Subunit Affinities and Stoichiometries of the Human Papillomavirus Type 11 E1:E2:DNA Complex†

Shih-Fong Chao,‡ Warren J. Rocque,§ Selwyn Daniel,‡ Linda E. Czyzyk,‡ William C. Phelps,|| and Kenneth A. Alexander*,‡,⊥

Division of Pediatric Infectious Diseases, Department of Pediatrics and Department of Microbiology, Duke University Medical Center, Durham, North Carolina 27710, and Departments of Molecular Sciences and Virology, Glaxo-Wellcome Inc., Research Triangle Park, North Carolina 27709

Received November 3, 1998; Revised Manuscript Received January 20, 1999

ABSTRACT: The association between the papillomavirus E1 and E2 proteins is an important regulatory interaction, imparting coordinated control of viral transcription and replication. Using fluorescence polarization, we have characterized the interactions between HPV-11 E1, HPV-11 E2, and DNA in solution at equilibrium. For these studies, two double-stranded fluorescein-labeled oligonucleotides were prepared. The first fluorescent oligonucleotide, designated Fl-E2BS and containing a single E2 binding-site palindrome ($ACCGN_6CGGT$), was used to determine the affinity of E2 for its DNA binding site. HPV-11 E2 bound Fl-E2BS with an apparent K_d of 0.84 nM. Binding was saturable and consistent with a single class of noninteracting sites. The second oligonucleotide, designated Fl-E1E2BS, contained both E1 and E2 sites in sequence derived directly from the HPV-11 origin of replication. Under titration conditions identical to those used for Fl-E2BS, the E2 protein exhibited reduced affinity for Fl-E1E2BS (K_d > 100 nM). E1 binding to Fl-E1E2BS was of very low affinity. Addition of excess HPV-11 E1 to Fl-E1E2BS lowered the dissociation constant for the E2:Fl-E1E2BS interaction to 2 nM. This effect was not dependent upon ATP or magnesium ion. Fluorescence polarization and other data suggest formation of a complex containing six E1 molecules and a single dimer of E2 bound to a single Fl-E1E2BS oligonucleotide; E2 dissociation from the final complex did not occur. In summary, physical interaction between E1 and E2 increases the DNA binding affinity of each. The role of this energy coupling may be to promote origin-specific binding of both E1 and E2 to DNA.

The *Papillomaviridae* are a large family of double-stranded DNA viruses that cause benign tumors (warts) in infected epithelia. Concerns about human papillomavirus (HPV)[1] infection increased when it was demonstrated that such infections were associated with genital condylomata, with dysplasia and carcinoma of the uterine cervix, and with some cancers of the head and neck (for review, see *1, 2*). A goal of HPV research is to understand the biochemical mechanisms underlying viral transcription and replication control. Such an understanding will be necessary for the development of effective papillomavirus antiviral therapies (*3*).

The papillomavirus proteins responsible for virus transcription and replication control are the E1 and E2 proteins. The E1 protein is a 70−80 kDa nuclear phosphoprotein which is required for viral DNA replication (*4, 5*). E1 binds DNA both specifically and nonspecifically and binds to the viral origin of replication (*ORI*). Sequence-specific DNA binding by E1 is most likely mediated by the papillomavirus E2 protein; association of E1 with E2 appears to enhance the affinity of E1 for DNA (*6*−*16*). E1 has significant structural and functional homology to the simian virus 40 (SV40) large T antigen protein, and possesses DNA helicase activity (*17*−*19*). E1 also interacts with DNA polymerase α (*20*), and in doing so, E1 may serve as a modulator of papillomavirus replication.

The papillomavirus E2 protein is a pivotal factor in both transcriptional modulation and control of papillomavirus replication. The 43−50 kDa E2 protein is comprised of two well-conserved functional domains joined by a small, poorly conserved hinge domain (reviewed in *21*). The amino-terminal domain of E2 is necessary for viral *trans*-activation and for direct association with the E1 protein. The carboxyl-terminal portion of E2, designated E2C, binds a 12 base pair palindromic DNA sequence, 5′-$ACCGN_4CGGT$-3′, which is present at several locations throughout the HPV genome. This sequence is repeated 4 times near the viral *ORI* (*22*−*24*). Studies of the HPV-11 E2C protein association with a 24 base pair fluorescein-labeled E2 binding-site oligonucleotide

† This work was supported by a grant from The Smokeless Tobacco Research Council, Inc. (K.A.A. and S.-F.C.), and by a grant from The Children's Miracle Network (K.A.A.).

* To whom correspondence should be addressed at the Department of Microbiology, Box 3020, Duke University Medical Center, Durham, NC 27710. Telephone: (919) 684-9590. Fax: (919) 684-8735. E-mail: kenalex@abacus.mc.duke.edu.

‡ Department of Pediatrics, Division of Pediatric Infectious Diseases, Duke University Medical Center.

§ Department of Molecular Sciences, Glaxo-Wellcome, Inc.

|| Department of Virology, Glaxo-Wellcome, Inc.

⊥ Department of Microbiology, Duke University Medical Center.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; BPV, bovine papillomavirus; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPV, human papillomavirus; *ORI*, origin of replication; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SV40, simian virus 40; TRIS, tris(hydroxymethyl)aminomethane.

10.1021/bi982616v CCC: $18.00
Published on Web 03/16/1999

(*25*) demonstrated a solution stoichiometry of one E2C dimer binding to one DNA double-stranded oligonucleotide. Binding occurred as a single class of noninteracting binding sites. Competitive E2C binding titrations between the same fluorescein-labeled E2 binding-site oligonucleotide and an unlabeled oligonucleotide of identical sequence yielded a native complex dissociation constant of 4.5×10^{-9} M. Similar titrations of oligonucleotides with sequences from the seven E2 binding sites within the HPV-11 genome demonstrated that all high-affinity E2 binding sites were located near the *ORI*, while sites distant from the *ORI* bound with only weak affinity.

Although E1 and E2 complex formation with DNA is well established, there has been little quantitative analysis of the E1:E2:DNA complex. Interaction between E1 and E2 with the HPV-11 genome leads to the formation of an E1:E2:*ORI* complex; HPV-11 E2 association with E1 appears to enhance the DNA binding affinity of E2 for DNA (*11*, *26*). Sedman and Stenlund (*15*) described how bovine papillomavirus (BPV) E2 and BPV E1 bind the *ORI* individually with low sequence specificity; however, together they bind the *ORI* with greatly increased sequence specificity. Subsequent work by these authors proposed that this E1:E2:*ORI* complex may serve as a precursor for the formation of a trimeric E1 complex (*16*), and then from this trimeric E1 complex to the formation of a hexameric E1 complex with helicase and ATPase activities (*27*).

Whether observations of BPV-E1 and E2 complex formation with DNA are transferable to HPV proteins is unclear. The model BPV E1:E2 binding DNA oligonucleotide studied by Sedman and Stenlund (*15*) contains a low-affinity E2 binding-site palindrome. In contrast, the E2 binding site used in our studies binds HPV-11 E2C with high affinity (*25*). For this paper, we sought to determine quantitatively the affinity of the HPV-11 E1 and E2 proteins for DNA. We also sought to determine the quantitative effects of E1:E2 association on the affinity of each protein for DNA, and to determine if E2 remained in the final equilibrium complex. Because the E1 protein has both helicase and ATPase activities, we assessed the role of ATP as a potential regulator of E1:E2:DNA complex formation. Finally, we determined the equilibrium stoichiometry of the E1:E2:DNA complex.

