A Fluorescence Anisotropy Study of DNA Binding by HPV-11 E2C Protein: A Hierarchy of E2-Binding Sites

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ABSTRACT: Association of the human papillomavirus (HPV) E2 protein with its palindromic DNA-binding site is a necessary step for transcriptional *trans*-activation. To study the interaction between DNA and E2, the carboxyl-terminal domain of HPV-11 E2 protein (E2C) was expressed in *Escherichia coli* and purified to homogeneity. The binding affinity of the recombinant E2C protein for a single palindromic DNA recognition site was determined using a 5′-fluorescein-labeled 24 base pair oligonucleotide. Competitive titrations between the fluorescein-labeled oligonucleotide and an unlabeled oligonucleotide of identical sequence yielded a native affinity of 4.5×10^{-9} M. Sequences from the seven E2-binding sites within the HPV-11 genome were titrated to establish a hierarchy of binding site affinities. All high-affinity E2-binding sites are located within or near the HPV-11 LCR. E2-binding sites distant from the LCR appear to have low affinity for E2. When the location and affinity of each E2-binding site are plotted in relation to a transcription map of HPV-11, it is apparent that the major RNA transcripts produced reflect the high-affinity E2-binding sites within the HPV LCR. To assess the E2C-binding contribution of specific base pairs within the oligonucleotide palindrome, additional double-stranded oligonucleotides were prepared in which the central nonpalindromic sequences were varied. While simple strand transposition of the A_4·T_4 center had a minimal effect upon the E2C−oligonucleotide binding affinity, replacement with TATA·ATAT or CGCG·GCGC centers substantially decreased the affinity of E2C for its binding site. Alteration of the canonical portions of the E2-binding palindrome reduced the DNA−protein binding affinity dramatically.

Eucaryotic gene transcription is frequently modulated through a series of *trans*-acting proteins which associate with specific DNA sequences. Human papillomaviruses (HPVs) encode a transcriptional modulator, E2, which is required for the control of HPV RNA transcription, and plays an auxiliary role in HPV replication (Mohr *et al.,* 1990; Chiang *et al.,* 1992a,b; Del Vecchio *et al.,* 1992). E2 binds a 12 base pair palindromic sequence, $ACCGN_4CGGT$, which is present at several locations throughout the HPV genome, and is repeated several times near the viral origin of replication (Androphy *et al.,* 1987; Li *et al.,* 1989).

The 50 kDa E2 protein is comprised of three broad functional domains (Giri *et al.,* 1984; McBride *et al.,* 1991; Prakash *et al.,* 1992). The amino-terminal domain of E2 is necessary for viral *trans*-activation, and for direct association with the E1 protein. The smaller carboxyl-terminal domain encodes the DNA-binding and dimerization functions. The primary structures of both the *trans*-activating and DNA-binding domains are well conserved across the many species-specific types of papillomaviruses. Linking the amino- and carboxyl-terminal domains is a small, poorly conserved "hinge" region.

The X-ray crystal structure of the carboxyl-terminal domain of the bovine papillomavirus (BPV) E2 protein bound to a double-stranded DNA palindrome has been solved by Hegde *et al.* (1992). Crystallography revealed that the DNA-binding domain of BPV has a novel, dimeric anti-parallel β-barrel structure, and that the associated DNA strand is draped across the barrel, causing compression of the major groove. The DNA segment is bent approximately 14° through the central bases of the palindrome. DNA contact with the E2 protein occurs through the 8 palindromic base pairs, 4 bp on each side of the central nonpalindromic region, resulting in approximate C_2 symmetry for the complex. No protein−DNA interactions have been ascribed to the four central nonpalindromic base pairs.

Association of HPV E2 protein with its target DNA-binding site may have activating or inhibitory effects upon viral transcription [reviewed by McBride *et al.* (1991)]. Chow *et al.* (1987) identified and mapped several RNA species from a condyloma acuminatum containing HPV type 11. The predominating RNA species identified had their 5′ ends near nucleotide 700, with a minority of transcripts beginning near nucleotides 100 or 1200. The authors suggested that HPVs have a complex system of coding region utilization, and that some open reading frames (ORFs)[1] may require internal reinitiation for successful translation. Because E2 is a central factor in the control of HPV transcription, and because there

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are seven potential E2-binding sites within the HPV-11 genome, precise characterization of the E2−DNA interaction with each E2-binding site should contribute to a more complete understanding of HPV transcriptional control.

Initial E2−DNA association studies were based upon BPV-1 E2 and its DNA-binding sites. Androphy *et al.* (1987) and Li *et al.* (1989) demonstrated high-affinity DNA binding by BPV E2. High-affinity binding was determined by the canonical portions of the $ACCGN_4CGGT$ palindrome. Variation of the four central, nonpalindromic bases appeared to have a lesser effect upon the E2−DNA binding affinity. Studies have been performed to determine the affinity of E2 for its palindromic DNA-binding site using the BPV-1 E2 protein (Monini *et al.*, 1991). The dissociation constant reported for an 86 amino acid polypeptide encompassing the C-terminal domain of BPV E2 protein binding to a 41 base pair single site oligonucleotide was 4×10^{-10} M. More recently, Sanders and Maitland (1994) measured the affinity between the bacterially-expressed HPV-16 E2 carboxyl terminus and several E2-binding-site oligonucleotides. An E2C−DNA K_d of $\sim 8 \times 10^{-11}$ M was reported; however, the K_d for an oligonucleotide with a mutant canonical sequence was 2×10^{-9} M, indicating that the "non-specific" DNA-binding affinity was also very high. Bedrosian and Bastia (1990) demonstrated that the affinity of HPV-16 E2C binding to DNA was in part determined by the A/T richness of the central four bases of the E2-binding-site palindrome. DNA bending studies indicated that the E2-binding site may exist in a naturally bent confirmation, and that it could bend further upon E2 binding.

