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In Vitro Evaluation of Phosphorothioate Oligonucleotides Targeted to the E2 mRNA of Papillomavirus: Potential Treatment for Genital Warts

LEX M. COWSERT,[1]* MAUREEN C. FOX,[1] GERALD ZON,[2]† AND CHRISTOPHER K. MIRABELLI[1]

ISIS Pharmaceuticals, 2280 Faraday Avenue, Carlsbad, California 92008,[1] and Applied Biosystems, Foster City, California 94404[2]

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Papillomaviruses induce benign proliferative lesions, such as genital warts, in humans. The E2 gene product is thought to play a major role in the regulation of viral transcription and DNA replication and may represent a rational target for an antisense oligonucleotide drug action. Phosphorothioate oligonucleotides complementary to E2 mRNAs were synthesized and tested in a series of in vitro bovine papillomavirus (BPV) and human papillomavirus (HPV) models for the ability to inhibit E2 transactivation and virus-induced focus formation. The most active BPV-specific compounds were complementary to the mRNA cap region (ISIS 1751), the translation initiation region for the full-length E2 transactivator (ISIS 1753), and the translation initiation region for the E2 transrepressor mRNA (ISIS 1755). ISIS 1751 and ISIS 1753 were found to reduce E2-dependent transactivation and viral focus formation in a sequence-specific and concentration-dependent manner. ISIS 1755 increased E2 transactivation in a dose-dependent manner but had no effect on focus formation. Oligonucleotides with a chain length of 20 residues had optimal activity in the E2 transactivation assay. On the basis of the above observations, ISIS 2105, a 20-residue phosphorothioate oligonucleotide targeted to the translation initiation of both HPV type 6 (HPV-6) and HPV-11 E2 mRNA, was designed and shown to inhibit E2-dependent transactivation by HPV-11 E2 expressed from a surrogate promoter. These observations support the rationale of E2 as a target for antiviral therapy against papillomavirus infections and specifically identify ISIS 2105 as a candidate antisense oligonucleotide for the treatment of genital warts induced by HPV-6 and HPV-11.

Papillomaviruses cause a spectrum of benign and malignant diseases in both animals (23) and humans (44). Despite its widespread occurrence as a sexually transmitted disease (28) and its association with malignant disease (44), no specific antiviral agents for papillomavirus exist. Current treatment of genital warts is nonspecific and consists primarily of surgical or chemical removal of the wart. These therapies are not curative because they are unable to produce a loss of the viral DNA episome that resides in the basal epithelium of latently infected patients.

Antisense oligonucleotides have been shown to be effective, specific inhibitors of gene expression (18) and to be active against a number viruses including Rous sarcoma virus (42), vesicular stomatitis virus (1), herpes simplex virus type 1 (31), influenza virus (43), and human immunodeficiency virus (41). Current information regarding gene structure and sequence and transcriptional regulation of a variety of papillomaviruses led us to adapt a number of papillomavirus gene expression and infection models in order to evaluate the potential use of antisense oligonucleotides as an approach to the treatment of human papillomavirus type 6 (HPV-6) and HPV-11-induced genital warts. We took advantage of the similarities in gene structure and function of bovine papillomavirus type 1 (BPV-1) and HPV-6 and HPV-11 to test the hypothesis that the E2 gene may represent a rational therapeutic target.

The E2 open reading frame of papillomaviruses encodes a family of sequence-specific DNA-binding proteins that regulate transcription of papillomavirus early genes (26, 35). This observation was first made in BPV-1 (34) and has been extended to other papillomaviruses including HPV-11 (21), HPV-16 (29), and HPV-18 (16). In BPV-1, at least three E2 gene products have been identified: the full-length E2 gene which functions as a transcriptional transactivator, a truncated E2 gene which is transcribed from the promoter at nucleotide (nt) 3080 functions as a transcriptional transrepressor (22), and a truncated E2 gene transcribed from the promoter at nt 890 that uses the splice donor/acceptor sites at nt 1235/3225 (6).

The activities of BPV-1 promoters are strongly dependent upon an E2-dependent transcriptional enhancer located in the long control region of the BPV-1 genome. The promoters are responsible for the expression of the transforming gene E6, as well as genes E7 and E1, which are involved in plasmid replication and which are regulated by the E2 responsive element E2RE1 located in the long control region (17, 33). In addition, transcription from P_{2443}, which directs transcription of the transforming gene E5 as well as that of gene E2, is under the control of E2RE1 (20). Thus, E2 regulates the expression of viral genes responsible for transformation, replication, and transcriptional regulation.

