levels are controlled, at least in part, by the virus transcription regulator E2.

Human papillomaviruses (HPVs) are a family of epitheliotropic viruses that infect both cutaneous and mucosal epithelia. HPV infection most commonly results in benign papillomas or warts; however, on rare occasions, malignant lesions can develop following infection with a high-risk HPV type and integration of the virus genome into the host genome (18). HPV-16 is the most significant member of this high-risk subgroup, being associated with approximately 60% of cervical carcinoma cases worldwide (52).

Transcription of the 8.0-kb virus genome generates a number of transcripts as a result of a complex program of alternative splicing and polyadenylation (44). Viral mRNAs are translated to yield six early proteins, expressed throughout the virus life cycle (primarily involved in episomal maintenance of the genome, transcriptional regulation, and cell transformation) (52) and two late proteins, the capsid proteins L1 and L2. Expression of the capsid proteins is restricted to cells undergoing terminal differentiation in the uppermost layers of the stratified epithelium (31) but because late transcripts are expressed in less-differentiated epithelial cells (43), control of late-gene expression is largely attributed to posttranscriptional mechanisms. *cis*-acting inhibitory elements identified within the late region of several papillomaviruses (40) appear to regulate gene expression via distinct RNA-based mechanisms. However, the overall effect is the same in that each element probably acts in a position- and orientation-dependent manner

through interactions with cellular factors (40) to reduce the levels of polyadenylated viral late mRNAs in the cytoplasm of undifferentiated cells. The best-understood example is bovine papillomavirus type 1 (BPV-1), in which regulation of late-gene expression by a short negative regulatory element (NRE) has been shown to involve the binding of a key splicing factor, U1 small nuclear ribonucleoprotein (U1snRNP), to a consensus 5' splice site in the NRE and inhibition of poly(A) polymerase by the U1 70K subunit of U1snRNP (12, 15). It is not known how such repression is alleviated to allow late-gene expression in differentiated papillomavirus-infected cells.

The HPV-16 NRE has been mapped to a 79-nucleotides sequence which overlaps the 3' end of the L1 coding region and extends into the late 3' untranslated region (UTR) (20). It is similar in its position within the late region to the BPV-1 element but is more complex, containing a 5' portion that contains four weak 5' splice sites and that is similar in length to the whole BPV-1 element but has a novel 3' GU-rich portion. Deletion and mutation analysis has revealed that both portions of the HPV-16 element contribute to full repression of gene expression; no simple mutation alleviates the repressive effect (9). The element has been shown to interact with a range of cellular RNA-processing factors, including the auxiliary splicing factor U2AF⁶⁵ (10), the polyadenylation factor CstF-64, and the elav-like shuttling protein HuR (23) through its 3' GU-rich portion and a U1snRNP-like complex through its 5' splice site-containing 5' portion (9). It has been implicated in controlling mRNA stability (21), nuclear retention (23), and 3'-end formation (K. McGuire and S. V. Graham, unpublished observations).

Splicing occurs in the spliceosome, a large multicomponent complex composed of U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) plus many associated

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polypeptides (35). Formation of the commitment, or early (E) splicing complex, involves U1 snRNP recognition of the 5' splice site and recognition of the branch site upstream of the 3' splice site by U2AF⁶⁵, leading to recruitment of the rest of the complex. Recruitment of these splicing factors to the 5' and 3' splice sites is mediated and promoted by SR proteins, which are essential splicing factors (5) so called because of their C-terminal arginine- and serine-rich domains (RS domains). Phosphorylation of these proteins on their RS domains regulates their well-documented roles in constitutive and alternative and enhancer-dependent splicing (13).

Binding of U1 snRNP to 5' splice sites is stabilized by SF2/ASF, probably through interaction with U1 70K (24), and SF2/ASF binds and recruits to the polypyrimidine tract the 35-kDa subunit of U2AF (50). SR proteins also bridge splicing factors across exons and help to define these units. As the terminal exon of most transcripts has no downstream 5' splice site, a regulatory interaction between splicing and polyadenylation complexes has been proposed (1). Indeed, mRNA 3'-end formation and splicing are linked in the nucleus, probably through association of the components of each process with the carboxyl-terminal domain of RNA polymerase II (33) and some splicing factor; for example, U1 snRNP, SRp75, and U2AF⁶⁵ have been shown to regulate 3'-end processing (14, 15, 22, 32, 46).