Many studies of E2:DNA interaction have utilized an oligonucleotide gel-shift method. Although gel retardation of labeled oligonucleotides by E2 is readily quantifiable, such measurements of the fractions of free and bound DNA are not made at equilibrium due to the utilization of the gel electrophoresis technique. In the course of electrophoresis, the relative E2 and DNA concentrations vary continuously, as do the solvent conditions. Furthermore, solutions used during the preequilibration steps often differ substantially from the TRIS−borate buffers used for electrophoresis. These factors could potentially result in specious measurements of the K_d for the DNA:protein complex. Therefore, because of shortcomings in the oligonucleotide gel-shift method, fluorescence anisotropy determination was chosen as the technique for measuring E2:DNA, E1:DNA, and E1:E2:DNA complex formation.

Fluorescence anisotropy determination is a spectroscopic technique that is particularly well suited to the study of protein binding to small oligonucleotides (*28*−*30*), and has been used to study a variety of protein:DNA associations (for example, see *31*, *32*) including HPV-11 E2C and DNA (*25*). By measuring the fluorescence anisotropy of fluorescein-labeled oligonucleotides as a function of added E1 and E2 protein, the equilibrium affinity constants for E1:DNA, E2:DNA, and E1:E2:DNA complexes may be determined in solution under equilibrium conditions. Furthermore, because measurements are made at equilibrium, factors such as the order of reagent addition and the possibility of multiple reaction pathways do not affect experimental outcomes.

EXPERIMENTAL PROCEDURES

HPV-11 E2 Expression. DNA encoding the entire HPV-11 E2 sequence plus an initiator methionine was obtained by polymerase chain amplification from an HPV-11 genome inserted into pUC plasmid pW97 (*33*). The PCR product encoding the full-length HPV-11 E2 protein was ligated into a bacteriophage T7 expression vector, pSS582 (*25*), containing a T7 gene 10 promoter (*34*). Plasmids were purified from recombinant colonies and used to transform BL21 DE3 *Escherichia coli.* BL21 DE3 *E. coli* expressing E2 were grown for 18 h at 34 °C in enriched medium containing 50 μg/mL kanamycin. PCR fidelity was confirmed by DNA sequencing. Aliquots of recombinant BL21 DE3 *E. coli* were frozen at −80 °C, and were used to inoculate larger scale cultures for E2 preparation.

DNA Affinity Column. A DNA affinity column was prepared using a 42 base pair double strand oligonucleotide containing two E2 binding-site palindromes. The oligonucleotide sequences were 5′-CCCCCCACCGAAAACGGTCCCCCCACCGAAAACGGTCCCCCC-3′ and 5′-GGGGGGACCGTTTTCGGTGGGGGGACCGTTTTCGGTGGGGGG-3′. One gram of cyanogen bromide-activated agarose (Pharmacia) bound 1.5×10^{-7} mol of annealed double-stranded oligonucleotide. After washing, the column was stored at room temperature in 20% ethanol.

E2 Purification. Five liter overnight cultures were grown without induction at 34 °C. Cells were collected by centrifugation at 5000 rpm for 15 min at 4 °C in a GSA rotor. The bacterial pellets were then resuspended in 50 mL of buffer containing 20 mM TRIS, pH 8.5, 5 mM EDTA, 10 mM DTT, and 1 mM PMSF. The resulting bacterial suspension was sonicated on ice at maximum power 2 times for 2 min intervals using a microtip. The sonicated bacteria were then centrifuged for 20 min at 18 500 rpm in an SS-34 rotor. After centrifugation, the supernatant was saved and frozen at −80 °C for later purification.

The thawed E2 supernatant was filtered through a 0.45 μm filter unit (Millipore), and then fractionated by FPLC at 4 °C using a 1.6 cm × 10 cm heparin−agarose (Sigma) column with a 0−1 M NaCl gradient in a buffer containing 20 mM TRIS, pH 8.5, 1 mM EDTA, and 5 mM DTT. Fractions containing E2 protein were analyzed by Western blot and pooled. No-salt DNA column buffer (20 mM TRIS, pH 8.0, 1 mM EDTA, 5 mM DTT, 0.1% Tween 20) was added to reduce the NaCl concentration to <100 mM. The diluted heparin−agarose column eluate was then applied at room temperature to a 1.5 cm × 8 cm DNA affinity column (described above). After loading, the column was washed extensively with no-salt buffer DNA column buffer. E2 was then eluted in a stepped gradient: first, a 0−0.12 M NaCl gradient in a buffer with 20 mM TRIS, pH 8, 1 mM EDTA,

0.1% Tween 20, 0 or 1 M NaCl. The column gradient was then held at 0.12 M NaCl for several column volumes. After the eluate absorbance returned to base line, the NaCl concentration was stepped immediately to 0.36 M. At this salt concentration, E2 protein eluted as a single sharp peak.

Samples from purified E2 protein fractions were analyzed by SDS−PAGE and Western blot. E2-containing fractions were pooled, aliquoted, and frozen at −80 °C for long-term storage. From each E2 preparation, a sample was removed for quantitative amino acid analysis.

HPV-11 E1 Purification. HPV-11 E1 protein purification from baculovirus-transfected Sf9 cells will be described elsewhere (*35*). Briefly, overexpressed E1 protein was purified to apparent homogeneity (>95% purity, no detectable nuclease activity) using heparin−agarose and immunoaffinity chromatography. For each E1 preparation, E1 concentration was determined by Lowry assay or by quantitative amino acid analysis.

Synthetic Oligonucleotides. PAGE-purified fluorescein-labeled and unlabeled oligonucleotides were obtained commercially (Genosys Corp.) and used without further purification. Commercially obtained 5′-fluorescein phosphoramidite-labeled oligonucleotides were prepared by the method of Schubert et al. (*36*). Contaminating unlabeled oligonucleotide was less than 2% (manufacturer's quality control report). Oligonucleotide concentrations were calculated from measured 260 nm absorbancies. Complementary oligonucleotides were annealed by combining equimolar amounts of each in 1× TBE buffer, and heating samples to 97 °C for 1 h. The oligonucleotide solutions were then allowed to cool at 5 °C/h to a temperature approximately 25 °C below the calculated melting point of each oligonucleotide pair and allowed to anneal for 48−72 h. PAGE analysis of samples showed near 100% annealing (data not shown). Annealed oligonucleotides were stored at −80 °C. The structures of the Fl-E2BS and Fl-E1E2BS are shown in Figure 2.

Fluorescence Measurements. All fluorescence measurements were made using an SLM-Aminco 8100 spectrofluorometer assembled in the "T" geometry and equipped with Glanz-Thompson polarizers in the excitation and emission beams. Samples were prepared in triplicate and were maintained at 25 ± 0.2 °C. A minimum of 10 min equilibration time was given prior to each measurement. The fluorescence anisotropy of each sample was determined by triplicate sets of 10 measurements per set. The dissociation constant for each complex was determined by measurement of the steady-state anisotropy of the fluorescein-labeled oligonucleotide as a function of added E2 and/or E1 protein. The anisotropy limits of the titrations, corresponding to free fluorescein-labeled oligonucleotide (A_f) and protein-bound fluorescein-labeled oligonucleotide (A_b), as well as the observed anisotropy (A) and the fractional change in fluorescein quantum yield upon binding (q), were used to calculate the fraction of fluorescein-labeled oligonucleotide associated with E1 and/or E2 as described in eq 1 (*29*):

$$\text{fraction oligonucleotide bound} = (A - A_f)/[(A_b - A)q + (A - A_f)] \quad (1)$$

All sample measurements were corrected for blank fluorescence. Even at the lowest oligonucleotide concentrations (1 nM), blank fluorescence was initially less than 20% and remained less than 30% of the sample fluorescence intensity throughout the titrations. The fractional change in fluorescein quantum yield was calculated by measuring the total fluorescence intensities of the free and bound oligonucleotides, subtracting the intensities of the blank controls, and then correcting for dilution as described in eq 2 (*29*):

$$q = [(I_{||} + 2I_{\perp})_{sample} - (I_{||} + 2I_{\perp})_{blank}]_{final} V_{final} / [(I_{||} + 2I_{\perp})_{sample} - (I_{||} + 2I_{\perp})_{blank}]_{initial} V_{initial} \quad (2)$$

where $I_{||}$ and $I_{\perp}$ are the fluorescence intensities with parallel and perpendicularly oriented excitation and emission polarizers.