In HPV the role of full-length E2 as a transcriptional transactivator versus its role as a transrepressor is less clear. Full-length E2 has been shown to be a transcriptional transactivator that acts through E2 conditional enhancers (26). Full-length E2 has also been demonstrated to act as a repressor of the E6 promoter for HPV-11 and for HPV-16 and HPV-18 (3, 5, 30). On the basis of these observations, it has been proposed that the major function of full-length E2 in HPV is repression of viral transcription, perhaps only at high concentrations of E2 such as those achieved by expres-

* Corresponding author.
† Present address: Lynx Therapeutics, Foster City, CA 94404.

sion from surrogate promoters. The relative roles for these two activities at physiologic concentrations and in the context of the viral genome are not yet known.

In addition to its central role in the regulation of viral transcription, E2 has also been identified as one of two viral gene products required for viral DNA replication for BPV-1, HPV-11, and HPV-16 (8, 27). On the basis of its critical position in the regulation of viral transcription and its direct role in viral DNA replication, E2 was selected as a target for antisense oligonucleotides. Oligonucleotides that are designed to bind to the E2 mRNA and, as a result, that inhibit the expression of E2 protein should disrupt the regulation of other papillomavirus gene products, should inhibit the life cycle of the virus, and may provide a novel class of selective antiviral compounds for the treatment of papillomavirus infection. HPV-6 and HPV-11 were selected because they are found in approximately 90% of genital condylomas (12, 13, 44).

In this report we describe the in vitro activities of a series of oligonucleotides designed to hybridize to specific sequences within the BPV-1 E2 and HPV-11 E2 mRNAs. Phosphorothioate oligonucleotides were used in the studies in order minimize the potential metabolic inactivation of serum and cellular nucleases. As a result of the present investigation, antisense oligonucleotides which inhibit BPV-1 transactivation and transformation and HPV-11 E2 transactivation were identified.

MATERIALS AND METHODS

Cells and viruses. C127 mouse cells (10) were maintained in antibiotic-free Dulbecco modified Eagle medium, high glucose (4,500 mg/liter) supplemented with glutamine to 40 mM, and heat-inactivated (65°C, 30 min) fetal bovine serum to 10%. I-38 cells are a single cell clone derived from a single focus after infection of C127 cells with BPV-1.

BPV-1 was purified from experimentally induced fibropapillomas by double banding in cesium chloride as described previously (7). Virions were characterized by electron microscopy (2) and enzyme-linked immunosorbent assay with a BPV-1 type-specific monoclonal antibody (7). Viral DNA was characterized by digestion with a panel of restriction endonucleases, and biological activity was assessed by quantitative focus formation on C127 cells (10).

Plasmids. Plasmid IPV100 contains the complete BPV-1 genome (7,945 bp) cloned at the *Bam*HI into PUC19. Plasmid PCH110 (Pharmacia, Piscataway, N.J.) constitutively expresses the β-galactosidase gene and was used to control for transfection efficiency. Plasmid IPV110 contains the E2RE1 of BPV-1 (nts 7200 to 7386) cloned into the *Sal*I site of pCAT-Enhancer (Promega, Madison, Wis.). E2RE1 was reconstructed by using overlapping oligonucleotides (32) and contains *Sal*I overhangs. Expression of the chloramphenicol acetyltransferase (CAT) gene was shown to be dependent on E2. Plasmid IPV118 contains the 2.3-kb *Xmn*I fragment of HPV-11 (nts 2665 to 4988) (12) cloned into the pSVL (Pharmacia, Piscataway, N.J.) simian virus 40-based expression vector. Plasmid IPV120 contains the long control region of HPV-11, amplified by polymerase chain reaction, cloned into pCAT-Enhancer. Expression of the CAT gene from IPV120 was shown to be dependent on expression of E2 from either HPV-11 E2 or BPV-1 E2 expression constructs.

Design and synthesis of oligonucleotides. Forty-one oligonucleotides were designed to be complementary to the BPV-1 E2 mRNA. All oligonucleotides were synthesized as phosphodiester or phosphorothioate. Oligonucleotides were designed to be complementary to such regions as the 5′ cap region, initiation of translation region, translation termination region, and polyadenylation signal. In addition, oligonucleotides were designed to be complementary to the 5′-untranslated region, the 3′-untranslated region, and various locations within the coding region. Oligonucleotides were synthesized by a solid-support phosphoramidite procedure at Applied Biosystems, Inc. (Foster City, Calif.), as described previously (39). The compounds used in these experiments were shown to be greater than 90% pure with reference to contaminating oligonucleotides of different chain lengths by polyacrylamide gel electrophoreses and densitometric scanning.