Here we show that, as well as a U1 snRNP-like complex and U2AF⁶⁵, phosphorylated SF2/ASF is also recruited to the HPV-16 NRE. The association of SF2/ASF with the NRE is indirect, and it is found in a complex with U2AF⁶⁵, suggesting that a splicing-associated complex forms in the late 3'UTR. The subcellular localization and levels of expression and phosphorylation of SF2/ASF change significantly during differentiation of epithelial cells containing episomal copies of the HPV-16 genome. By comparing undifferentiated and differentiating cell populations harboring episomal and integrated copies of the HPV-16 genome, we show that expression of SF2/ASF is directly regulated by virus infection and that the virus transcription factor E2 is at least partly responsible for this control. It is likely that SF2/ASF interaction with the NRE regulates mRNA processing of viral late transcripts.

MATERIALS AND METHODS

Cell culture. The W12 line, obtained from Margaret Stanley (Cambridge, United Kingdom), is an HPV-16-positive cervical epithelial cell line established from a low-grade lesion (41). The 20863 (W12E) and 20861 (W12G) lines are subclones of the W12 line and contain episomal and integrated copies of HPV-16 DNA, respectively (19). The HaCaT cell line is a spontaneously immortalized aneuploid human keratinocyte line that retains the ability to differentiate (2, 39). All W12 lines were cultured on mitomycin-C treated J2 3T3 fibroblast feeder layers and maintained at low density (2×10^5 to 2.5×10^5 cells/100-mm dish) with medium changes on alternate days for up to 5 days to limit the onset of differentiation. Cells were induced to differentiate by either culturing for up to 10 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, cholera toxin (8.4 ng/ml), hydrocortisone (0.4 µg/ml), and epidermal growth factor (10 ng/ml) and containing 1.2 mM Ca^{2+} with medium changes on alternate days or by culturing in F medium followed by growth in F medium, 20% fetal calf serum, and 1.68% methylcellulose for 24 h as described (38). HeLa cells and HaCaT cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine (Life Technologies).

Establishment of U2-OS cells stably expressing HPV-16 E2. We plated out 10^6 cells on a 100-mm tissue culture plate. The following day, these cells were transfected with no DNA or with 0.16, 0.32, 0.69, or 1.3 µg of pCMV alone or

with various amounts of pCMV-E2₁₆ (0.5, 1.0, 2, and 4 µg) with Lipofectamine plus reagent (Invitrogen) or calcium phosphate. The following day the cells were washed twice with phosphate-buffered saline and then refed. Twenty-four hours later the cells were split 1 in 4 and plated in medium containing 0.75 mg of G418 (Invitrogen) per ml. After 2 weeks the control cells had all died. The cells transfected with pCMV alone and those with pCMV-E2₁₆ grew under selection and were clonally expanded into permanent cell lines. E2 expression was assessed by reverse transcription (RT)-PCR, by Western blot with an anti-HPV-16 E2 monoclonal antibody, and by a transcription assay with an E2-responsive ptk6E2-luciferase reporter construct.

Preparation of cell extracts. For the preparation of cellular protein extracts, cells were washed twice with phosphate-buffered saline and lysed either by resuspension in boiling 2× sodium dodecyl sulfate (SDS) sample buffer [63 mM Tris-HCl, pH 6.8, 1% (wt/vol) SDS, 10% glycerol, 5% (vol/vol) β-mercaptoethanol, 0.004% (wt/vol) bromophenol blue] or by resuspension in 1 ml of E-buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% NP-40, protease inhibitor cocktail [Roche Molecular Biochemicals]) per 10^7 cells. Extracts were sonicated on ice, cell debris was pelleted, and the protein concentration was determined by the Bradford assay (Bio-Rad). For W12 cells, feeder cells were first removed by treatment with 0.1% trypsin–0.5 mM EDTA. Nuclear and cytoplasmic extracts were prepared as described (47) or nuclear extracts were purchased from 4C Biotech, Senefte, Belgium. Nuclear and cytoplasmic extracts were dialyzed against buffer D (20 mM HEPES, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol).

Western blotting. For Western blot analysis, proteins fractionated on SDS-polyacrylamide gel electrophoresis (PAGE) gels were electroblotted onto polyvinylidene difluoride membranes (Amersham). Blots were blocked overnight in phosphate-buffered saline with 5% (wt/vol) dried milk powder at 4°C. Primary antibodies were diluted in phosphate-buffered saline with 0.05% Tween 20 and 1% dried milk powder and incubated with the blots for 2 h at room temperature with shaking. Mouse monoclonal antibodies against HPV-16 L1 (CamVir 1; Abcam) and HPV-16 E1/E4 (TVG 402; gift of J. Doorbar, National Institute for Medical Research, London) were used at dilutions of 1:10,000 and 1:500, respectively. Monoclonal antibody against involucrin (SY5; Sigma) was used at a dilution of 1:5,000. Antibody MC3 raised against U2AF⁶⁵ (gift of M. Carmo-Fonseca, University of Lisbon, Portugal) was used at a dilution of 1:100. Mouse monoclonal antibodies against SF2/ASF (Mab 96; Zymed), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6CS; Biodesign International) were used at a dilution of 1:1,000. Anti-E2 antibody was a rabbit polyclonal antibody raised against a glutathione S-transferase fusion containing the carboxyl terminus of HPV-16 E2 and was a gift of L. Banks, (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy). Following washing in phosphate-buffered saline with 0.05% Tween 20, blots were incubated with secondary antibody at a dilution of 1:1,000 in phosphate-buffered saline with 0.05% Tween 20 and 1% dried milk powder for 1 h at room temperature with shaking. Secondary antibody was either anti-mouse- or protein A-conjugated horseradish peroxidase (Sigma). Membranes were washed in phosphate-buffered saline with 0.05% Tween 20 and visualized with ECL reagents (Amersham) according to the manufacturer's instructions.

