A Human Papillomavirus E2 Transcriptional Activator
THE INTERACTIONS WITH CELLULAR SPlicING FACTORS AND POTENTIAL FUNCTION IN PRE-mRNA PROCESSING*

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The papillomaviruses are a family of small, nonenveloped, double-stranded DNA viruses that cause epithelial and fibroepithelial lesions (1). With few exceptions, papillomaviruses are highly species- and lesion-specific. A large group (≈20 types) of human papillomaviruses are specifically linked with epidermodysplasia verruciformis (EV),1 which is a rare hereditary, lifelong disease characterized by the development of multiple cutaneous warts. The sequence of EV-HPV E2 hinge contains multiple arginine/serine (RS) dipeptide repeats which are characteristic of a family of pre-messenger RNA splicing factors, called SR proteins. Here we show that the HPV-5 (an EV-HPV) E2 protein can specifically interact with cellular splicing factors including a set of prototypical SR proteins and two snRNP-associated proteins. Transiently expressed HPV-5 E2 protein colocalizes with a nuclear matrix associated-splicing coactivator in nuclear speckled domains. The RS-rich hinge is essential for E2 transactivator interaction with splicing factors and for its subnuclear localization. Moreover, we present functional evidence for the HPV-5 E2 transactivator, which shows that the RS-rich hinge domain of the E2 protein can facilitate the splicing of precursor messenger RNA made via transactivation by E2 itself. Our results, therefore, suggest that a DNA binding transactivator containing an RS-rich sequence can play a dual role in gene expression.

The papillomaviruses are a family of small, nonenveloped, double-stranded DNA viruses that cause epithelial and fibroepithelial lesions (1). With few exceptions, papillomaviruses are highly species- and lesion-specific. A large group (≈20 types) of human papillomaviruses are specifically linked with epidermodysplasia verruciformis (EV),1 which is a rare hereditary, lifelong disease characterized by the development of multiple cutaneous warts (2). Infection by EV-associated HPV types 5 and 8 carries a high risk of developing squamous cell carcinoma, implicating these viruses in oncogenicity (2). Papillomavirus gene expression is controlled by the products encoded by the viral E2 gene (3). The papillomavirus E2 proteins recognize the palindromic sequence ACCN6GGT, which occurs in multiple copies within the long control region of viral genomes and function as transcriptional activators. The E2 protein contains two defined functional domains that are relatively conserved among different types of papillomaviruses. The N-terminal conserved domain, consisting of approximately 200 amino acids, is crucial for transcriptional activation, whereas the domain at the C terminus consisting of approximately 100 amino acids contains the DNA binding and dimerization properties of the protein. These two domains are linked by a hinge region that lacks conservation in amino acid sequence and varies in length among papillomaviruses. The hinge region of the bovine papillomavirus (BPV) E2 protein was predicted to adopt a random coil structure and could confer flexibility to the E2 molecule (3). Unlike the two well defined terminal domains, the function of the hinge region remains largely unclear.

In comparison with several well studied E2 gene products, the E2 protein of EV-associated HPVs harbors a relatively long hinge region (≈200 amino acid residues) and the hinge sequence is rich in arginine, serine, and glycine residues (Fig. 1). The arginine/serine (RS) dipeptide repeat in the hinge is characteristic of a superfamily of proteins, which are primarily involved in the splicing of precursor mRNA (4–6). A group of prototypical SR proteins containing an extensive RS domain at the C terminus can be recognized by monoclonal antibody (mAb) 104, which stains lateral loops corresponding to sites of RNA pol II transcription on amphibian lampbrush chromosomes (7). Individual SR proteins can complement splicing deficient cytoplasmic S100 extract, indicating that they are essential splicing factors but have redundant functions in splicing (8). SR proteins are also crucial players in alternative splicing by modulating splice site choice; different SR proteins affect splice site selection with different efficiencies and with different mechanisms (4–6). In addition to the prototypical SR proteins, the RS domain of its analogous arginine alternating domain (9) is also present in other essential splicing factors and regulators including U2AF subunits, U1 and U5 snRNP-associated SR proteins, and Drosophila Tra and Tra-2 proteins (4). Using a variety of methods, some RS domains have been shown to mediate protein-protein interaction between SR proteins (10). Some RS domains can influence RNA binding (5) or promote RNA-RNA annealing (11, 12).

In addition, short stretches of RG or RGG repeats scatter within the hinge region of EV-HPV E2 proteins; some of these repeats neighbor a serine residue as SRG or SRGG (Fig. 1). The RG-rich sequence of the E2 hinge is reminiscent of the RGG box that is present in a number of RNA-binding proteins, including some nucleolar proteins, the ICP27 protein of herpes simplex

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1 The abbreviations used are: EV, epidermodysplasia verruciformis; snRNP, small nuclear ribonucleoprotein; PAGE, polyacrylamide gel electrophoresis; HPV, human papillomavirus; BPV, bovine papilloma virus; mAb, monoclonal antibody; pol, polymerase; PBS, phosphate-buffered saline; TBST, Tris-buffered saline with Tween 20; DTT, dithiothreitol; FAS, protein A-Sepharose; aa, amino acid(s); PCR, polymerase chain reaction; ORF, open reading frame; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; TFIIID, transcription factor IID; CPSF, cleavage poly(A) specificity factor; CTD, C-terminal domain; SR, serine/arginine-rich.