Determination of the E1 and E2 Stoichiometries in the E1:E2:Fl-E1E2BS Complex. To determine the stoichiometry of E2 in the E1:E2:Fl-E1E2BS complex, concentrated solutions of E1 and Fl-E1E2BS in fixed ratios of 12 mol of E1 to 1 mol of Fl-E1E2BS (300 nM E1, 25 nM Fl-E1E2BS) and 6 mol of E1 to 1 mol of Fl-E1E2BS (300 nM E1, 50 nM Fl-E1E2BS) were prepared. Aliquots of purified E2 protein were then added, and the fluorescence anisotropy of the solutions was determined following each E2 addition. Because fluorescent oligonucleotide was presumed to be the limiting reagent for E1:E2:Fl-E1E2BS complex formation, the stoichiometry of E2 in the E1:E2:Fl-E1E2BS complex could be determined.

Similarly, the stoichiometry of E1 in the E1:E2:Fl-E1E2BS complex was determined by combining high concentrations of E1 and Fl-E1E2BS in fixed ratios of 3 mol of E1 to 1 mol of Fl-E1E2BS (300 nM E1, 100 nM Fl-E1E2BS) and 1 mol of E1 to 1 mol of Fl-E1E2BS (300 nM E1, 300 nM Fl-E1E2BS). Aliquots to E2 were then added, and the fluorescence anisotropy of the labeled oligonucleotide was measured. In this case, E1 was held as the reagent limiting E1:E2:Fl-E1E2BS complex formation. By determining how much E1:E2:Fl-E1E2BS complex could form in the presence of limiting E1, and assuming an E2 binding stoichiometry of 1 E2 dimer per Fl-E1E2BS oligonucleotide (see Results), the stoichiometry of E1 in the E1:E2:Fl-E1E2BS complex could be measured.

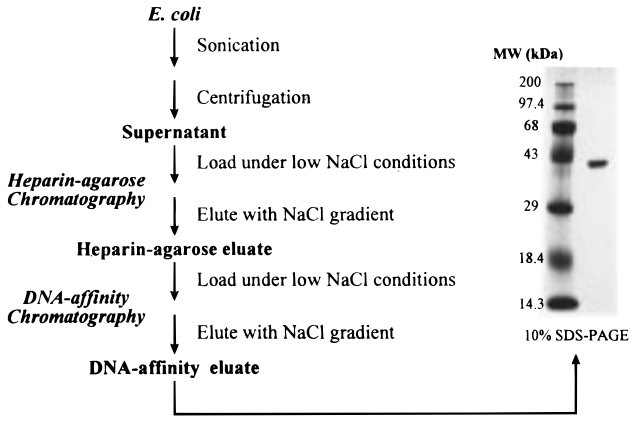

FIGURE 1: Purification of HPV-11 E2 from recombinant *E. coli.* Eluted fraction from DNA affinity column.

RESULTS

HPV-11 E2 Expression and Purification. Milligram quantities of homogeneously pure native sequence HPV-11 E2 protein could be prepared reproducibly (Figure 1). Using two

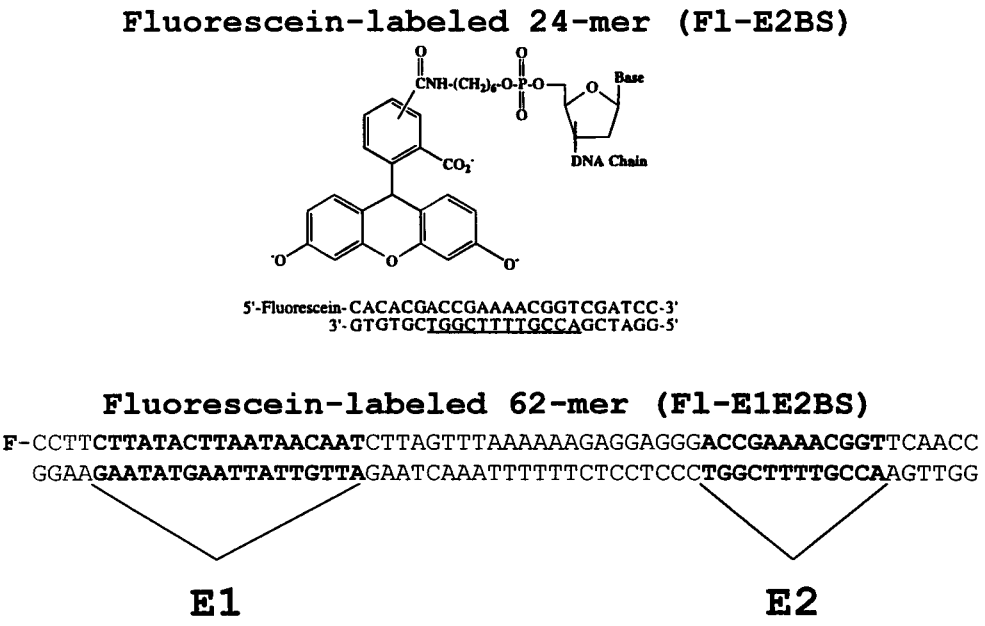

FIGURE 2: Structures of fluorescein-labeled oligonucleotides Fl-E2BS and Fl-E1E2BS. Illustrated are the structure of the phosphoramidite-linked fluorescein moiety and the sequences of the Fl-E2BS and Fl-E1E2BS oligonucleotides. The sequence of the smaller 24 base pair fluorescent oligonucleotide, Fl-E2BS, contains the full 12 base pair E2 binding-site palindrome (*25*). Flanking base pairs were added to increase oligonucleotide duplex stability and to prevent formation of stable hairpin structures. The sequence of the 62 base pair Fl-E1E2BS was derived from base pairs 7924−0052 of the HPV-11 origin of replication, and includes the putative E1 binding-site palindrome and a single E2 binding-site palindrome.

affinity chromatography steps, HPV-11 E2 was prepared without the use of denaturing or harsh buffer conditions. Degradation of E2 was not observed under routine storage conditions. Quantitative amino acid analysis was used to determine the concentration of E2 (data not shown). DNA sequencing confirmed the anticipated nucleotide sequence of E2 (*11*; *25*).

Affinity of HPV-11 E2 Protein for Fluorescein-Labeled Oligonucleotides. To determine the affinity of E2 for its palindromic DNA binding site, fluorescein-labeled oligonucleotides were titrated with purified E2 protein. E2:oligonucleotide associations were determined by measuring changes in fluorescein fluorescence anisotropy. For this purpose, two 5′-fluorescein-labeled oligonucleotides were prepared and annealed with their respective unlabeled complementary strands. These fluorescein-labeled double-stranded oligonucleotides were designated Fl-E2BS (fluorescent E2 binding site) and Fl-E1E2BS (fluorescent E1 and E2 binding sites) (Figure 2).