Purification of NRE binding proteins from agarose beads. Proteins binding to NRE RNA were purified as described (7). Essentially, 500 pmol of RNA prepared by *in vitro* transcription was oxidized by treatment with sodium periodate and then incubated with 400 µl of adipic acid dihydrazide beads for 12 h at 4°C to link the RNA to the beads. The beads were washed three times with 2 M NaCl and equilibrated with buffer D (20 mM HEPES-KOH, pH 7.6, 5% glycerol, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol); 250 µl of HeLa nuclear extract in buffer D was applied, and the mixture was incubated for 20 min at 30°C. The beads were washed four times in buffer D with 400 mM NaCl and 4 mM MgCl_2 , and bound proteins were eluted in protein loading buffer by heating to 95°C for 5 min.

Coimmunoprecipitation of NRE-binding proteins. Mammalian expression constructs containing a luciferase reporter gene were derived from pCIneo (Promega). The simian virus 40 late poly(A) site, f1 *ori*, simian virus 40 enhancer, *neo* gene, and synthetic poly(A) site were removed by digestion with BamHI and NotI, and the sites were blunt ended and religated. A *Renilla* luciferase gene was inserted into NheI- and XbaI-digested, religated plasmid. Finally, the HPV-16 late 3'UTR from pCATPE445 or pCATΔNRE (9) was inserted into the new XbaI- and SalI-digested luciferase plasmid to give pRL+NRE and pRL–NRE. CsCl-purified plasmids were transfected into HeLa cells with Lipofectamine plus reagent according to the manufacturer's instructions (Invitrogen). Nuclear extract (90 µg) was incubated with 20 µl of MC3 anti-U2AF⁶⁵ antibody or anti-involucrin antibody in 150 µl of buffer E (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP-40, 0.5% sodium deoxycholate,

FIG. 1. Differentiation of W12 cells in monolayer culture. Western blot analysis with antibodies against the differentiation-specific proteins (A) involucrin (cellular), (B) E1[^]E4, and (C) L1 (viral) of W12E, W12G, and HaCaT cells grown in high-Ca²⁺ medium and harvested after 5 (D5) or 10 (D10) days in culture.

0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride) overnight at 4°C on a rotating wheel; 75 μ l of a 50% (vol/vol) slurry of protein A-Sepharose in buffer E was added to the sample and mixed for 1 h at 4°C on a rotating wheel. The Sepharose beads were pelleted by centrifugation in an Eppendorf microcentrifuge at 10,000 rpm for 10 min. Beads were washed twice with EB buffer (buffer E with bovine serum albumin [2 mg/ml]), once with EN buffer (buffer E with 500 mM NaCl), and four times with buffer E. Precipitated complexes were eluted by addition of protein loading buffer and boiling for 3 min.

Isolation of phosphoproteins and dephosphorylation of cellular proteins. Phosphoproteins were purified from HeLa and W12E cells with a Qiagen phosphoprotein purification kit according to the manufacturer's instruction and in the presence of phosphatase inhibitors. Dephosphorylation was carried out with calf intestinal alkaline phosphatase (Promega) exactly as described (8).

Immunocytochemistry of raft tissue. Organotypic raft tissue was fixed in 10% neutral-buffered formalin overnight and paraffin embedded; 4- μ m cross sections were cut and placed on poly-L-lysine (Sigma)-coated slides. Immunocytochemistry was performed with the ABC Elite kit (Vector Laboratories) following the protocol provided. Briefly, sections were deparaffinized by incubating in xylene and rehydrated in a graded series of alcohol (100, 95, 75, and 50% ethanol). For antigen retrieval, the tissue was heated in citrate buffer at pH 6.0 for 10 min in a microwave. Monoclonal antibody against SF2/ASF (clone 96) was added at 1:250 dilution for 1 h at room temperature. Diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories) was used as the chromogen.