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virus, and the EBNA1 protein of Epstein-Barr virus (13). The realization that EV-HPV E2 proteins contain an RS-rich hinge domain suggests that they may exert a function similar to that of cellular SR proteins or interact with SR proteins through their RS repeat sequence. In the present study, we show that the HPV type-5 (an EV-HPV) E2 protein can specifically interact with several cellular splicing factors of the SR protein family. Our indirect immunofluorescent study revealed that transiently expressed HPV-5 E2 protein in transfected HeLa cells colocalizes with a splicing factor in nuclear matrix speckles. More importantly, we provide evidence suggesting that the HPV-5 E2 protein likely participates in pre-mRNA splicing as well as in transcriptional regulation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Plasmids HPV5/9 and pSV2-neo HPV-16 were obtained from E.-M. de Villiers (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and W. C. Y. Yu (Academia Sinica, Taipei, Taiwan), respectively, and they were used as template for polymerase chain reaction (PCR) amplification of the HPV-5 or HPV-16 E2 coding region. The PCR product containing HPV-5 E2 ORF cloned into pGEM-1 (Promega), generating plasmid pSV5E2. Likewise, plasmid pSV16E2H was constructed. These two plasmids were used as template for *in vitro* synthesis of HPV-5 and HPV-16 E2 proteins, respectively. Bacterial expression vector pAR5E2H was constructed as follows. The PCR product containing HPV-5 E2 ORF was inserted into pAR3800–1 (14) to generate pAR5E2. The restricted fragment of pAR5E2 coding for amino acid residues 228–315 in full-length HPV-5 E2 was replaced by the PCR product spanning amino acid residues 397–515, generating pAR5E2H. The pAR5E2H plasmid was used to overproduce the hinge-deleted E2 protein.

Plasmids pAD5E2 and pAD5E2H used for yeast two-hybrid assays were constructed as follows. The coding sequences for full-length and hinge-deleted HPV-5 E2 proteins were in-frame placed downstream of the GAL4 activation domain in pACT2 (CLONTECH) using restriction enzymes BamHI and EcoRI. To construct pEGUS–100kD, the DNA fragment encoding the U5–100kD protein was generated by PCR using pBluescriptSK+100KHeLa (gift of R. Luhrmann, Philips-Universit{"a}t, Marburg, Germany) as template and then inserted into pEG202 (15). The resulting plasmid produced the U5–100kD protein fusion with the LexA DNA binding domain. The remaining pEG202-derived plasmids including pEG5ASF, pEGSC35, and pEGU1–70K were generous gifts of J. Y. Wu (Washington University, St. Louis, Missouri) and W. C. Y. Yu (Academia Sinica, Taipei, Taiwan), respectively. When used as probes, unincorporated labels were removed by chromatography on a NAP column (Amersham Pharmacia Biotech).

**Preparation of Anti-E2 Antibodies**—Plasmid pAR5E2H was transformed into *E. coli* (BL21(DE3)); overproduction of the hinge-deleted HPV-5 E2 (E2H) protein was induced by isopropyl-1-thio-β-D-galactopyranoside. Overproduced E2H protein was solubilized from inclusion bodies with 8 M urea followed by SDS-polyacrylamide gel purification and then used to immunize rabbits. To purify antibodies, recombinant E2H was coupled to Sepharose according to the method published by Biotech. Antibodies were subjected to chromatography on E2H- or Sepharose essentially according to Harlow and Lane (18). The concentration of affinity-purified antibodies was 5 mg/ml.

**In Vitro Transcription-Translation**—*In vitro* transcription-translation-reaction coupled reactions were performed according to the manufacturer's instructions (Promega). Plasmids used in this experiment were all derived from pGEM-1 (Promega); each contained the coding region for HPV-5 E2, HPV-16 E2, ASF/SF2, SC35, U1–70K, U5–100kD, and GAL4 VP16 (gift of Y.-S. Lin, Academia Sinica, Taipei, Taiwan), respectively. When used as probes, unincorporated labels were removed by chromatography on a NAP column (Amersham Pharmacia Biotech).

**Commmunoprecipitation**—To co-immunoprecipitate HPV-5 E2 and SR proteins, 25 μl each of *in vitro* transcription-translation mixtures containing unlabeled HPV-5 E2 and 35S-labeled SH protein were incubated for 30 min at 4 °C. Ten micrograms of affinity-purified anti-E2 antibodies were coupled to 2.5 mg of PAS in NET-2 buffer as described above. The mixture containing HPV-5 E2 and an SR protein was then incubated with anti-E2 coupled PAS at 4 °C for 1 h. Unbound proteins were removed, and the resin was then washed four times with 1 ml of NET-2 buffer. Bound proteins were recovered, resolved on SDS-PAGE and visualized by autoradiography.