To determine if the full-length E2 protein bound DNA with different affinity than E2C, Fl-E2BS was titrated with full-length E2 under conditions identical to those used to determine the E2C:Fl-E2BS dissociation constant (*25*). Fl-E2BS binding by E2 appeared to be saturable and consistent with a single class on noninteracting binding sites (Figure 3). The K_d for this interaction was 0.84 nM. This dissociation constant was substantially lower than the 45 nM K_d reported for the E2C:Fl-E2BS interaction.

To assess the contributions of size and flanking base sequences on the E2:oligonucleotide binding affinity, a 62 base pair oligonucleotide which contained single E1 and E2 sites, Fl-E1E2BS, was titrated with E2 in the presence of EDTA and 10 base pairs of poly(dI-dC) per oligonucleotide base pair. The affinity of E2 for Fl-E1E2BS was poor, with a K_d exceeding 100 nM (Figure 4). Results obtained from titrations performed in the presence of 0.1 mg/mL salmon testis DNA and also with 0.0146 mg/mL poly(dI-dC) [10 base pairs of poly(dI-dC) per base pair fluorescent oligonucleotide] were very similar (data not shown).

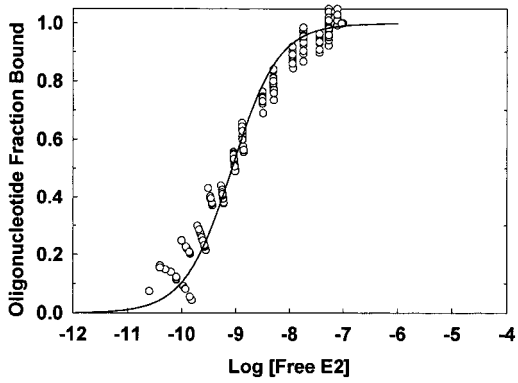

FIGURE 3: Association of HPV-11 E2 with Fl-E2BS in EDTA. The affinity of HPV-11 E2 protein for Fl-E2BS was determined by measuring the increase in Fl-E2BS fluorescence anisotropy as a function of added E2. 1×10^{-9} M Fl-E2BS in a buffer containing 20 mM TRIS, pH 8.0, 150 mM KCl, 5 mM EDTA, 5 mM DTT, and 0.1 mg/mL salmon testis DNA was titrated with a stock solution of E2. Samples were excited with 480 nm light, with the excitation slit at 8 mm. Emitted light was collected through 3 mm thick OG-515 filters (Schott). The fluorescence anisotropy of the solution was determined after each addition of E2, as described under Experimental Procedures. Using the observed fluorescence anisotropy and the anisotropies of the free and bound oligonucleotide, the fraction of oligonucleotide bound to E2 could be calculated as described above. The data were fit to the Michaelis−Menton equation using a Newton−Gauss iterative least-squares regression analysis assuming a single class of noninteracting E2 binding sites (SigmaPlot). The fraction of Fl-E2BS bound by E2 was then plotted as a function of the log of the free E2 concentration. The binding curve shown corresponds to a K_d of 0.84 nM. Titrations performed in the presence of poly(dI-dC) [10 base pairs of poly(dI-dC) per base pair of oligonucleotide] gave similar results (data not shown).

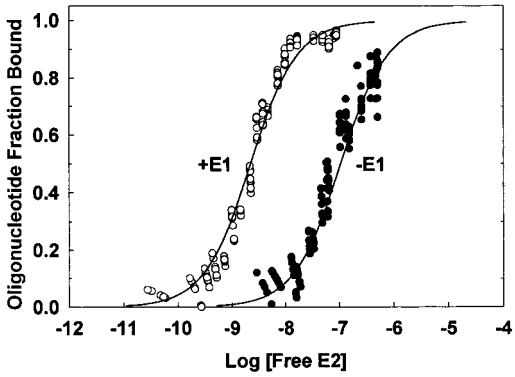

FIGURE 4: Association of HPV-11 E2 with Fl-E1E2BS in the presence and absence of excess HPV-11 E1 (+EDTA). 3.9×10^{-8} M Fl-E1E2BS (−E1 titration) or 1×10^{-9} M Fl-E1E2BS (+E1 titration) in a buffer containing 20 mM TRIS, pH 8.0, 150 mM KCl, 5 mM EDTA, 5 mM DTT, and poly(dI-dC) [10 base pairs of poly(dI-dC) per base pair of oligonucleotide] was titrated with a stock solution of E2. For titrations in the presence of E1, 244 nM E1 was added to the titration buffer at the beginning of each experiment. Samples were excited with 480 nm light, with the excitation slit at 8 mm. Emitted light was collected through combined 3 mm thick OG-515 and 2 mm thick BG-28 filters. Because the E2:Fl-E1E2BS interaction did not reach saturation, the affinity for the E2:Fl-E1E2BS interaction could only be estimated. Data were fit to a binding curve as described above. The binding curves shown correspond to E2:Fl-E1E2BS dissociation constants of greater than 100 nM (−E1) and 2.2 nM (+E1).

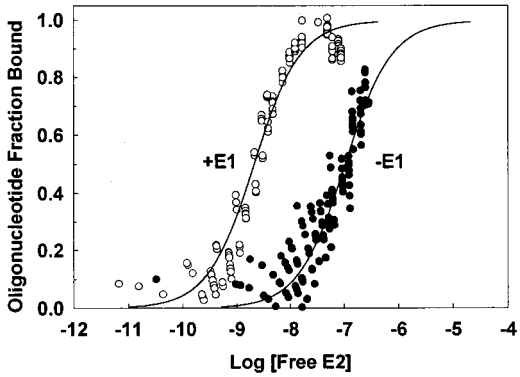

FIGURE 5: Association of HPV-11 E2 with Fl-E1E2BS in the presence and absence of excess HPV-11 E1 (+Mg^{2+} and ATP). 3.9×10^{-8} M Fl-E1E2BS (−E1 titration) or 1×10^{-9} M Fl-E1E2BS (+E1 titration) in a buffer containing 20 mM TRIS, pH 8.0, 150 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ATP, 5 mM DTT, and poly(dI-dC) [10 base pairs of poly(dI-dC) per base pair of oligonucleotide] was titrated with a stock solution of E2. A stoichiometric amount of $MgCl_2$ was also added to the E2 stock solution to bind EDTA present therein. For titrations in the presence of E1, 244 nM E1 was added to the titration buffer at the beginning of each experiment. Samples were excited with 480 nm light, with the excitation slit at 8 mm. Emitted light was collected through combined 3 mm thick OG-515 and 2 mm thick BG-28 filters. Because the E2:Fl-E1E2BS interaction did not reach saturation, the affinity for the E2:Fl-E1E2BS interaction could only be estimated. Data were fit to a binding curve as described above. The binding curves shown correspond to E2:Fl-E1E2BS dissociation constants of greater than 100 nM (−E1) and 2.1 nM (+E1).

Effect of Saturating HPV-11 E1 on E2:Fl-E1E2BS Association. Because papillomavirus E2 proteins promote association of E1 with DNA, thermodynamic theory predicts that the converse would also be true. To assess the effect of E1 on E2 binding to DNA, the affinity of E2 for Fl-E1E2BS was determined in the presence of a large excess of purified HPV-11 E1. Addition of 244 nM purified HPV-11 E1 protein at the start of the titration lowered the E2:Fl-E1E2BS dissociation constant dramatically from >100 nM in the absence of E1 to 2.2 nM in the presence of E1 (Figure 4). E2 binding to the oligonucleotide was again consistent with a single class of noninteracting E2 sites on the oligonucleotide.