RESULTS

Epithelial differentiation in monolayer culture. The W12 cell line provides a good model system for analysis of cervical epithelial differentiation in monolayer culture. In the W12E (20863) subclone (19), around 100 copies of the genome are maintained episomally (a model for the infected cell), while another subclone, W12G (20861), contains only integrated genomes (a model for the virus-transformed cell). At low cell density in the presence of 1.2 mM Ca²⁺ differentiation occurs spontaneously after around 5 days in monolayer culture (Fig. 1). At 5 days, the W12E cells showed only low levels of expression of the suprabasal cell marker involucrin, but when cultured for a further 5 days, there was a significant increase in expression of involucrin (Fig. 1A) and the viral late proteins E1[^]E4 (Fig. 1B) and L1 (Fig. 1C). Immunofluorescence data

indicate that around 10 to 20% of the cells were positive for involucrin at day 5, while this increased to 75 to 85% at day 10 (data not shown). With the same protocol with W12G cells, there was only a modest increase in involucrin expression (Fig. 1A), and no expression of the viral late proteins was detected, as these are no longer expressed when the virus genome becomes integrated (52) (data not shown). The HaCaT human keratinocyte cell line, which contains no virus genomes (2, 39), was also able to differentiate with this method and displayed a marked increase in involucrin expression after 10 days in culture (Fig. 1A) but no expression of virus late product (Fig. 1B and C).

HPV-16 NRE binds an SF2/ASF-containing complex. The HPV-16 NRE binds several RNA-processing factors, including a U1snRNP-like complex (9) and U2AF⁶⁵ (10, 23), both key proteins in the formation of splicing complexes when linked with SR proteins, in particular SF2/ASF. Such complexes have been shown to be involved in terminal exon definition and thus could have a role in regulating mRNA 3'-end formation (1). We have evidence that the NRE can regulate polyadenylation (McGuire and Graham, unpublished observations), and so we investigated whether SF2/ASF could bind the NRE. The NRE sequence does not contain any good consensus SF2/ASF binding sites (with ESE finder: <http://exon.cshl.edu/ESE>; data not shown) and UV cross-linking experiments, followed by Western blotting with monoclonal antibody 96 against SF2/ASF (17), failed to show evidence of direct binding to the NRE (data not shown). However, UV cross-linking usually only detects proteins that contact the RNA directly, and, in splicing complexes, SF2/ASF bridges between U1snRNP and U2AF and does not bind the RNA directly.

We investigated whether SF2/ASF bound the NRE indirectly with affinity chromatography. The protein was readily affinity purified in 400 mM Na⁺ from HeLa nuclear extracts on NRE RNA bound to agarose beads but was not affinity purified by poly(U) RNA-linked beads or beads alone (Fig. 2A). In addition, we found that SF2/ASF binds to the 3' GU-rich portion of the NRE and not the 5' portion that contains four weak 5' splice sites (Fig. 2A, tracks 5 and 6), making it likely that it binds via interaction with U2AF (1), which also binds the 3' portion of the NRE (Fig. 2B) (9).

To investigate NRE-SF2/ASF binding further, we carried out coimmunoprecipitation with an anti-U2AF⁶⁵ antibody to pull out U2AF⁶⁵/SF2/ASF complexes in HeLa cells transfected with phRL+NRE and phRL-NRE. These constructs are identical except that the former contains the 79-nucleotide NRE sequence, while in the latter it has been precisely deleted. As observed previously (9), deletion of the NRE resulted in a 20-fold increase in reporter gene expression in HeLa cells, demonstrating the inhibitory function of the NRE (data not shown). In splicing, U2AF⁶⁵ can exist in a complex with SF2/ASF through interaction of the latter with the 35-kDa subunit of U2AF. However, if the NRE acts as a binding site for U2AF complexed with SF2/ASF, then the presence of many copies of the NRE in the nuclei of cells transfected with phRL+NRE should result in more efficient pulldown of U2AF⁶⁵/SF2/ASF complexes than in HeLa cells transfected with the construct that does not contain NRE sequences.