Oligonucleotide modulation of E2-dependent transactivation. For BPV-1 E2 experiments, I-38 cells were plated 24 h prior to transfection at 5×10^4 cells per cm^2 in 60-mm petri dishes. After attachment, cells were treated overnight with oligonucleotide by adding oligonucleotides to the medium at the stated concentrations. The next day, the medium was aspirated and replaced with fresh medium without oligonucleotide and was incubated for 1 h at 37°C in a humidified chamber. Cells were cotransfected with 10 μg each of IPV110 and PCH110 by the calcium phosphate coprecipitation method (15). Four hours after transfection, cells were treated with 15% glycerol for 1 min (11), refed with medium containing oligonucleotide at the original concentration, and incubated for 48 h. After incubation, cells were washed with phosphate-buffered saline, scraped and transferred to a microcentrifuge tube, and centrifuged. Cells were suspended in 100 μl of 250 mM Tris-HCl and disrupted by three freeze-thaw cycles. Extracts were cleared by centrifugation, transferred to a fresh tube, and stored at −80°C until assayed for CAT and β-galactosidase activities.

For inhibition of HPV-11 E2 transactivation, C127 cells were pretreated with oligonucleotide as described above; this was followed by cotransfection with 13 μg of IPV118 HPV-11 E2 expression plasmid, 5 μg of IPV120-15 E2-CAT reporter plasmid, and 2 μg of PCH110 as described above. Following transfection, cells were treated with oligonucleotide and were incubated for 48 h. Cells were harvested and processed for CAT and β-galactosidase assays as described above.

CAT activity was determined by standard protocols (14). Acetylated and nonacetylated reaction products were separated by thin-layer chromatography and were quantitated by using a Molecular Dynamics PhosphoImager (Molecular Dynamics, Sunnyvale, Calif.). β-Galactosidase activity was determined by standard procedures (19). To control for transfection efficiencies, data were normalized to β-galactosidase activity.

Inhibition of BPV-1 focus formation. C127 cells were plated at subconfluence (5×10^4 cells per cm^2) in 60-mm petri dishes. Cells were either infected with 50 focus-forming units per plate of purified BPV-1 or transfected with cloned BPV-1 DNA. Twenty-four hours after infection or transfection, oligonucleotides were added to the medium. The medium was changed every 72 h, with fresh oligonucleotide added at each medium change. At 25 days postinfection, cells were fixed in 10% formalin in phosphate-buffered saline for 5 min and stained with 0.14% methylene blue aqueous solution for 10 min. Plates were washed with water and foci were counted.

Oligonucleotide-mediated, site-specific cleavage of HPV-11 E2 transcripts. HeLa cell nuclear processing extract was prepared by the procedure of Dignam et al. (9). Extracts were stored at −80°C in processing extract buffer (20%

TABLE 1. Antisense oligonucleotides tested for inhibition of E2 transactivation[a]

Oligomer	Sequence[b]	Target[c]	Activity
1751	AGGTTTGCACCCGACTATGCAAGTACAAAT	BPV-1 E2 mRNA cap	Active
1753	CGTTCGCATGCTGTCTCCATCCTCTTCACT	BPV-1 E2 mRNA AUG-TA	Active
1755	AAATGCGTCCAGCACCGGCCATGGTGCAGT	BPV-1 E2 mRNA AUG-TR	Active
2324	CTGTCTCCATCCTCTTCACT	BPV-1 E2 AUG	Active
2325	TGTCTCCATCCTCTTCAC	BPV-1 E2 AUG	Active
2326	GTCTCCATCCTCTTCA	BPV-1 E2 AUG	Active
2327	TCTCCATCCTCTTC	BPV-1 E2 AUG	Nonactive
2328	CCATCCTCTTCACT	BPV-1 E2 AUG	Nonactive
2105	TTGCTTCCATCTTCCTCGTC	HPV-6 and HPV-11 E2 mRNA AUG	Active

[a] Oligonucleotides were synthesized on an automated DNA synthesizer as described previously (31). After synthesis, oligonucleotides were routinely analyzed by electrophoresis through polyacrylamide gels to monitor chain length integrity. The linkage for all oligomers was P=S.
[b] Boldface type indicates positions of the initiation codon.
[c] AUG-TA and AUG-TR, initiation for transactivator and transrepressor, respectively.

glycerol, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.9], 0.1 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol). Purified *Escherichia coli* RNase H was purchased from Pharmacia and was diluted in processing extract buffer prior to use.