Presence of E2 in the E1:E2:Fl-E1E2BS Complex. To ascertain whether E2 remained associated with Fl-E1E2BS in the final complex when excess E1 was present, anti-HPV-11 E2 monoclonal antibody was added. Addition of E2 binding antibody to a solution containing 1 nM E2, 6 nM E1, and 1.25 nM Fl-E1E2BS led to an increase in Fl-E1E2BS anisotropy, suggesting that E2 remained associated with the fluorescein-labeled oligonucleotide. Similarly, addition of anti-HPV-11 E1 monoclonal antibody prompted an increase in fluorescence anisotropy. These observations were also confirmed at higher concentrations of E1, E2, and fluorescent oligonucleotide (data not shown).

Effect of Mg^{2+} and ATP on the Association of E2 with DNA in the Presence and Absence of HPV-11 E1. Because E1 is a helicase/ATPase required for HPV replication, the effects of Mg^{2+} and ATP on E1 and E2 binding were assessed. The presence of 0.5 mM $MgCl_2$ and 0.1 mM ATP had little or no effect on the E2:Fl-E1E2BS interaction (Figure 5). Addition of 244 nM purified HPV-11 E1 protein at the beginning of the titration again lowered the E2:Fl-E1E2BS dissociation constant from >100 nM in the absence of E1 to 2.1 nM in the presence of E1 (Figure 5). E2 binding to the oligonucleotide remained consistent with a single class of noninteracting E2 sites on the oligonucleotide.

A free magnesium ion concentration of 0.5 mM was selected in keeping with observations that the intracellular free magnesium ion concentrations range between 200 and 600 μM (*37*, *38*). During preliminary experiments, it was noted that higher concentrations of magnesium ion increased the free anisotropy of the Fl-E1E2BS oligonucleotide and appeared to increase the affinity of E2 for DNA (data not shown). Unfortunately, addition of magnesium ion to the unbound oligonucleotide prompted a substantial increase in the fluorescence anisotropy of the free oligonucleotide, thereby precluding accurate determination of the E2:Fl-E1E2BS affinity in high magnesium concentrations. This effect may have been due to magnesium ion-induced aggregation of oligonucleotide strands (*39*).

Determination of the E2 Stoichiometry in the E1:E2:Fl-E1E2BS Complex. Because the addition of E1 dramatically increases the affinity of E2 for the Fl-E1E2BS oligonucleotide, we were able to determine the stoichiometry of E2 binding to the E1:Fl-E1E2BS oligonucleotide complex. By measuring the fluorescence anisotropy of the Fl-E1E2BS oligonucleotide as a function of the molar ratio of E2 dimer to Fl-E1E2BS, we determined the number of moles of E2 associated with E1:Fl-E1E2BS in solution at equilibrium without the use of bifunctional cross-linking reagents (Figure 6). Because Fl-E1E2BS was at concentrations 23- and 11-fold over the K_d of E2 association with the E1:Fl-E1E2BS complex, and because E1 was in 6- and 12-fold excesses over the concentration of Fl-E1E2BS (300 nM HPV-11 E1, 50 nM Fl-E1E2BS and 300 nM HPV-11 E1, 25 nM Fl-E1E2BS), binding of E2 to the E1:Fl-E1E2BS complex was stoichiometric rather than hyperbolic. With addition of

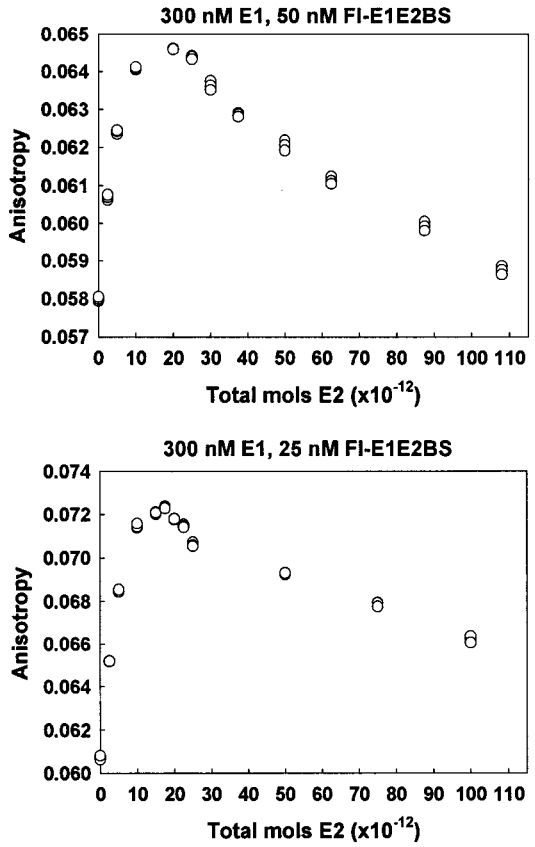

FIGURE 6: Determination of the E2 stoichiometry in the E1:E2:Fl-E1E2BS complex. Samples containing 3×10^{-7} M E1 and 5×10^{-8} M Fl-E1E2BS (6:1) and 3×10^{-7} M E1 and 2.5×10^{-8} M Fl-E1E2BS (12:1) were prepared in a buffer containing 20 mM TRIS, pH 8.0, 150 mM KCl, 5 mM EDTA, 5 mM DTT, and 0.034 mg/mL poly(dI-dC) [10 base pairs of poly(dI-dC) per base pair of oligonucleotide]. Total sample volume was 5×10^{-4} L. The samples and appropriate fluorescence blanks were titrated with a stock solution of E2. The binding curves above show anisotropy maxima at 2×10^{-11} mol (6:1) and 1.75×10^{-11} mol (12:1) E2 (expressed as E2 dimer), corresponding to E2 stoichiometries of 0.8 (6:1) and 1.4 (12:1) in the E1:E2:Fl-E1E1BS complex.

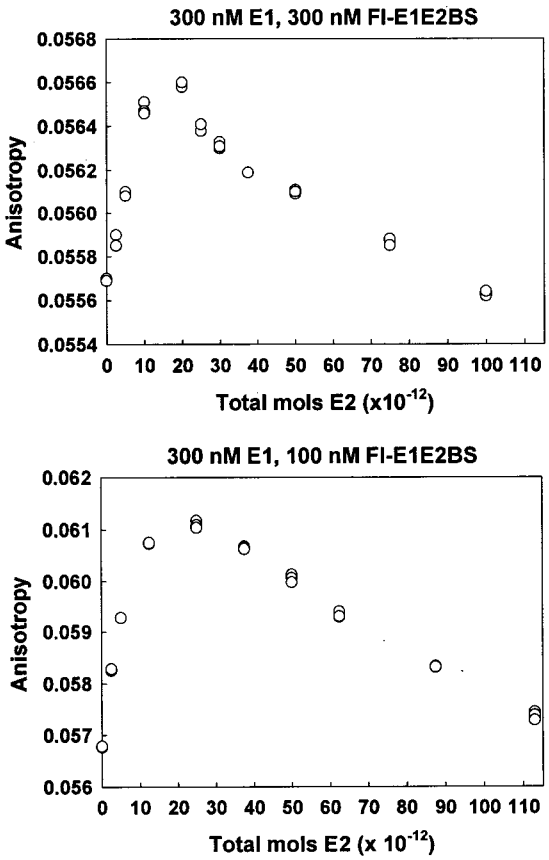

FIGURE 7: Determination of the E1 stoichiometry in the E1:E2:Fl-E1E2BS complex. 3×10^{-7} M E1 and 3×10^{-7} M Fl-E1E2BS (1:1 titration) and 3×10^{-7} M E1 and 1×10^{-7} M Fl-E1E2BS (3:1 titration) were combined in a buffer containing 20 mM TRIS, pH 8.0, 150 mM KCl, 5 mM EDTA, 5 mM DTT, and 0.034 mg/mL poly(dI-dC) [10 base pairs of poly(dI-dC) per base pair of oligonucleotide]. Total sample volume was 5×10^{-4} L. Each sample and an appropriate fluorescence blank were titrated with a stock solution of E2. The binding curves above show anisotropy maxima at 2×10^{-11} mol (1:1) and 2.5×10^{-11} mol (3:1) E2 (expressed as E2 dimer), corresponding to E1 stoichiometries of 7.5 (1:1) and 6 (3:1) in the E1:E2:Fl-E1E2BS complex.