Figure 2C (lanes 2 and 3) shows that no complex was precipitated in the absence of antibody from HeLa cells trans-

FIG. 2. HPV-16 NRE binds SF2/ASF in a complex with U2AF through its 3' GU-rich portion. Western blotting of affinity-purified (400 mM Na⁺) proteins from HeLa cell nuclear extracts that interact with NRE RNA. (A) Western blotting with monoclonal antibody 96 against SF2/ASF. (B) Western blotting with monoclonal antibody MC3 against U2AF⁶⁵. Lane 1, NRE-binding proteins. Lane 2, poly(U)-binding proteins. Lane 3, proteins purified with beads alone. Lane 4, 2 μ g of HeLa nuclear extracts. Lane 5, 5'NRE-binding proteins. Lane 6, 3'NRE-binding proteins. (C) Western blot with monoclonal antibody 96 against SF2/ASF of coimmunoprecipitated U2AF⁶⁵ and SF2/ASF in HeLa cells transfected with phRL+NRE or phRL-NRE. Lane 1, nuclear extract from HeLa cells transfected with phRL+NRE, protein A-Sepharose, with anti-involucrin antibody. Lane 2, nuclear extract from HeLa cells transfected with phRL-NRE, protein A-Sepharose, no antibody. Lane 3, as for lane 2 but cells transfected with phRL+NRE. Lane 4, nuclear extract from HeLa cells transfected with phRL-NRE, protein A-Sepharose with anti-U2AF⁶⁵ antibody. Lane 5, nuclear extract from HeLa cells transfected with phRL+NRE, protein A-Sepharose, with anti-U2AF⁶⁵ antibody. Control Western blots to show input levels of SF2/ASF and U2AF⁶⁵ in HeLa nuclear extracts are shown beneath the main panels.

fected with either construct. In the presence of anti-U2AF⁶⁵ antibody, no complex was detectable in precipitates from extracts of HeLa cells transfected with the construct lacking the NRE (lane 4). However, a complex was easily detected in cells transfected with the construct containing the NRE (lane 5). No complex was precipitated from HeLa cell extracts transfected with phRL+NRE when an anti-involucrin antibody was used instead of the anti-U2AF⁶⁵ antibody (lane 1). The band below the SF2/ASF band in lanes 1, 4, and 5 is a nonspecific antibody-related fragment. The experiment was repeated with anti-SF2/ASF antibody as the primary antibody in immunoprecipitation, with very similar results. These data indicate that SF2/ASF and U2AF⁶⁵ are part of a complex that is pulled out on HPV-16 NRE RNA.

Expression of SF2/ASF is upregulated during W12E cell differentiation. If the HPV-16 NRE regulates late-gene expression in a differentiation stage-specific manner, it might be expected that expression of NRE-binding proteins that affect this regulation might respond to differentiation. We assessed the levels of all the NRE-binding RNA processing proteins

that we had identified in undifferentiated and differentiated W12E cells where the HPV-16 genome is episomal (model for the infected epithelial cell), W12G cells where the HPV-16 genome is integrated into the host genome (model for the transformed epithelial cell) (19), and HaCaT cells, which are immortalized epithelial cells containing no virus genomes. W12E cells of low passage number were used (<passage 17), and Southern blot analysis was used to confirm that the genomes were largely episomal in W12E cells and largely integrated in W12G cells (data not shown).

Protein extracts were prepared by lysis in SDS sample buffer and analyzed by Western blotting. In order to assess protein loading, membranes were stripped and reprobed with the 6CS antibody against GAPDH. Levels of GAPDH have been shown to decrease modestly upon epithelial differentiation (42), and our data are consistent with this (Fig. 3A). For most

FIG. 3. SF2/ASF is upregulated with differentiation in W12E cells. (A) Western blot analysis of levels of SF2/ASF in W12E (episomal genomes), W12G (integrated genomes), and HaCaT (no virus genomes) cells cultured for 5 days (U) and 10 days (D). Twofold titrations are shown. Each membrane was stripped of the first antibody and reprobed with GAPDH to assess protein loading. (B) Section of a normal human keratinocyte organotypic raft culture stained with monoclonal antibody 96 against SF2/ASF. (C) Quantification by densitometry scanning of levels of SF2/ASF in undifferentiated and differentiated W12E cells, showing the mean and standard deviation from the mean of three separate experiments.

FIG. 4. SF2/ASF expression and phosphorylation are upregulated during W12E cell differentiation. Phosphorylated and unphosphorylated protein fractions were prepared by column fractionation from undifferentiated (U) and differentiated (D) W12E cells. (A) Western blot analysis of phosphorylated forms (B, bound fraction) of SF2/ASF and a Western blot showing unphosphorylated GAPDH protein (U, unbound fraction). (B) Limiting alkaline phosphatase treatment of HeLa, day 5 W12, and day 10 W12 cellular extracts was carried out, and untreated (–) and treated (+) extracts were electrophoresed and Western blotted with the anti-SF2/ASF antibody. (C) Western blot of nuclear (NE) and cytoplasmic (CE) protein extracts from undifferentiated and differentiated W12E and HaCaT cells probed with the anti-SF2/ASF antibody. Arrows indicate the positions of hyper- and hypophosphorylated SF2/ASF. (D) Western blot of nuclear (NE) and cytoplasmic (CE) protein extracts from HeLa cells and undifferentiated and differentiated W12E cells probed with antibodies against involucrin (invol.) to demonstrate differentiation, U2AF⁶⁵ to demonstrate good nuclear fractionation, and GAPDH, which is located mainly in the cytoplasm but is also present in the nucleus.