**Far-Western and Western Blot Analyses**—To analyze protein-protein interaction by Far-Western blotting, SR and snRNP proteins were fractionated on 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The blots were incubated with binding buffer (25 mM HEPES (pH 7.9), 3 mM MgCl2, 4 mM KCl, and 1 mM DTT) containing 6 M guanidine hydrochloride for 10 min at 4 °C. The blot was subsequently incubated with binding buffers containing decreasing concentrations of guanidine hydrochloride for 5 min per change. Blockage of the blot was performed by incubation with 5% skim milk and 1 mM DTT in TBST (100 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20) for 1 h, followed by 1% skim milk and 1 mM DTT in TBST for 1 h. The blot was incubated with 0.5–1 × 10̇ 6 cpm/ml of 35S-labeled E2 protein in TBST containing 1% skim milk and 1 mM DTT at 4 °C overnight. To remove unbound proteins, the blot was washed with TBST containing 1% skim milk twice and then with TBST once for 10 min per wash. The blot was subjected to autoradiography.

For Western blotting analysis, proteins were transferred onto nitrocellulose (Schleicher & Schuell) or polyvinylidene difluoride membranes (Bio-Rad). The blots were probed with anti-U1–70K antibody or anti-U5–100kD anti-serum (generous gifts of R. Luhrmann), and signals were detected using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

** Yeast Two-hybrid Assays**—A pACT2-derived bait plasmid and a pEG202-derived prey plasmid were co-transformed with the LacZ reporter plasmid pMA3A into Matα and Matα strains of *S. cerevisiae* EGY48 (Matα trp1 ura3 his3 leu2:lsp606-L612) using the modified lithium acetate transformation protocol provided by CLONTECH. Plasmid DNA was recovered from the transformants and verified by Southern blot analysis. Five independent clones of each transformants were subjected to the liquid β-galactosidase assay using o-nitrophenyl-β-D-galactopyranoside as substrate according to the protocol recommended by CLONTECH; each clone was assayed three times.
Indirect Immunofluorescence—HeLa cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transfected using calcium phosphate. Twenty-four hours after transfection, slides were plated onto Lab-Tek chamber slides (Nunc) and incubated for another 24 h. For double-immunofluorescence experiments, cells grown on chamber slides were rinsed twice with PBS and fixed with 2% formaldehyde for 30 min followed by permeabilization with 0.5% Triton X-100 for 10 min. After washing with PBS, cells were blocked by 3% skim milk in PBS for 30 min and then incubated with primary antibodies in PBS for 1 h at room temperature. The primary antibodies used were affinity-purified anti-U1 antibody (5 μg/ml; mAb BA111) (1 μg/ml; Calbiochem), and anti-HA antibody (1:20 dilution from the supernatant of hybridoma culture medium; gift of S.-C. Cheng, Academia Sinica, Taipei, Taiwan). Cells were then washed with PBS, followed by incubation with appropriate secondary antibodies, fluorescein-conjugated anti-mouse IgG (7.5 μg/ml; Cappel Laboratories) or rhodamine-conjugated anti-rabbit IgG (12 μg/ml; Cappel Laboratories), in PBS for 1 h at room temperature. After extensive washing with PBS, cells on slides were mounted immediately with mounting medium (Biomeda). The specimens were observed using a laser confocal microscope (MRC 600 model; Bio-Rad) coupled with an image analysis system.

Transient Transfection and CAT and β-Galactosidase Activity Assays—HeLa cells were grown in Dulbecco’s modified Eagle’s medium as described above. Cells were seeded 18 h before transfection and transfection using LipofectAMINE (Life Technologies, Inc.) was performed essentially according to the manufacturer’s instructions. For transfection, the amount of plasmids used was indicated in each figure legend. Cells were collected 48 h after transfection. One tenth of the cells were lysed in a denaturing buffer and subjected to Western blot analysis, and the remaining cells were lysed in 120 μl of lysis buffer containing 0.25 M Tris-HCl (pH 7.5) and 0.5% Triton X-100 (2.5 × 108 cells/ml of buffer) to obtain the cell extract for CAT assay or for both CAT and β-galactosidase activity assays. The method for the CAT assay is described by Carey et al. (19). For the β-galactosidase activity assay, the reaction was performed using the chemiluminescent substrate GalactoStar (CLONTECH) according to the manufacturer’s instructions, and the luminescent enzyme activity was measured according to the method described by Schoneich et al. (20) with minor modifications.

RNA Protection and Northern Blot Analysis—For RNA protection assays, all RNA samples were prepared from 60-mm dish cultures 24 h after transfection. Total RNA was analyzed using Trizol reagent (Life Technologies, Inc.) and then treated with DNase (1 unit/μg of RNA; Roche Molecular Biochemicals). Five (pSV40 plasmid) or 15 (pE2Sp1 plasmids) micrograms of DNase-treated RNA were combined with 2 × 1010 cpm anti-CAT probe (specific activity 3.4 × 108 cpm/μg) in a 30-μl mixture containing 40 μM PIPES (pH 6.7), 0.4 M NaCl, 1 mM EDTA, and 80% formamide. The RNA-probe mixture was heated at 85 °C for 5 min and then incubated at 45 °C for 5 h to allow annealing. The digestion was performed by adding 300 μl of a buffer containing 10× Tris-HCl (pH 7.4), 0.5 M NaCl, 5 mM EDTA, and RNase T1 (0.6 unit/μl of reaction; Roche Molecular Biochemicals), and the reaction mixture was incubated at 30 °C for 1 h. The samples were analyzed by electrophoresis on a 6% polyacrylamide denaturing gel.