HPV-11 E2 transcript was prepared by T7 transcription from plasmid IPV130-20 which contains the 2.3-kb *Xmn*I fragment (nts 2665 to 4998) inserted into the *Sma*I site of pGEM-1 vector (Promega).

Oligonucleotides and transcript were mixed at a 10:1 molar ratio in a buffer consisting of 12.5 mM ATP, 50 mM creatine phosphate, and 8.25 mM $MgCl_2$ and were heated to 60°C for 20 min. After equilibration to room temperature, 15 μl of nuclear processing extract (approximately 10 μg of total protein) or 0.18 U of *E. coli* RNase H was added and the mixture was incubated at 37°C for 15 min. The mixture was deproteinized by phenol-chloroform-isoamyl alcohol extraction and was ethanol precipitated with 10 μg of glycogen as a carrier. Reaction products were fractionated on 0.8% agarose–formaldehyde gels and were visualized by ethidium bromide staining.

RESULTS

Modulation of BPV-1 E2 transactivation. To identify antisense-sensitive regions along the E2 transactivator mRNA, antisense oligonucleotides were designed to hybridize to various regions along the full-length E2 transactivator mRNA of BPV-1 and were tested for the ability to inhibit E2 transactivation in BPV-1-infected cells. Of the 82 oligonucleotides synthesized, only those oligonucleotides with significant and specific activity at 1 μM are listed in Table 1.

In initial screens, two phosphorothioate oligonucleotides complementary to E2 mRNA(s) were identified as being among the most active in reducing E2 transactivation in BPV-1-transformed rodent cells. These two compounds were targeted to the mRNA cap region (ISIS 1751) and the initiation of translation codon (ISIS 1753). In dose-response experiments, ISIS 1753 had a 50% inhibitory concentration (IC_{50}) of 25 to 50 nM, while ISIS 1751 had an IC_{50} of approximately 500 nM (Fig. 1). The shapes of the dose-response curves for the two compounds differed. ISIS 1751 activity was concentration dependent to 500 nM, where it plateaued and exhibited no further activity to 5,000 nM. The activity of ISIS 1753 was dose dependent throughout the entire range tested and was able to reduce E2-dependent CAT activity to background levels.

ISIS 1755, which is complementary to the first initiation of translation codon distal to the promoter identified at nt 3080, increased E2-dependent transactivation in a concentration-dependent manner (Fig. 1).

Inhibition of focus formation. Two compounds that inhibited E2 transactivation, ISIS 1751 and ISIS 1753, also inhibited BPV-1 focus formation on C127 cells (Fig. 2). This effect was found to be concentration dependent with an IC_{50} of 10 nM for ISIS 1753. A clear IC_{50} for ISIS 1751 was not achieved, but it appeared to be in the range of 500 to 1,000 nM. ISIS 1755, which increased E2-dependent transactivation, had no effect on BPV-1 focus formation.

Optimization of chain length. To assess the effect of chain length on efficacy and potency, oligonucleotides of decreasing chain length, complementary to the initiation of the translation region of the E2 transactivator, were synthesized and tested in the E2 transactivation assay (Fig. 3). Comparison of the IC_{50}s revealed that an oligonucleotide of 20 residues was the most potent inhibitor of E2 transactivation and that by the time oligonucleotides of 14 residues were used, all activity was lost. Increasing the chain length to 30 oligonucleotides did not result in increased activity (Fig. 1).