of the NRE-binding cellular proteins, there was little significant difference in levels of expression between undifferentiated and differentiated cells (data not shown). However, in the W12E line, SF2/ASF was significantly upregulated with differentiation to a level of four- to eightfold (Fig. 3A and C). In the W12G cell line, which contains integrated copies of the HPV-16 genome, and the HaCaT cell line, which does not contain any virus genomes, SF2/ASF was downregulated with differentiation.

In case the changes that we observed were specific to the particular method of differentiation used, all experiments were carried out with extracts made from W12E, W12G, and HaCaT cells induced to differentiate by suspension in methylcellulose for 24 h. Very similar results were obtained in all cases (data not shown). A similar level of induction of SF2/ASF in differentiated W12E cells was observed when cellular proteins were extracted in E buffer, although phosphorylated forms of SF2/ASF were not seen, as expected (17). We could not detect alternative phosphorylated forms of the protein in W12G cells even with lysis in boiling SDS buffer. Finally, we established raft cultures of normal human keratinocytes, sectioned them, and performed immunocytochemistry to detect SF2/ASF. Figure 3B shows that SF2/ASF is abundant in the basal layer cells but that levels decrease in the upper epithelial levels, mirroring our observations with monolayer HaCaT cells. As a decrease in expression of SF2/ASF was observed in differentiated normal human keratinocytes, W12G, and HaCaT cells, our data indicate that upregulation of SF2/ASF in W12E cells is due to the presence of episomal HPV-16 genomes rather than epithelial differentiation.

Levels of phosphorylation of SF2/ASF change in the nucleus and cytoplasm during W12E cell differentiation. Phosphoryla-

tion of SR proteins is essential for their activities in constitutive and alternative splicing (6, 51), and unphosphorylated SF2/ASF is not normally detected *in vivo* (17). Column purification of phosphorylated proteins followed by Western blotting confirmed that all the SF/ASF protein was phosphorylated in undifferentiated and differentiated W12E cells, as none could be detected in the unbound fraction from the column (Fig. 4A). In contrast, GAPDH was present only in the unbound fraction, as expected. Consistently more SF2/ASF protein could be detected in the differentiated W12E cell lysates, as we observed previously, and this appeared to have a lower electrophoretic mobility than the SF2/ASF protein in undifferentiated W12E cells, indicating a possible difference in phosphorylation between the two cell types.

Limiting alkaline phosphatase treatment of cellular extracts demonstrated that SF2/ASF was hyperphosphorylated in HeLa cells and undifferentiated W12 cells (Fig. 4B). Dephosphorylation of the protein in differentiated W12 cells was not clearly demonstrated. However, as we found that SF2/ASF is mostly highly phosphorylated in these cells (Fig. 4C), it is possible that the treatment was too mild to dephosphorylate to the extent that different forms of the protein could be seen on the gel. We examined further the phosphorylation status of SF2/ASF by Western blotting of nuclear and cytoplasmic fractions of W12E and HaCaT cells (Fig. 4C). A significant difference was found in the subcellular localization, abundance, and phosphorylation levels of SF2/ASF upon W12E cell differentiation. Again, much more SF2/ASF was detected in differentiated W12E cells, and levels of phosphorylation of the protein were clearly much higher in these cells also. While there was a similar distribution of the protein between the nucleus and cytoplasm in undifferentiated cells, in differentiated cells most of the

protein was located in the nuclear fraction. In contrast, in undifferentiated HaCaT cells, most of the SF2/ASF was located in the nucleus and was hyperphosphorylated, while the levels of the protein declined significantly when the cells were differentiated. Figure 4D demonstrates good separation of the nuclear and cytoplasmic compartments for the subcellular fractions used in Fig. 4C. This comparison indicates that the changes in subcellular levels of expression and phosphorylation of SF2/ASF upon differentiation of W12E cells are not due to epithelial differentiation but could be due to the presence of episomal virus genomes.

Virus transcription factor E2 regulates expression of SF2/ASF. The HPV-16 genome expresses several proteins that can regulate transcription. E6 and E7 are overexpressed in W12G cells compared to W12E cells and not expressed in HaCaT cells. Thus, these are not good candidates for upregulation of SF2/ASF in W12E cells or, conversely, downregulation in W12G cells. However, the viral transcription factor E2 is expressed in W12E cells but not in W12G or HaCaT cells and has been shown previously to interact with a number of cellular transcription factors and transcriptional coactivators (3, 27, 25, 30, 37). In addition, expression of E2 is upregulated during differentiation of HPV-infected cervical epithelial tissue (29).