Northern blot analysis was performed essentially as described by Tarn et al. (21) except that each sample contained 0.5 μg of RNA and anti-U1 riboprobe was used.

RESULTS

EV-HPV E2 Proteins Contain RS Dipeptide Repeats in the Hinge—As described above, the E2 protein of EV-HPVs has an unusual feature in its primary structure, i.e. a long hinge region containing multiple RS dipeptide repeats and RG or RGG repeats (for HPV type-5, see Fig. 1). A phylogenetic analysis by comparing the hinge amino acid sequences revealed distinct groupings of E2 proteins encoded by EV-HPVs (data not shown). In the group of EV-HPVs (including HPV-5), the hinge region of E2 is the longest (200 aa) among all papillomavirus E2 proteins, in general, whereas the E2 hinge in another group of EV-HPVs is moderate in length (150 aa) and contains fewer RS repeats (12 in average). In sharp contrast, the hinge of the E2 protein encoded by non-EV-HPVs is relatively short in length (60 aa) and lacks repeated RS or RG sequences (Fig. 1).

In Vitro Interactions of the HPV-5 E2 Protein with RS Domains—RS domains are known to be involved in protein-protein interaction between pre-mRNA splicing factors (4–6). This notion prompted us to ask whether an E2 protein containing the RS-rich hinge could specifically interact with cellular splicing factors that also harbor an RS domain. A mixture of mAb104 reactive SR proteins was isolated from HeLa cells by a two-step salt precipitation as described by Zahler et al. (8). The SR protein preparation contained six major polypeptides, two of which are of ~30 kDa (Fig. 2A, lane 2), as reported (8). For Western blot analysis, SR proteins were fractionated by SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane, followed by incubation with in vitro translated, 35S-labeled HPV-5 or HPV-16 E2 protein. The HPV-5 E2 protein appeared to interact with all members of this set of SR proteins (lane 3), but no significant interaction was detected with the labeled HPV-16 E2 protein (lane 4) or with the HPV-5 E2 deletion mutant lacking the hinge region (data not shown). This result suggests that interactions occur between the HPV-5 E2 protein and SR proteins, likely via their RS domain.

We next examined whether the HPV-5 E2 protein could interact with components of spliceosomal snRNPs. Proteins associated with Sm snRNPs were enriched from HeLa cell nuclear extracts by precipitation with 65% saturated (NH4)2SO4, followed by immunoprecipitation with anti-Sm antibody. snRNP-associated proteins are shown in Fig. 2B (lane 2). Using 35S-labeled HPV-5 E2 protein as a probe, snRNP-associated polypeptides of approximately 100 and 70 kDa were reproducibly detected on Far-Western blots (lanes 6 and 10). The triplet 70-kDa bands probably correspond to U1–70K (lane 4), which is an integral component of the U1 snRNP and contains an arginine alternating domain at the C terminus. Recently, two additional snRNP-associated SR proteins were identified: the 27-kDa protein specific to the U4/U6/U5 tri-snRNP and the 100-kDa U5 snRNP protein, which is also present in the tri-snRNP complex (22–24). Both proteins contain an RS or an arginine alternating domain at their N terminus. Antiserum against the U5–100K protein specifically recognized a doublet band in the snRNP protein enriched fraction (lane 8), but not in the mock immunoprecipitate (lane 7). The doublet comigrated with the ~100-kDa HPV-5 E2-interacting protein (lane 10), suggesting that the U5–100kD protein is possibly the other snRNP-associated protein that can interact with the HPV-5 E2 protein (see below for further analyses).
This result therefore suggests that the HPV-5 E2 protein can interact with snRNP-associated proteins, which contain an arginine alternating domain.

To examine whether interactions between HPV-5 E2 and SR proteins could occur in solution, we performed in vitro association assay by coimmunoprecipitation. The immunoprecipitation efficiency was first evaluated by incubation of the reticulocyte lysate mixture containing in vitro synthesized, $^{35}$S-labeled HPV-5 E2 protein ($\approx$0.7 pmol) with anti-E2 antibody-coupled protein A-Sepharose. Approximately 10% of loaded E2 protein was immunoprecipitated (Fig. 3, top panel). For coimmunoprecipitation experiments, HPV-5 E2 and individual SR proteins were synthesized separately in in vitro translation; only SR proteins but not the E2 protein were labeled with $^{35}$S)methionine in order to avoid confusing data resulted from comigration of U1–70K and E2 on SDS-PAGE. The E2 protein was then mixed with an SR protein in the reticulocyte lysate, followed by immunoprecipitation. As shown in Fig. 3 (bottom panel), all four SR proteins were coimmunoprecipitated with the HPV-5 E2 protein by anti-E2 antibodies, but not by control antibodies. However, SR proteins alone failed to be precipitated by anti-E2 antibodies, and the control protein, GAL4-VP16, had no detectable interaction with the E2 protein (Fig. 3, bottom panel), suggesting that the immunoprecipitation of SR proteins can be attributed to their specific association with the HPV-5 E2 protein. Consistent with the results of Far-Western blot analysis, the E2 protein lacking the RS-rich hinge had no significant interaction with ASF/SF2 (data not shown). In summary, the result of the association assay provides additional evidence for in vitro interactions of the HPV-5 E2 protein with at least four cellular RS domain-containing splicing factors.