E2 to the E1:Fl-E1E2BS complex, Fl-E1E2BS was set as the limiting reagent. If the stoichiometry of E2 (as dimer) in the E1:E2:oligonucleotide complex were 1, then oligonucleotide would not be depleted until the ratio of E2 dimer to oligonucleotide was 1. If the stoichiometry of E2 dimer in the E1:E2:oligonucleotide complex were 2, then oligonucleotide would not be depleted until the ratio of E2 dimer to oligonucleotide was 2. Thus, the anisotropy peaks correspond to the stoichiometry of E2 in the E1:E2:Fl-E1E2BS complex. Our data indicate that 0.8 mol of dimeric E2 protein bound Fl-E1E2BS in the presence of a 6-fold excesses of E1 protein, while 1.4 mol of E2 dimer bound Fl-E1E2BS in the presence of a 12-fold excess of E1. These results imply that a single dimer of E2 is present in the equilibrium E1:E2:Fl-E1E2BS complex. At E2 quantities in excess of 0.8 and 1.4 mol of E2 dimer/mol of oligonucleotide, respectively, the fluorescence anisotropy of the solutions declined gradually. This may be due to DNA-independent association of E2 with the E1 protein (see Discussion).

Determination of the E1 Stoichiometry in the E1:E2:Fl-E1E2BS Complex. Because the affinity of E1 for the E2:Fl-E1E2BS complex is not high (data not shown), direct titrations of high concentrations of E2:Fl-E1E2BS with E1 to determine the E1 stoichiometry were not feasible. Therefore, an alternate form of the experiment was performed.

The stoichiometry of E1 in the E1:E2:Fl-E1E2BS complex was determined by combining Fl-E1E2BS and HPV-11 E1 in varying molar ratios and then titrating aliquots of HPV-11 E2 (Figure 7). It was hypothesized that at low ratios of E1 to Fl-E1E2BS, the availability of E1 protein would limit formation of the complete E1:E2:Fl-E1E2BS complex. As in the experiments to determine the E2 stoichiometry in the E1:E2:Fl-E1E2BS complex (above), it was assumed that, because Fl-E1E2BS were at concentrations 136- and 45-fold over the K_d of E2 association with the E1:Fl-E1E2BS complex, binding of E2 to the E1:Fl-E1E2BS complex would be stoichiometric rather than hyperbolic. These titrations were designed with E1 as the limiting reagent for complex formation. When stoichiometric quantities of E1 were available, complex formation would proceed to completion. Specifically, if the stoichiometry of E1 in the E1:E2:oligonucleotide complex were 6, then E1 would not be depleted until the ratios of E1 to oligonucleotide and E1 to E2 dimer were both 6:1. If the E1 stoichiometry in the E1:E2:DNA complex were 3, then E1 would not be depleted

Table 1: Affinities of E2 Proteins for Fluorescein-Labeled Oligonucleotides in the Presence and Absence of E1[a]

proteins	oligonucleotide	K_d (nM)
HPV-11 E2C	E2BS (+EDTA)	4.5
HPV-11 E2C	F1-E2BS (+EDTA)	45
HPV-11 E2C	F1-E2BS (+Mg^{2+})	38
HPV-11 E2	F1-E2BS (+EDTA)	0.84
HPV-11 E2	F1-E1E2BS (+EDTA)	>100
HPV-11 E2	F1-E1E2BS (+Mg^{2+}/ATP)	>100
HPV-11 E2 + HPV-11 E1	F1-E1E2BS (+EDTA)	2.2
HPV-11 E2 + HPV-11 E1	F1-E1E2BS (+Mg^{2+}/ATP)	2.1

[a] The table summarizes results for titrations of fluorescein-labeled oligonucleotides with E2C and E2. Experimental conditions, including addition of magnesium ion, ATP, and E1 protein, are as described in the table and under Experimental Procedures. Data for E2C association with E2BS and F1-E2BS are from Alexander and Phelps (*25*).

by E2 addition until the ratios of E1 to oligonucleotide and E1 to E2 dimer were both 3:1. If the E1 stoichiometry in the E1:E2:DNA complex were 1, then E1 would not be depleted by E2 addition until the ratios of E1 to oligonucleotide and E1 to E2 dimer were both 1:1.

The titrations performed at 1:1 and 3:1 E1:Fl-E1E2BS ratios show distinct anisotropy maxima at points where the molar ratios of E1 to E2:Fl-E1E2BS complex are 7.5:1 and 6:1, respectively. The anisotropy maximum for the titrations at the 6:1 and 12:1 E1:Fl-E1E2BS ratios occurs at roughly equimolar concentrations of Fl-E1E2BS and E2 (expressed as dimer). These results support the assertion that a hexamer of E1 is present in the equilibrium E1:E2:Fl-E1E2BS complex.

As noted in the experiment to determine the E2 stoichiometry in the E1:E2:Fl-E1E2BS complex, addition of E2 protein in excess of that required for complex formation prompted a decrease in the solution anisotropy. This again may be due to DNA-independent association of E2 with the E1 protein (see Discussion).

DISCUSSION

Fluorescence anisotropy determination was selected as a method to study the interaction of HPV-11 E1 and E2 proteins with DNA in solution at equilibrium without need of protein cross-linking reagents. To this end, two 5′-fluorescein-labeled oligonucleotides, designated Fl-E2BS and Fl-E1E2BS, were prepared and annealed with unlabeled complementary oligonucleotides. The structures of these oligonucleotides are shown in Figure 2. Because Fl-E2BS and Fl-E1E2BS fluorescence anisotropies vary directly as a function of E1 or E2 binding, the affinities of E1 or E2 for each oligonucleotide were determined.

The affinities of HPV-11 E2C and full-length HPV-11 E2 for the Fl-E2BS and Fl-E1E2BS oligonucleotides are listed in Table 1. Under identical titration conditions, full-length E2 protein bound the Fl-E2BS oligonucleotide with 50-fold higher affinity (Figure 3) than did the E2C protein (*25*). In contrast, the affinity of E2 for the longer native-sequence Fl-E1E2BS oligonucleotide, determined under identical conditions, was markedly reduced in comparison to Fl-E2BS binding (Figure 4). Similar to observations reported by Pepinsky et al. (*40*) for BPV E2 protein, these data indicate that protein structures present in E2 but outside of the E2C dimer domain interact directly or indirectly with components of Fl-E2BS and Fl-E1E2BS. These interactions may increase or decrease the affinity of E2 for DNA, depending upon the DNA sequence. Similarly, variation of DNA structure surrounding the E2 binding-site palindrome appears to be a determinant of E2:DNA binding affinity (*24*). Thain et al. (*41*) demonstrated that DNA binding by HPV-16 E2 was also affected by structures extending beyond the 12 base pair E2 binding-site palindrome. The presence of the fluorescein label may affect the apparent affinity of E2 for the labeled oligonucleotide. The addition of different nonspecific carrier DNAs, such as poly(dI-dC) or salmon sperm DNA, did not affect the affinity of E2 for each labeled oligonucleotide (data not shown).