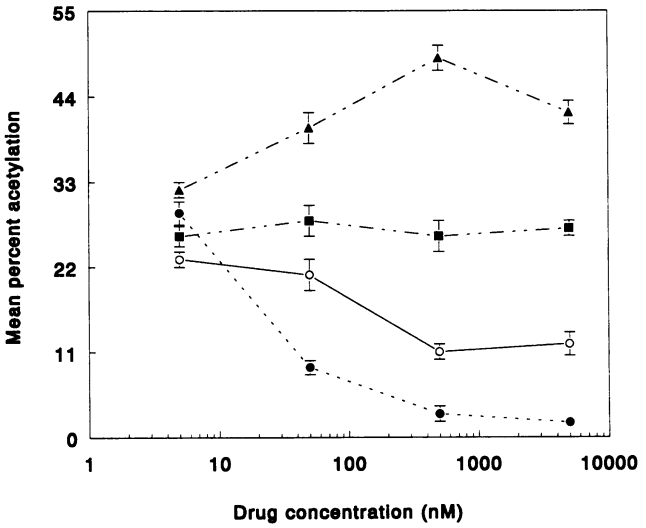

FIG. 1. The E2-dependent CAT expression assay was used to test the concentration dependence of the antisense effect. Each oligonucleotide was tested in triplicate over a 3-log-unit range of oligonucleotide concentrations. Results are the means of triplicate experimental points and are expressed as percent acetylation. Results are normalized to β-galactosidase activity. ○, ISIS 1751; ●, ISIS 1753; ▲, ISIS 1755; ■, ISIS 2105.

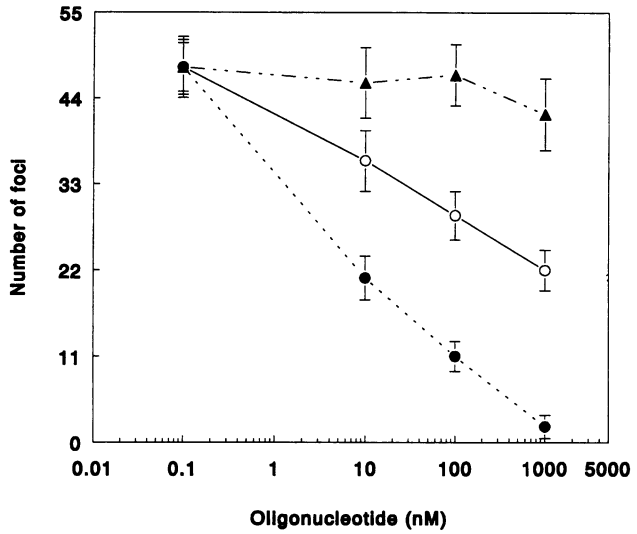

FIG. 2. Inhibition of BPV-1 focus formation by E2 antisense oligonucleotides. C127 cells were plated in 60-mm petri dishes and were infected with 50 focus-forming units of purified BPV-1. At 24 h postinfection, cells were treated with oligonucleotide over a 2-log-unit concentration by direct addition of oligonucleotide to the culture medium. The medium was aspirated and replaced with medium containing oligonucleotide every 72 h. After 25 days, cells were fixed and stained and the foci were counted. Data are the means of triplicate experimental points and are presented as the number of foci per plate. ○, ISIS 1751; ●, ISIS 1753; ▲, ISIS 2105.

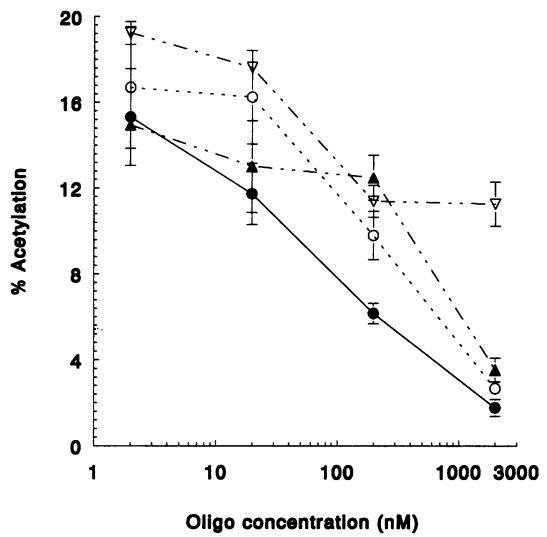

FIG. 3. Optimization of oligonucleotide length. Oligonucleotides centered on the AUG codon of the BPV-1 E2 transactivator of decreasing length were synthesized and tested in the E2 transactivation assay. Cells were pretreated with oligonucleotide and were then transfected with the E2-dependent CAT reporter and constitutive β-galactosidase expression vector. Results are the means of triplicate experimental points and are normalized to β-galactosidase activity. ●, ISIS 2324–20-mer; ○, ISIS 2325–18-mer; ▲, ISIS 2326–16-mer; ▽, ISIS 2327–14-mer.