**Detection of Specific Interactions between HPV-5 E2 and SR Proteins in Vivo by the Yeast Two-hybrid Assay**—We next examined whether the HPV-5 E2 protein interacts with SR proteins in vivo by the yeast two-hybrid assay. The full-length or hinge-deleted HPV-5 E2 (Fig. 1) was fused to the GAL4 activation domain, and SR proteins were fused to the LexA DNA binding domain. Recombinant plasmids as well as the Lac Z reporter were transformed into the yeast strain EGY48 and the transformants were assayed for the $\beta$-galactosidase activity. As shown in Table I, the full-length HPV-5 E2 protein interacted with all the tested SR proteins, whereas deletion of the hinge in the E2 protein dramatically reduced its interaction with SR proteins. Western blot analysis using anti-E2 antibodies revealed that the level of the GAL4-E2A fusion protein was severalfold higher than that of the full-length E2 fusion protein (data not shown). Thus, we conclude that the RS-rich
hinge region of the HPV-5 E2 protein is essential for E2 interactions with RS domain-containing splicing factors. However, in the two-hybrid assay, the two snRNP proteins appeared to have only weak interaction with the E2 protein. This may in part be attributed to the low amount of U5–100kD and U1–70K fusion proteins yielded in yeast (data not shown). Moreover, it is important to note that interactions between SR proteins can be influenced by phosphorylation of RS domains (24). Phosphorylation of RS proteins in the reticulocyte lysate may differ from that in yeast, thus resulting in different relative strength of interaction observed in the various assays. Nevertheless, both in vitro and in vivo analyses demonstrated the importance of the hinge domain for the interactions of the HPV-5 E2 protein with SR proteins.

**Localization of Transiently Expressed HPV-5 E2 Protein in Nuclear Speckles** —Localization studies using antibodies specific for snRNPs and non-snRNPs SR proteins revealed that these nuclear factors are concentrated in nuclear speckles, the interchromatin granule-related clusters (4). Since the HPV-5 E2 protein contains an RS-rich sequence and can interact with several splicing factors, we examined whether the E2 protein behaves similarly to cellular SR proteins with regard to the cellular localization. HeLa cells were transfected with a vector that expressed the HPV-5 E2 protein under the control of the human cytomegalovirus enhancer-promoter. Double immunofluorescent staining was then performed using anti-E2 antibodies and mAb B4A11, which was selected because it gives prominent punctate immunofluorescent staining pattern in interphase nuclei and its antigen is colocalized with Sm antigens and SC35 in nuclear speckle domains (25). Polyclonal anti-E2 antibodies were raised against the hinge-deleted E2 protein expressed in *E. coli*; thus, the antibodies should not cross-react with any cellular SR proteins. In untransfected HeLa cells, immunofluorescent staining using purified anti-E2 antibodies revealed only very weak, diffuse signals throughout the whole cell (data not shown). Fig. 4a shows that transiently expressed HPV-5 E2 protein, like cellular SR proteins, displayed a speckled staining pattern in the nucleus. Colocalization experiments revealed that the majority of punctate spots of the E2 protein appears to overlap with foci labeled with mAb B4A11 within the optical section (panels a–c). At least 50 transfected cells scored from several separate experiments were analyzed. Although E2 and B4A11 fluorescent signals displayed a high degree of overlap in the overlaid image shown in panel c, 10–30% of speckles were stained either by anti-E2 or by B4A11 antibodies in a small fraction of transfected cells (data not shown). To exclude the possibility that overexpressed E2 protein aggregated to form a speckled pattern, a vector that does not replicate to high copy number was used to express the HPV-5 E2 protein in HeLa cells. A similar result was obtained (data not shown). Moreover, a similar staining pattern was also observed in cells expressing the HA epitope-tagged HPV-5 E2 protein (panel i). Thus, it appears very unlikely that a cellular protein cross-reacting with anti-E2 antibodies was induced and accumulated in nuclear speckles upon overexpression of E2.

Deletion of the hinge prevented the E2 protein from accumulating in nuclear speckles, and instead resulted in uniform nucleoplasmic distribution but with exclusion of nucleoli (panel e). Localization of the hinge-deleted HPV-5 E2 protein is therefore coincident with that of the BPV-1 E2 protein (Ref. 26 and data not shown). In summary, the HPV-5 E2 protein is localized in nuclear speckle domains where an SR-related splicing coactivator is concentrated, and the RS-rich hinge is critical for targeting the E2 protein to such domains.

Dramatic changes in nuclear ultrastructure have been observed upon infection of a variety of viruses or upon transient expression of a viral protein (27–29). Distribution of nuclear speckles reacting with mAb B4A11 was apparently not affected by overexpression of the HPV-5 E2 protein (Fig. 4, panels a–c, and data not shown). Nor was a change in the number or size of coiled bodies observed under the same conditions (data not shown). Nevertheless, it is still possible that infection of EV-HPV could cause reorganization of splicing factors in the nucleus of host cells.