Our data indicate that the affinity of E2 for native-sequence DNA is only modest, rendering significant E2:DNA association unlikely in the absence of other modulating factors (e.g., transcription factors or the E1 protein). Thus, significant E2:DNA association is likely to occur only with the concurrent association of the E1 protein. Therefore, to assess the energetic contribution of E1:DNA association to the promotion of E2 binding, E2:Fl-E1E2BS binding titrations were performed in the presence of excess E1 protein.

To determine the effects of E1:DNA binding on the E2:DNA association, the affinity of E2 for Fl-E1E2BS was determined in the presence of excess E1. Addition of excess E1 at the beginning of the E2:Fl-E1E2BS titrations prompted a substantial increase in the affinity of E2 for DNA, as well as the predicted increase in the bound fluorescence anisotropy of Fl-E1E2BS (Figure 4). E1, therefore, increases the affinity of E2 for DNA in the same manner that E2 improves E1:DNA association. While these experiments do not preclude the formation of stable "intermediate" states of E1:E2:DNA complexes (e.g., a dimer of E2 bound to DNA and a monomer or trimer of E1), the close adherence of the E2 binding isotherm to that predicted for a single class of noninteracting binding sites points strongly to an absence of stable intermediates.

That E1 would increase the affinity of E2 for DNA is predictable based upon the work of other investigators. Many investigators have demonstrated that the affinity of the E1 protein for DNA is weak and that E1:DNA binding may be relatively nonspecific. Addition of E2 facilitates E1 binding to DNA, and promotes site-specific binding of E1 on the DNA strand. If E2 association with DNA promotes E1:DNA association, the principle of conservation of Gibb's free energy (*42*) dictates that E1 association with DNA must enhance E2:DNA binding. Therefore, complete E2-mediated association of E1 with DNA would not subsequently promote dissociation of E2 from the complex without input of energy from another source (e.g., ATP hydrolysis or binding of an additional factor). Without the input of additional energy, E2 must remain in the final E1:E2:DNA complex. Our observation that an anti-HPV-11 E2 antibody specifically increases the anisotropy of the equilibrium E1:E2:DNA complex provides experimental evidence against the loss of E2 from the fully assembled complex.

To assess the potential role of magnesium ion and ATP binding to E1 as a modulator of E1's ability to promote E2:DNA association, the affinity of E2 for Fl-E1E2BS was determined in the presence of ATP, magnesium ion, and excess E1 (Figure 5). In the absence of E1, the affinity of E2 for Fl-E1E2BS was unaffected by ATP and magnesium.

Addition of excess E1 again prompted the predicted increase in the bound fluorescence anisotropy of Fl-E1E2BS, but failed to produce a substantial change in the affinity of E2 for DNA beyond that seen in the absence of ATP and free divalent cation. These data indicate that, while ATP and magnesium are requirements for E1 helicase activity, the energy-dependent steps in the helicase activity cycle do not include the initial DNA binding process. Furthermore, ATP and magnesium ion do not promote allosteric changes that affect the affinity of E2 for the E1:Fl-E1E2BS complex.

Because the addition of E1 dramatically increases the affinity of E2 for the Fl-E1E2BS oligonucleotide, we were able to determine the stoichiometry of both E1 and E2 in the E1:E2:Fl-E1E2BS complex in solution without benefit of bifunctional cross-linking reagents. Using high concentrations of Fl-E1E2BS and 6- and 12-fold molar excess concentrations of E1 protein, the stoichiometry of E2 in the E1:E2:Fl-E1E2BS complex was determined to be a single E2 dimer per double-stranded Fl-E1E2BS oligonucleotide.

By restricting the molar ratio of E1 to 1 mol of E1/mol of Fl-E1E2BS and 3 mol of E1/mol of Fl-E1E2BS, titrations were performed that allowed the determination of the E1 stoichiometry in the E1:E2:Fl-E1E2BS complex. Because the amount of E1 available for E1:E2:Fl-E1E2BS complex formation was limited, incremental addition of E2 promoted E1:E2:Fl-E1E2BS complex formation (with a resultant increase in fluorescence anistropy) until E1 was depleted. The anisotropy maximum for titrations performed at 1:1 and 3:1 molar ratios of E1 and Fl-E1E2BS yielded E1 stoichiometries of 6 E1 molecules per mole of Fl-E1E2BS.

We find that our data are generally in agreement with those of Sedman and Stenlund (*15*), Sedman et al. (*43*), and Sedman and Stenlund (*27*), obtained for the BPV system. Our results show that E2 binding to DNA, in the absence of free E1 protein, is of low affinity, and the addition of E1 protein increases the affinity of E2 for DNA. Furthermore, E1 binding to DNA is, in the absence of the free E2 protein, of lower affinity, and that the addition of E2 protein promotes higher affinity sequence-specific binding of E1 to DNA (data not shown). There are, however, a few notable differences between the BPV system and the HPV-11 system. First, and in contrast to observations made in the BPV system, the HPV-11 E2 protein remains present in the equilibrium E1:E2:DNA complex. Because the presence of E1 increases the affinity of E2 for Fl-E1E2BS, the conservation of free energy dictates that the release of E2 would occur only if the E1 concentration on the DNA strand were reduced or if some other event (e.g., binding of DNA polymerase α, ATP hydrolysis) interfered with the E1:E2 interaction. Second, we find that the stoichiometry of the equilibrium E1:E2:DNA complex does not vary, and that the complexes formed in the presence of high E1 concentrations contain a single (double-stranded) oligonucleotide bound to a single E2 dimer and a hexamer of E1 molecules. We see no evidence for individual monomers or trimers of E1 binding to Fl-E1E2BS in the presence of E2. It should be noted that while a variety of E1:E2 complexes may form in solution, the spectrofluorometer measures fluorescence anisotropy of the labeled oligonucleotide, providing direct information only about DNA-bound complexes.

Extrapolating from observations made on the SV40 virus large T antigen (*44*−*46*) and BPV E1 (*16*), we suggest that E1 binds to DNA either as a hexameric structure or as a pair of trimeric ring-like structures. The palindromic nature of the E1 binding site and observations of hexamer formation by large T antigen on DNA suggest that HPV-11 E1 binds to DNA as a pair of trimers; however, we have no specific data to support this assertion.

It is interesting to note the unanticipated finding that the solution anisotropy of the E1:E2:Fl-E1E2BS complexes decreased with further addition of E2 protein. The reason for the anisotropy decline is unclear, but it may be a result of DNA-independent E2 binding of E1. Addition of E2 above the stoichiometric amount required for E1:E2:Fl-E1E2BS complex formation may promote dissociation of E1 from the fluorescent oligonucleotide.

The experiments described above were performed under equilibrium conditions; therefore, no specific conclusions may be drawn about the mechanisms or kinetics of E1, E2, and DNA interaction. The order of binding that leads to E1:E2:DNA complex formation cannot be determined from these data, and the order of protein addition is immaterial to the experimental results. The structure of the E1 component of the HPV DNA replication complex cannot be inferred from these data. It should also be noted that our studies were not performed under conditions that would promote procession of the E1 helicase; E1:E2 complex and/or E2:DNA dissociation would presumably be required before helicase activity could begin. Because the E1:E2:DNA complexes appeared to be stable in solution, our data do suggest that dissociation of E2 from the E1:E2:DNA complex requires an additional biochemical step beyond complete E1 association. The lack of effect from addition of ATP and magnesium ion suggests that this additional biochemical step is unlikely to be ATP binding or hydrolysis. This lack of ATP dependence for HPV-11 E1 complex formation contrasts with the behavior of SV40 large T antigen, which requires ATP binding, but not ATP hydrolysis for oligomer formation (*45*, *46*), and with BPV-1 E1 protein, which also appears to require ATP for hexamer formation (*27*).