ISIS 2105 inhibits HPV-11 E2 transactivation. On the basis of the observations presented above, a 20-mer phosphorothioate oligonucleotide, ISIS 2105, was designed to hybridize to the AUG region of the HPV-11 E2 transactivator mRNA covering nts 2713 to 2732. ISIS 2105 was found to inhibit HPV-11 E2-dependent transactivation in a concentration-dependent, sequence-specific manner, with an IC_{50} in the range of 5 to 7 μM (Fig. 4). Within a concentration range in which ISIS 2105 inhibited E2 transactivation up to 70%, ISIS 2324, a BPV-1 E2-specific oligonucleotide, did not produce a significant inhibitory effect in the HPV-11 E2 transactivation assay. This observation was expected since ISIS 2324 has minimal sequence homology with HPV-11. Similarly, ISIS 2105 had no effect on BPV-1 E2 transactivation, as measured in BPV-1-transformed cells (Fig. 1).

ISIS 2105-induced, site-specific cleavage of HPV-11 E2 transcript by RNase H. RNase H is an endoribonuclease that specifically degrades the RNA strand of an RNA-DNA hybrid. This activity was used to test the ability of ISIS 2105 to hybridize to its target in a sequence-dependent manner. The sequence to which ISIS 2105 hybridizes is approximately 117 bases from the 5′ terminus of the T7-produced HPV-11 transcript. If ISIS 2105 hybridized to its target RNA in a sequence-specific manner, then RNase H should cleave off the 5′-terminal 117 bases. Both *E. coli* RNase H and HeLa cell nuclear extracts resulted in specific cleavage of the T7 transcript, consistent with the location of the ISIS 2105-binding site on the HPV-11 transcript (Fig. 5). ISIS 2423 did not induce cleavage of the HPV-11 T7 transcript, demonstrating the requirement for sequence homology. ISIS 2105 did not induce cleavage of a heterologous transcript, human 5-lipoxygenase, for which there is no sequence homology, demonstrating the binding specificity of ISIS 2105 (data not shown).

DISCUSSION

One of the major difficulties in the study of the papillomavirus life cycle and the development of specific antiviral agents has been the lack of an in vitro system permissive to viral replication. In human genital warts caused by HPV-6 or

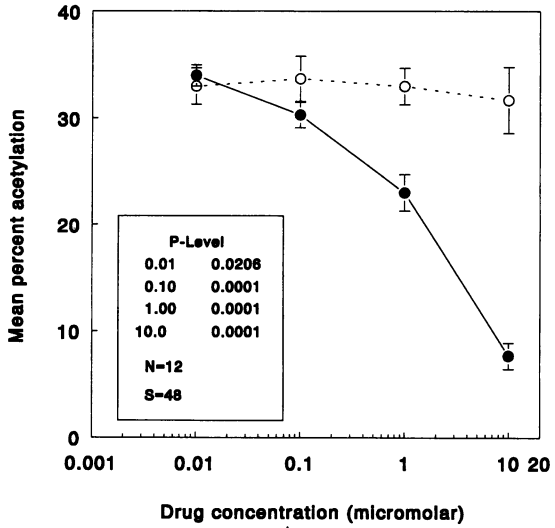

FIG. 4. Inhibition of HPV-11 E2-dependent transactivation. C127 cells were pretreated with oligonucleotide and were then co-transfected with IPV118, HPV-11 E2 expression constructs, IPV128 HPV-11 E2-dependent CAT expression reporter, and PCH110. Four separate experiments were carried out. An analysis of variance was performed on the portion of acetylated chloramphenicol. The data are expressed as the mean ± standard deviation percent acetylation of the four experiments. ●, ISIS 2105; ○, ISIS 2324.

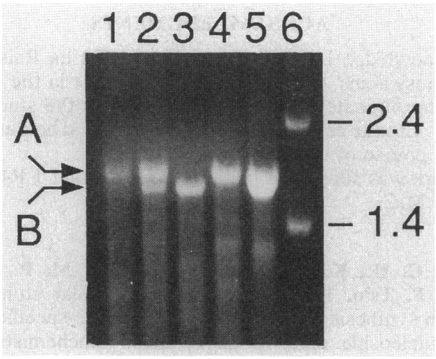

FIG. 5. ISIS 2105 directs RNase H cleavage of HPV-11 E2 RNA. ISIS 2105 was hybridized to HPV-11 E2 RNA. Following hybridization, RNA was treated with RNase H and the reaction products were analyzed by gel electrophoresis. Lane 1, untreated RNA; lane 2, RNA plus ISIS 2105 incubated with HeLa cell nuclear extract; lane 3, RNA plus ISIS 2105 incubated with purified *E. coli* RNase H; lane 4, RNA plus ISIS 2324 incubated with HeLa cell nuclear extract; lane 5, 5-LO RNA plus ISIS 2105 incubated with HeLa cell nuclear extract; lane 6, molecular weight markers.