**The HPV-5 E2 Protein Transactivates an Intron-containing Reporter More Efficiently than Its Hinge-deleted Mutant**—The various assays described above indicated that the HPV-5 E2 protein could interact with several RS domain-containing splicing factors through its hinge. We therefore hypothesized that the E2 protein functions not only in transcriptional activation but also in the splicing of primary transcripts. To test this possibility, we first determined the ability of the HPV-5 E2 protein to transactivate its responsive promoter and further examined whether deletion of the hinge would have any effect on E2’s transactivation ability. Previously, BPV-1 and HPV-16 E2 proteins were shown to transactivate an E2-dependent reporter, pE2x2-Sp1x2-tk38-cat (abbreviated herein as pE2Sp1-CAT; Ref. 16), which contains two consensus E2 sites and two Sp1 sites in the HSV-1 thymidine kinase (tk) gene minimal promoter upstream of the CAT reporter gene (Fig. 5A). Like those two E2 proteins, HPV-5 E2 activated transcription at such a defined promoter in a concentration-dependent fashion and at a comparable level as HPV-16 E2 when the same amounts of DNA per culture were employed (data not shown). Fig. 5B, upper panel, shows that the transactivation activity of the hinge-deleted E2 protein (lane 3) was similar to that of full-length E2 (lane 2), suggesting that the RS-rich hinge domain apparently makes no significant contribution to transcriptional activation.

Next, to test whether the E2 hinge plays some role in the splicing of pre-mRNA, we inserted a modified human β-globin intron 1 into the CAT coding region (Fig. 5A). The resulting plasmid is referred to as pE2Sp1-CAT(in1). The presence of an in-frame stop codon within the β-globin intron would impede production of active enzyme without splicing of the CAT pre-mRNA. When the pE2Sp1-CAT(in1) construct was used as a reporter, 5-fold more extract was required in the assay to achieve levels of CAT activity comparable to those of the reporter lacking the intron. This indicated that CAT mRNA production was suppressed in cis by the insertion of the β-globin intron. Nevertheless, Fig. 5B shows that the transactivation activity of full-length E2 was ~4-fold higher than that of hinge-deleted E2 (upper panel, lanes 5 and 6). Expression of the transactivators was examined by Western blot analysis using anti-E2 antiserum and showed that the level of E2 or E2ΔH
protein was similar in transfectants with different reporters (Fig. 5B, bottom panel, compare lanes 2 and 5 for E2, and lanes 3 and 6 for E2ΔH). Moreover, transactivation activity was monitored by cotransfection of either of the CAT reporters with a β-galactosidase construct that did not contain the intron. Expression of the β-galactosidase gene was driven by the E2-responsive promoter, the same as that used in the CAT reporter constructs (Fig. 5A). Regardless of whether the CAT reporter contained the β-globin intron, extracts from E2 and E2ΔH transfectants exhibited similar levels of β-galactosidase activity (data not shown). The results from three cotransfection experiments are summarized in Fig. 5C, emphasizing that the transactivation activity of hinge-deleted E2 became significantly lower than that of full-length E2 only when the E2-responsive reporter contained the intron. It is most noteworthy that the level of the CAT activity transactivated by E2 was only ~10-fold higher than the background when pE2Sp1-CAT was used as a reporter (Fig. 5B, upper panel, lane 2); however, transactivation by E2 appeared to increase synergistically to 30-fold when the reporter used contained the β-globin intron (lane 5). This result indicated that, besides transcriptional activation, at least another mechanism involves to achieve the synergistic effect of the full-length E2 protein on the transactivation of an intron-containing reporter, which is thus likely the splicing of pre-mRNA.

The HPV-5 E2 Protein Facilitates the Splicing of Pre-mRNA Transactivated by E2 Itself—We then analyzed the splicing of the CAT primary transcript transactivated by full-length and hinge-deleted E2. Duplicate transfections were performed. Total RNA from one dish of transfected cells was collected and subjected to RNase T1 protection assay using the probe spanning the entire β-globin intron and its downstream CAT gene (Fig. 5A). Transfected cells in the duplicate dish were assayed for CAT activity in order to evaluate CAT production. No significant difference was observed between the amounts of the CAT mRNA transactivated by E2 and E2ΔH while normalized by the level of U1 small nuclear RNA (Fig. 6A, lanes 2 and 3). This confirmed that transcriptional activation was of nearly equal efficiency with these two transactivators when pE2Sp1-CAT was used. Examination of the CAT pre-mRNA splicing from three independent experiments revealed that a 2-fold difference in the ratio of spliced to unspliced CAT transcript between full-length and hinge-deleted E2 (Fig. 6A, lanes 5 and 6, and 6B, lane 1). CAT production at the protein level was simply represented by CAT activity, i.e., percentage of chloramphenicol conversion without subtraction of the background obtained from transfection with the empty expression vector. The level of the CAT protein in E2ΔH transfected cells was ~36% that of the CAT protein produced by full-length E2 when the intron-containing reporter was used (Fig. 6B, lane 4). A greater difference between E2 and E2ΔH in producing CAT protein than in promoting splicing can be rationalized by the fact that the transactivation activity of hinge-deleted E2 reduced slightly by the presence of the β-globin intron in the reporter (Fig. 5B, compare lane 6 to lane 3 for the activation (n-fold) of E2ΔH). Nevertheless, all above results can support our previous speculation that the E2 transactivator plays a role in assisting pre-mRNA splicing through its RS domain. However, RS domain-containing proteins may have some other biological activities (30, 31); thus, the possibility that HPV-5 E2 can act through other mechanisms to regulate gene expression cannot be excluded.