In summary, the physical interactions between E1 and E2 serve to increase the DNA binding affinity of each. The role of this energy coupling may be to promote *ORI*-specific binding of both E1 and E2 to DNA. In the case of HPV-11 proteins, ATP binding to E1 does not modulate this form of energy coupling. Equilibrium binding of HPV-11 E1 and E2 to Fl-E1E2BS results in formation of a stable E1:E2:Fl-E1E2BS complex, comprised of one E2 dimer and a hexameric aggregate of E1 on the DNA strand. This observation is consistent with the predominantly hexameric solution structure observed for purified HPV-11 E1 (*35*) and contrasts with observations of the BPV system, wherein one E2 dimer associates with a monomer of E1. Furthermore, and in contrast to reports on the BPV E1:E2:DNA complex (*27*), HPV-11 E2 protein-mediated association of E1:DNA complex formation does not result in E2 dissociation from the DNA strand.

It is the complex interplay between E1, E2, host cell transcription factors, and host cell DNA replication proteins that coordinately regulates papillomavirus transcription and replication. To develop further a thermodynamic model of E1:E2:DNA complex formation, we are now studying the interactions between the E1:E2 complex, host cell transcription factors, and host cell DNA replication proteins.

ACKNOWLEDGMENT

We acknowledge Drs. W. K. Joklik and J. Keene for their guidance and encouragement.

REFERENCES

1. Zur Hausen, H., and de Villers, E.-M. (1994) *Annu. Rev. Microbiol. 48*, 427−447.
2. Sugerman, P. B., and Shillitoe, E. J. (1997) *Oral Dis. 3*, 130−147.
3. Phelps, W. C., and Alexander, K. A. (1995) *Ann. Int. Med. 123*, 368−382.
4. Clertant, P., and Seif, I. (1984) *Nature 311*, 276−279.
5. Botchan, M., Berg, L., Reynolds, J., and Lusky, M. (1986) *Ciba Found. Symp. 120*, 53−67.
6. Mohr, I. J., Clark, R., Sun, S., Androphy, E. J., MacPherson, P., and Botchan, M. R. (1990) *Science 250*, 1694−1699.
7. Blitz, I. L., and Laimins, L. A. (1991) *J. Virol. 65*, 649−656.
8. Ustav, M., Ustav, E., Szymanski, P., and Stenlund, A. (1991) *EMBO J. 10*, 4321−4329.
9. Wilson, V. G., and Ludes-Meyers, J. (1991) *J. Virol. 65*, 5314−5322.
10. Yang, L., Li, R., Mohr, I. J., Clark, R., and Botchan, M. (1991) *Nature 353*, 628−633.
11. Bream, G. L., Ohmstede, C. A., and Phelps, W. C. (1993) *J. Virol. 67*, 2655−2663.
12. Seo, Y. S., Muller, F., Lusky, M., Gibbs, E., Kim, H. Y., Phillips, B., and Hurwitz, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2865−2869.
13. Frattini, M. G., and Laimins, L. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12398−12402.
14. Lusky, M., Hurwitz, J., and Seo, Y.-S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8895−8899.
15. Sedman, J., and Stenlund, A. (1995) *EMBO J. 14*, 6218−6228.
16. Sedman, J., and Stenlund, A. (1996) *EMBO J. 15*, 5085−5092.
17. Seo, Y. S., Muller, F., Lusky, M., and Hurwitz, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 702−706.
18. Yang, L., Mohr, I., Fouts, E., Lim, D. A., Nohaile, M., and Botchan, M. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5086−5090.
19. Jenkins, O., Earnshaw, D., Sarginson, G., Del Vecchio, A., Tsai, J., Kallender, H., Amegadzie, B., and Browne, M. (1996) *J. Gen. Virol. 77*, 1805−1809.
20. Park, P., Copeland, W., Yang, L., Wang, T., Botchan, M. R., and Mohr, U. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8700−8704.
21. McBride A. A., Romanczuk, H., and Howley, P. M. (1991) *J. Biol. Chem. 266*, 18411−18414.
22. Dartmann, K., Schwarz, E., Gissmann, L., and zur Hausen, H. (1986) *Virology 151*, 124−130.
23. Androphy, E. J., Lowy, D. R., and Schiller, J. T. (1987) *Nature 325*, 70−73.
24. Li, R., Knight, J., Bream, G., Stenlund, A., and Botchan, M. R. (1989) *Genes Dev. 3*, 510−526.
25. Alexander, K. A., and Phelps, W. C. (1996) *Biochemistry 35*, 9864−9872.
26. Sun, Y. N., Lu, J. Z., and McCance, D. J. (1996) *Virology 216*, 219−222.
27. Sedman, J., and Stenlund, A. (1998) *J. Virol. 72*, 6893−6897.
28. Weber, G., and Bablouzian, B. (1966) *J. Biol. Chem. 241*, 2558−2561.
29. Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, pp 111−153, Plenum Press, New York.
30. Weber, G. (1997) *Methods Enzymol. 278*, 1−15.
31. LeTilly, V., and Royer, C. A. (1993) *Biochemistry 32*, 7753−7758.
32. Cicero, M. P., Alexander, K. A., and Kreuzer, K. N. (1998) *Biochemistry 37*, 4977−4984.
33. Gutman, L. T., St. Claire, K., Herman-Giddens, M. E., Johnston, W. W., and Phelps, W. C. (1992) *Am. Dis. Child. 146*, 694−699.
34. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
35. Rocque, W. J., Porter, D. J. T., Barnes, J. A., Dixon, E., Lobe, D. C., Su, J.-L., Willard, D. H., Gaillard, R., Condreay, J. P., Clay, W. C., Hoffman, C. R., Overton, L. K., Pahel, G., Kost, T. A., and Phelps, W. C., personal communications.
36. Schubert, F., Ahlert, K., Cech, D., and Rosenthal, A. (1990) *Nucleic Acids Res. 18*, 3427.
37. Blatter, L. A., and McGuigan, J. A. (1986) *Q. J. Exp. Physiol. 71*, 467−473.
38. Golding, E. M., Dobson, G. P., and Golding, R. M. (1996) *Magn. Reson. Med. 35*, 174−185.
39. Duguid, J. G., and Bloomfield, V. A. (1995) *Biophys. J. 69*, 2642−2648.
40. Pepinsky, R. B., Prakash, S. S., Corina, K., Grossel, M. J., Barsoum, J., and Androphy, E. J. (1997) *J. Virol. 71*, 828−831.
41. Thain, A., Webster, K., Emery, D., Clarke, A. R., and Gaston, K. (1997) *J. Biol. Chem. 272*, 8236−8242.
42. Weber, G. (1975) *Adv. Protein Chem. 29*, 1−83.
43. Sedman, T., Sedman, J., and Stenlund, A. (1997) *J. Virol. 71*, 2887−2896.
44. Borowiec, J. A., and Hurwitz, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 64−68.
45. Mastrangelo, I., Hough, P. V., Wall, J., Dodson, M., Dean, F., and Hurwitz, J. (1989) *Nature 338*, 658−662.
46. Borowiec, J. A., Dean, F. B., Bullock, P. A., and Hurwitz, J. (1990) *Cell 60*, 181−184.

BI982616V