HPV-11, the viral genome is maintained as a nuclear plasmid. This has not been achieved in cell culture for an HPV. BPV-1, however, is able to transform rodent cells and maintain its genome as a nuclear plasmid (10, 24). Because of these properties, BPV-1 has been used as a model for latent infection and to study papillomavirus transcription and transformation. This model has been used to test the hypothesis that antisense oligonucleotides can provide papillomavirus-specific antiviral effects and that the BPV-1 model can be used to predict targets in analogous HPV RNA sequences that are sensitive to oligonucleotide-induced inhibition. Two such regions were identified: the mRNA cap region and the initiation of translation region, which are targeted by ISIS 1751 and ISIS 1753, respectively.

Inhibition of E2 transactivation by ISIS 1751 and ISIS 1753 was concentration dependent. ISIS 1751 is targeted to the 5′ cap of the mRNA produced by the promoter at nt 2443 and is complementary to nts 2443 to 2472 (40). Two additional mRNAs capable of producing full-length E2 transactivator have been identified in BPV-1-transformed cells (36, 38). One is transcribed by the promoter located at nt 7940, and the other is transcribed by the promoter located at nt 890. Both of these mRNA species utilize the splice acceptor at nt 2558. Therefore, the sequences targeted by ISIS 1751 (nts 2443 to 2472) are not present in these mRNAs. This suggests that ISIS 1751 is capable of inhibiting only one species of E2 transactivator mRNA. BPV-1 can produce low levels of these alternate mRNAs, thus sustaining E2 expression in the absence of the mRNA produced from P_{2443}. In dose-response experiments, ISIS 1753 was able to reduce E2-dependent CAT expression to background levels. This compound was targeted to the AUG of the full-length E2 transactivator mRNA located at nt 2608. ISIS 1753 was complementary to nts 2598 to 2627. This region is contained in all species of E2 transactivator mRNAs identified. This suggests that, unlike ISIS 1751, ISIS 1753 is capable of inhibiting E2 expression from all known E2 mRNAs, thus accounting for the differences in the dose-response curves in both the E2 transactivation assay and the focus formation assay.

The chain length of oligonucleotides is thought to affect not only the specificity of hybridization but also cellular uptake. Therefore, the effect of oligonucleotide chain length on activity was assessed by synthesis of a series of truncated oligonucleotides centered on the AUG of the E2 transactivator. In the present study, a minimum phosphorothioate oligonucleotide chain length of 20 residues was required to maintain maximum efficacy. Activity decreased significantly with each truncation until all activity was lost with a 14-mer. This apparent optimization of potency in a 20-nt oligonucleotide may be specific for this particular site in the RNA, the type of oligonucleotide, and/or the cell type in which it is evaluated. We are investigating the relationship between oligonucleotide length and activity in a number of antisense target models.

To test the effectiveness of BPV-1-specific oligonucleotides in the disruption of the virus life cycle, oligonucleotides that inhibited E2 transactivation were evaluated for their ability to inhibit BPV-1 focus formation. Both ISIS 1751 and ISIS 1753 were shown to inhibit focus formation in a concentration-dependent fashion. These results are consistent with reports that have shown that genetic mutation of E2 results in replication- and transformation-defective mutants (17). Collectively, these findings provide further support that E2 is an essential gene in the life cycle of papillomaviruses and, as such, represents a rational target for papillomavirus therapy.

The nucleotide sequence of the RNAs that code for HPV-11 E2 protein differs significantly from those that code for the BPV E2 protein sequence. Therefore, oligonucleotides targeted to equivalent regions on the HPV and BPV RNAs have different nucleotide sequences. ISIS 2105, a sequence analog of the most potent and minimal-length phosphorothioate oligonucleotide targeted to the BPV-1 E2, was shown to produce dose-dependent inhibition of HPV-11 E2 transactivation. This compound had no inhibitory effect on BPV-1 transactivation. Conversely, ISIS 2324, the compound targeted to the BPV-1 E2 RNA, inhibited BPV-1 E2 transactivation with no effect on HPV-11 E2 activity. Taken together these data, which demonstrate that each phosphorothioate oligonucleotide was active in its homologous model and inactive in its heterologous model, provide evidence for (i) the requirement for oligonucleotide-RNA target complementarity and (ii) the sequence-specific inhibitory effect of ISIS 2105 on HPV-11 E2 expression.