Next, in order to examine whether the HPV-5 E2 protein can function in trans to facilitate splicing, we replaced the promoter of the pE2Sp1-CAT(In1) reporter with the SV40 enhancer-promoter (Fig. 5A), which should be independent of E2 transactivation. Cotransfection of full-length E2 or hinge-deleted E2 with the SV40-CAT(In1) reporter resulted in similar level of the CAT activity (Fig. 6B, lane 5). RNase protection assays revealed that splicing of the CAT pre-mRNA transactivated through the SV40 promoter was of similar efficiency in E2 and E2ΔH transfected cells (Fig. 6A, lanes 7 and 8, and 6B, lane 2). Thus, the results suggest that the E2 protein could not transactivate splicing without activation of gene transcription.
The HPV-5 E2 Protein Probably Assists Pre-mRNA Splicing in a Distance-dependent Manner—

As described above, by insertion of the \(\beta\)-globin intron into a E2-responsive CAT reporter at the position 209 base pairs downstream of the trans-
An HPV E2 Transactivator Can Function Post-transcriptionally

The E2 protein encoded by EV-associated HPVs contains an RS-rich sequence in the central hinge region, implying its role in pre-mRNA splicing. As predicted, we demonstrate here that the RS-rich hinge is essential for HPV-5 E2’s interaction with cellular splicing factors and also for its colocalization with SR proteins in nuclear speckles. More importantly, functional evidence showed that the RS-rich hinge of the HPV-5 E2 transactivator can facilitate the splicing of a primary transcript transactivated by E2 itself probably in a distance-dependent manner.

The various assays showed that the HPV-5 E2 protein can interact with at least four SR proteins (Figs. 2 and 3 and Table I). Although it is presently unclear whether all these proteins represent physiologically relevant targets for E2 in vivo, the following reasons may argue against that the E2-SR protein interactions detected in this study lacked specificity. First, SR proteins used in our in vitro protein-protein interaction assays were either isolated from HeLa cells or synthesized in the reticulocyte lysate, thus preventing nonspecific interaction between unphosphorylated RS domains as reported previously (32). Second, in the presence of RNase, the interactions between E2 and SR proteins on Far-Western blots were still detectable, albeit relatively weak (data not shown). This rules out the possibility that E2 and SR proteins only nonspecifically anchor an RNA molecule. Third, only two SR proteins from a pool of snRNP proteins were identified on Far-Western blots by using E2 as a probe, indicating the specificity of the interactions. Moreover, the recombinant HPV-5 E2 protein purified from the baculovirus system can be detected by mAb 104 (data not shown), suggesting that E2, like SR proteins, is phosphorylated in vivo. Phosphorylated RS domain may thus mediate specific interactions between E2 and cellular SR proteins under physiological conditions (32, 33). Finally, since in this study we examined the interactions of the HPV-5 E2 protein with subsets of SR proteins, which are either recognized by mAb104 or associated with the Sm snRNPs, it is still possible that the E2 protein can interact through its hinge with other cellular RS domain-containing proteins, for example, SR-like CTD-associated factors (30, 34), SR-related splicing coactivators (35), or a set of high molecular weight SR proteins recognized by mAb 16H3 (36).

** både in vitro (Fig. 3) and in vivo (Table I) protein-protein interaction assays provided an indication that the E2 protein has higher affinity to ASF/SF2 than to SC35. Both these two SR proteins can activate constitutive splicing and influence selection of alternative splice sites (4–6). However, their distinct RNA binding specificity suggests that they behave differently by binding to specific RNA elements (37). It is also noteworthy that ASF/SF2 can assist the recruitment or stabilization of the U1 snRNP to the functional 5' splice site (38). Thus, E2's apparent preference for ASF/SF2 may suggest that the E2-SR protein interaction experiment that the HPV-5 E2 protein interacted significantly with two snRNP-associated SR proteins, U1–70K and U5–100kD; the former appeared to be the most preferred protein bound by E2 among all those tested (Fig. 3). Conceivably, the U1–70K protein becomes near the 5' splice site of the intron in the early splicing complex. The U5–100kD protein has been speculated to replace U1–70K in its interactions with other SR
proteins when U5 and U6 snRNPs take over the 5′ splice site following destabilization of U1, because it, like U1–70K, possesses an alternating charge domain instead of a typical RS domain (23). Our results may therefore indicate that the HPV-5 E2 protein is capable of interacting with the SR proteins, which favor association with the 5′ splice site of pre-mRNA intron. Next, it will be of interest to test whether the E2 protein can direct SR proteins to modulate 5′ splice site selection and thus participate in alternative splicing.