Two different cell lines expressing HPV-16 E2 at a low level in the absence of other viral proteins were used to investigate whether E2 regulated SF2/ASF. The first was the HaCaT keratinocyte cell line, and the second was the U2-OS osteosarcoma cell line, both stably transformed with pCMVE2. Initially, similar results were observed with both cell types. However, it proved difficult to maintain E2 expression in the transformed HaCaT cells in culture, probably because E2 expression is toxic to these cells (45, 49), and we were unable to carry out sufficient replicate experiments to quantify our data. In contrast, three clones of the U2-OS cell line maintained stable expression of E2 over time. Cellular extracts were prepared from these cells and from control lines transformed with the empty vector, pCMV, and levels of SF2/ASF were analyzed by Western blotting in three separate experiments with each clone. As expected, no E2 expression was observed in the control pCMV-transformed cells while there was clearly E2 expression in the cells stably transformed with pCMVE2. Expression of E2 caused a three- to fourfold upregulation of SF2/ASF in the pCMVE2-transformed U2-OS cells, clone A4 (Fig. 5B), (similar results were obtained with clones B1 and B3), indicating that virus infection may contribute to induction of expression of the alternative splicing factor SF2/ASF in differentiating epithelial cells. Only minor differences in the other NRE-binding splicing factors were observed (data not shown).

DISCUSSION

Papillomaviruses have limited coding potential, and it is likely that they depend upon interaction with cellular factors to regulate epithelial differentiation stage-specific viral late gene expression. Papillomavirus late gene expression is regulated posttranscriptionally, and it has been proposed that the NRE is key to this regulation in HPV-16 (20). Elucidation of the proteins bound by the element in undifferentiated and differentiated epithelial cells should shed light on the mechanisms by

FIG. 5. Western blot analysis of expression of SF2/ASF in a clonal population of U2-OS cells stably expressing HPV-16 E2 protein. (A) Cell extracts in E buffer were prepared from U2-OS cells transformed with the E2 expression vector (E2) and from cells stably transformed with the vector alone (V). The protein concentration was measured by the Bradford assay, and equal quantities (20 μ g) were electrophoresed in each SDS-PAGE track. Panels were Western blotted with monoclonal antibodies against the proteins shown to the right. Each experiment was carried out three times with very similar results. (B) Quantification by densitometry scanning of levels of SF2/ASF in pCMV-transformed (V) and pCMVE2-transformed (E2) U2-OS cells, showing the mean and standard deviation from the mean of three separate experiments.

which this viral RNA element acts. The NRE has been shown previously to interact with several RNA-processing factors, including the auxiliary splicing factor U2AF⁶⁵ (10), the polyadenylation factor CstF-64, the *elav*-like HuR protein, which regulates mRNA stability (23), and a U1snRNP-like splicing complex (9). It is unlikely that all these NRE-binding proteins bind to the element simultaneously. However, the NRE has been shown to regulate 3'-end formation (McGuire and Graham, unpublished), nuclear export of late transcripts (23), and mRNA stability (21), suggesting that it may bind different protein complexes that regulate these events in the nucleus and the cytoplasm. In addition, because regulation of late-gene expression is tightly linked to epithelial differentiation, NRE-protein complexes would be expected to alter during differentiation. This study aimed to determine the components of one complex that may form upon the NRE during epithelial differentiation.

Our data demonstrate that the HPV-16 NRE binds the splicing factor SF2/ASF, which has pivotal roles in constitutive and alternative splicing (5). It is likely that this protein interacts with the late regulatory element through protein-protein interactions with U2AF, which binds its 3' GU-rich portion directly. We suggest that SF2/ASF is important in the life cycle of HPV-16, as we have observed virus regulation of abundance, subcellular localization, and phosphorylation of this protein during differentiation of virus genome-containing epithelial cells. By electrophoretic mobility shift assays, we found previ-

ously that Sm proteins, central components of U1snRNP, are present in the same NRE-binding complexes as U2AF (9, 23). U1snRNP binds the 5' portion of the NRE via four weak 5' splice sites, while U2AF binds the 3' portion. The two portions of the element have separate inhibitory activities on gene expression, but both are required for full repression (9). Since U2AF is known to recruit SF2/ASF (50), an interaction between U1snRNP and U2AF may be achieved by SF2/ASF bridging these factors across the NRE and optimizing its function.