The precise mechanism by which ISIS 2105, ISIS 2324, and the other active oligonucleotides modulate E2 transactivation has not been elucidated. At one level, the sequence-specific inhibitory effects described above are consistent with the initial design rationale of an antisense mechanism in which synthesis of E2 proteins is inhibited as a result of the oligonucleotide binding to their complementary receptor-binding site on the E2 mRNA.

At the level of the RNA target itself, a definitive mechanism of action for the oligonucleotides has not been assigned. One potential mechanism is hybridization arrest, in which protein synthesis is inhibited as a result of the binding of the oligonucleotide to the RNA, causing disruption of the protein translation process (25). This mechanism is consistent with the finding that many potent oligonucleotides are targeted to sites associated with the initiation of translation.

Another possible molecular mechanism for these compounds is cleavage of the oligonucleotide-RNA complex by RNase H, leading to degradation of the RNA (18). While ISIS 2105 was shown to support RNase H cleavage of E2 mRNA in vitro, it cannot be concluded that this process is responsible for the activity action of the compound observed in cells. Recent data suggest that phosphorothioate oligonu-

cleotides can produce antisense-mediated effects on RNA via multiple molecular mechanisms and that the role played by specific mechanisms may be dependent on the host cell and specific target site on the mRNA. For example, in human umbilical vein endothelial cells, phosphorothioate antisense oligonucleotides targeted to ICAM-1 mRNA have been found to function through at least two mechanisms (4). In one case, expression of ICAM-1 was attenuated without any effect on steady-state mRNA levels, while an oligonucleotide targeted to the 3′-untranslated region resulted in the degradation of ICAM-1 mRNA.

Expression of BPV-1 (and HPV-6 and HPV-11) early genes is thought to be tightly regulated by the ratio of E2 transactivator:E2 transrepressor with expression of full-length E2 transactivator subject to autoregulation (20, 26). This model suggests an intricate balance of transregulatory factors in which net transcription is dependent on excess E2 transactivator. Therefore, even minimal inhibition of E2 transactivator expression could result in complete down-regulation of early gene expression. The sensitivity of the BPV-1 models to nanomolar concentrations of E2-targeted oligonucleotides is consistent with this hypothesis and the fact that perturbation of the balance in favor of E2 transrepression can disrupt the life cycle of the virus. This postulated mechanism is consistent with the reduced potency of ISIS 2105 as measured in the HPV-11 E2 transactivation model. Within this model, no E2 transrepression exists. Therefore, the HPV-11 E2 RNA, which is expressed from a surrogate promoter, continues to be transcribed in the presence of oligonucleotides that inhibit expression of the E2 protein. This difference in the E2 transactivation models is a likely explanation for the 50- to 100-fold greater potency of ISIS 2324 over that of ISIS 2105.

The suitability of E2 as a target for antiviral therapy where the strategy is inhibition of DNA replication is well supported by genetic data for both BPV and HPV. E1 is an obvious alternative molecular target for this strategy. Currently, no assays exist for the evaluation of E1. The issue of whether the primary function of E2 is transactivation or transrepression in HPV is unresolved. Discrimination between these two activities will likely require analysis in the context of the entire viral genome. An in vitro system for HPV-11 that may allow this analysis as well as testing of E1 has recently been reported (8). Other genes, E6 and E7, have been proposed and tested as potential therapeutic targets (37). These genes may be suitable targets for high-risk viruses such as HPV-16 and HPV-18 in which inhibition or reversion of the malignant phenotype is the therapeutic strategy. On the basis of the current understanding of the functions of these genes, they do not appear to be suitable targets for therapeutic strategies designed to inhibit replication of episomal viral DNA of low-risk HPV types such as HPV-6 and HPV-11.

In summary, the data described in this report provide evidence to support the hypothesis that the E2 transactivator protein of papillomaviruses is a sensitive target for antisense oligonucleotide antiviral drug action. ISIS 2105, a phosphorothioate oligonucleotide targeted to a site in the E2 RNA of HPV-6 and HPV-11, is a compound that can inhibit E2 transactivation in cell culture models. The precise mechanism of action of the compound and its therapeutic utility are under investigation. ISIS 2105 is in phase I clinical trials for the treatment of genital warts.

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