Here, we demonstrate that the RS-rich hinge is required for E2 protein's colocalization with splicing factors in nuclear speckles (Fig. 4) and also for its function in promoting pre-mRNA splicing (Fig. 5). However, questions still remain as to whether the E2 transactivator functions within nuclear speckles and whether the speckle pattern of E2 expression is critical for E2's function in facilitating splicing. Recent data have revealed that precursor mRNA is transcribed and processed at foci dispersed throughout the nucleoplasm, supporting the idea that speckles may function as reservoirs that supply factors for gene expression (39, 40). Therefore, it is reasonable to predict that the HPV-5 E2 transactivator may leave speckles, coordinate with splicing factors, and accumulate at the sites of transcription for function. Moreover, phosphorylation of the RS-rich hinge region may regulate the sub-nuclear localization and function of the E2 protein, thereby modulating its function. All these hypotheses remain to be tested.

This study provides evidence that the HPV-5 E2 protein can facilitate the splicing of CAT primary transcripts containing the β-globin intron; however, the intron’s distance relative to the 5′ end of the transcript can determine the efficacy of the RS-rich hinge domain in assisting splicing. In fact, we observed in this study that insertion of the β-globin intron at different positions of the CAT reporter resulted in different degrees of reduction in transactivation by E2, and among all the constructs transactivation of pE2Sp1-CAT(In1) (the intron at position 209) was affected most severely. Accordingly, the synergistic effect of E2 in transactivation appeared to be most pronounced with this reporter. This is also partially in concert with our another observation that the insertion of an adenovirus intron (41) at position 209 markedly activated expression of the CAT reporter but completely neglected E2′ effect in splicing (data not shown). We therefore conclude from the apparent results that the HPV-5 E2 transactivator may conditionally facilitate the splicing of precursor mRNA in which the intron is inefficiently spliced, or located close to the 5′ end of the transcript. However, the possibility that the E2 protein controls the nuclear export of incompletely spliced mRNA or even the translation cannot be excluded. Although an artificial system was used, this study provides some clues to the mechanistic action of the HPV-5 E2 transactivator on assisting the splicing of pre-mRNA.

Recently, increasing evidence indicates previously unexpected roles for transcription factors in linking transcription and pre-mRNA processing (42, 43). The largest subunit of RNA pol II was first implicated in pre-mRNA splicing and polyadenylation through its C-terminal domain (44–46). Many lines of evidence suggest that the interaction of splicing factors with phosphorylated RNA pol II CTD is probably essential for spliceosome function (30, 47). Moreover, polyadenylation factors and the capping enzymes are also targeted onto pre-mRNA by binding to the phosphorylated CTD of RNA pol II (46, 48, 49). More recently, a holon-TFIID complex containing cleavage poly(A) specificity factor (CPSF) was found, indicating that TFIID not only nucleates transcription initiation but also assists polyadenylation by recruiting the CPSF complex to the promoter (50). In this study, we showed that pre-mRNA splicing may be mechanistically coupled to transcription through an RS-rich sequence-containing transactivator. Therefore, besides RNA pol II and TFIID, another category of transcription factors, DNA binding transactivators, can play a role in coordinating transcription and RNA processing. Conceivably, RNA pol II is physically proximal to the growing nascent transcript while RNA synthesis proceeds, whereas TFIID only binds to the core promoter during transcription initiation (51). Thus, CPSF recruited by TFIID prior to the elongation step of transcription may need to be delivered to the nascent RNA through phosphorylated RNA pol II (50). More like TFIID, transactivators bind to a sequence-specific element nearby the promoter and primarily play a role in initiation/reinitiation of transcription. The mechanism whereby the E2 transactivator functions post-transcriptionally needs further investigation. At present, a plausible mechanism is that the RS-rich hinge of the E2 transactivator recruits essential splicing factors including non-snRNP SR proteins and spliceosomal snRNPs, thereby increasing their concentration surrounding the promoter of an actively transcribed gene (Fig. 8). Whether the HPV-5 E2 transactivator can pass SR proteins to the RNA polymerase or even become incorporated into the spliceosome remains to be examined.

Expression of HPV genes is regulated via promoter activity and RNA processing (52). Complex splicing patterns produce an amazing variety of viral transcripts. Regulation of pre-mRNA splicing has been studied in more detail for BPV-1. The environment of a differentiating squamous epithelium plays an important role in alternative splicing of viral late transcripts, thus determining the fate of virus (53). In addition, multiple cellular SR proteins including ASF/SF2 are likely involved in modulating 3′ splice site selection of late pre-mRNAs through their binding to exonic sequences (54). Our finding revealing that an HPV E2 protein can interact with cellular SR proteins and likely couple transcription and pre-mRNA splicing may in future lead to new insights into the regulation of viral gene expression. Finally, our observation that E2 assists the splicing of a primary transcript in a distance-dependent manner (Fig. 7) may reflect that an intron closer to the E2 binding sites would be preferentially targeted by E2’s regulation. Future experiments will be aimed at determining how the E2 transactivator mediates the control of viral mRNA processing through its RS domain.

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