While U1snRNP has been found to associate with the 3'-terminal exon or with the 3'UTR of certain viral genes (28, 48), in particular the BPV-1 inhibitory element, this is the first demonstration of the association of components of an entire splicing-like complex with a 3'UTR control element. Repression of gene expression by the BPV-1 inhibitory element occurs by inhibition of poly(A) polymerase via the U1 70K subunit of U1snRNP that binds the single consensus 5' splice site in this element (15). However, the HPV-16 NRE has four weak 5' splice sites that repress gene expression, all of which are necessary for efficient binding of a U1snRNP that appears to lack U1 70K (9). Despite this, the HPV-16 NRE may also act to inhibit poly(A) polymerase in undifferentiated epithelial cells, as it binds U1A and U2AF, which can also efficiently inhibit poly(A) polymerase (22).

Furth et al. proposed that the BPV-1 element may interfere with terminal exon definition (12), a process that relies on molecular interactions across the last exon between splicing factors bound at the upstream 3' splice site and the polyadenylation site downstream. Binding of U1snRNP to a strong 5' splice site in a 3'UTR, as in the BPV-1 inhibitory element, inhibits the efficiency of splicing of the terminal exon, but weak interactions of U1snRNP with a 5' splice site couple splicing and polyadenylation, leading to efficient terminal exon definition (48). The HPV-16 NRE-protein complex contains the key nuclear factors (1) found in exon definition complexes, and the NRE, with its weak 5' splice sites and a U2AF binding site downstream, could clearly mimic a mini-intron and regulate terminal exon definition.

In undifferentiated epithelial cells, the NRE-protein complex may enhance the efficiency of binding of U1snRNP to the four weak 5' splice sites, leading to poor exon definition and low production of late transcripts, while in differentiated epithelial cells, changes in the complex could render the NRE-U1snRNP interaction weak, resulting in production of efficiently processed late transcripts that possess unusually long (>2 kb) terminal exons. Thus, one would expect to observe changes in the NRE-protein complex upon epithelial differentiation. One major change that we have noted is increased nuclear abundance and levels of phosphorylation of SF2/ASF, a key component of exon definition complexes, upon differentiation of HPV-16 episomal genome-containing epithelial cells. In differentiated HPV16-infected epithelial cells, phosphorylation of SF2/ASF may alter the composition of the NRE-binding complex to abolish the inhibitory effect.

Our data suggest that one factor responsible for controlling expression of SF2/ASF is the HPV-16 transcription and replication factor E2, which is essential for the virus life cycle. Apart from its key role in virus genome replication, E2 regulates RNA polymerase II-mediated transcription by binding to

E2 response elements in the virus long control region (16). E2 binds a number of cellular transcription factors (3, 25, 27, 30, 37), implying that it has the potential to regulate cellular gene expression. Previously, E2 has been shown to downregulate the human telomerase reverse transcriptase promoter, thus interfering with host cell growth regulation (26). In E2-transformed U2-OS cells, we found a three- to fourfold increase in SF2/ASF, and this is the first evidence that the viral transcription factor controls host cell RNA-processing factors. The increase that we observed in U2-OS cells is lower than the four- to eightfold increase that we found in HPV-16-infected epithelial cells. However, this may be because the basal levels of SF2/ASF protein in U2-OS cells are higher than in undifferentiated epithelial cells (data not shown), and we were unable to obtain replicate data from E2 stably transformed HaCaT cells to be certain of the levels of induction in epithelial cells.

Clearly, it is possible that other viral factors are also involved in the regulation of SF2/ASF. During an infectious life cycle, expression of E2 increases at late stages of infection (29), presumably to aid amplification of genome numbers during vegetative viral DNA replication. As concentrations of SF2/ASF are known to regulate 5' splice site selection (5), abundant, phosphorylated SF2/ASF in differentiated infected epithelial cells may be required to regulate alternative splicing of late transcripts from amplified virus genomes in cells that would normally have downregulated RNA-processing functions (11). Thus, concomitant regulation of expression of SF2/ASF could facilitate virus life cycle completion. Indeed, changes in expression and activity of SF2/ASF are known to regulate gene expression and the life cycle in adenovirus and human immunodeficiency virus (34, 36). It remains to be determined whether the amounts of SF2/ASF expressed are sufficient and how E2 carries out this regulation.

ACKNOWLEDGMENTS

We thank J. Caceres (Human Molecular Genetics Unit, Edinburgh) for advice and anti-SF2/ASF antibodies. We are very grateful to Margaret Stanley and Paul Lambert for the W12 cell line and its clones, respectively. Saveria Campo and Pablo Cordano supplied the HaCaT E2 stably transformed cell line.

This work was carried out with funding from the Biotechnology and Biological Science Research Council and the Wellcome Trust. M.M. was the recipient of a Medical Research Council studentship, and T.V. received studentship funding from the Thai government.

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