In each study described above, the authors used an oligonucleotide gel shift method. Although gel retardation of a labeled oligonucleotide by a DNA-binding protein is readily quantifiable, measurements of the fractions of free and bound DNA are not made at equilibrium due to the utilization of the gel electrophoresis technique. During the course of electrophoresis, the relative DNA and protein concentrations, as well as the solution conditions for binding, continually change. In addition, solutions used during preequilibration steps are typically different from the TRIS−borate buffers used during electrophoresis. These factors could potentially result in specious measurements of the K_d of the DNA−protein complex. Therefore, an alternative method for measuring the DNA−E2C affinity was explored.

Fluorescence anisotropy determination is a spectroscopic technique which is particularly well suited to the study of protein binding to small oligonucleotides (Weber, 1975; Lakowicz, 1983), and has been used to study a variety of protein−DNA associations [for example, see LeTilly and Royer (1993)]. By measuring the fluorescence anisotropy of a fluorescein-labeled 24 base pair E2-binding site oligonucleotide (designated Fl-E2BS) as a function of the added E2C protein, the association of the truncated E2 protein with the fluorescein-labeled oligonucleotide can be measured directly in defined solutions under equilibrium conditions. Once the affinity of the E2C protein for the fluorescein-labeled oligonucleotide is determined, the labeled oligonucleotide may be used as an indicator to measure dissociation constants for E2C association with unlabeled oligonucleotides. The E2C binding affinities of oligonucleotides containing sequences from all seven of the potential E2-binding sites of HPV-11 were determined, and a hierarchy of binding site affinities was established. In addition, to ascertain the relative contributions of the central four base pairs and the palindromic portions of the binding site oligonucleotides to the free energy of E2C−DNA association, systematic alterations in the E2BS(35,50) sequence were made, and the affinity of each modified binding site with E2C was determined.

EXPERIMENTAL PROCEDURES

E2C Cloning and Expression. DNA encoding the carboxyl-terminal 88 amino acids of the HPV-11 E2 protein plus an initiator methionine was obtained by polymerase chain amplification from an HPV-11 inserted into pUC plasmid pW97 (Gutman *et al.*, 1992). PCR primer oligonucleotides were obtained commercially. Their sequences were 5′-CCGCCATATGCATAGTGCAGCTACGCCTATAGTGC-3′ and 5′-CGTAGGATCCCGGGTTACAATAAATGTAATGACATAAACC-3′. The resulting DNA strand was cut with the restriction endonucleases *Nde*I and *Bam*HI and ligated into a bacteriophage T7 expression vector, pSS582 (S. Short, unpublished experiments), containing a T7 gene 10 promoter (Studier & Moffett, 1986). Plasmids were purified from recombinant colonies and used to transfect BL21 DE3 *E. coli.* BL21 DE3 *E. coli* expressing E2C were grown for 20 h at 30 °C in enriched medium with 50 μg/mL added kanamycin. Aliquots of recombinant BL21 DE3 *E. coli* were frozen at −80 °C, and were used to inoculate larger scale cultures for E2C preparation.

E2C Purification. The expressed C-terminal portion of HPV-11 E2 was purified in a manner similar to that used by Hegde *et al.* (1992). After growth was completed, the culture was centrifuged at 13 000 rpm for 30 min at 4 °C in a GSA rotor. The pelleted *E. coli* were resuspended in 2.5 volumes of buffer containing 50 mM TRIS, pH 7.5, 100 mM NaCl, 2 mM EDTA, 10 mM DTT, and 0.5 mM PMSF. The bacterial suspensions were divided into two aliquots and sonicated on ice at maximum power 4 times for 2 min intervals using a microtip. After sonication, the samples were centrifuged for 60 min at 20 000 rpm in an SS-34 rotor. After centrifugation, the bacterial pellet was discarded, and the supernatant was frozen at −80 °C for later purification.

The thawed recombinant *E. coli* supernatant was filtered through a 1.2 μm filter, and the pH was adjusted by the addition of 5 mL of 1 M unbuffered MES per 100 mL. The samples were then fractionated by FPLC using a Mono S 16/10 cation exchange column (Pharmacia) and a 0−1 M NaCl gradient in a buffer containing 20 mM MES, pH 6.2, 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. The E2C protein, which was obtained in a single sharp peak, was concentrated and applied to a 95×1.5 cm BioGel P-60 size exclusion column (BioRad). The sample was eluted at 8 mL/h in a buffer containing 20 mM MOPS, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT. The purified protein solution was again filtered through a 1.2 μm filter. Prior to freezing the purified protein for storage, an aliquot was removed for quantitative amino acid analysis. Amino

[1] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; LCR, long control region; ORF, open reading frame; *ORI*, origin of replication; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 2-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TRIS, tris(hydroxymethyl)aminoethane.

acid and DNA sequencing confirmed the predicted primary structure of E2C, including lysine to arginine and histadine to glutamine polymorphisms at HPV-11 E2 amino acid numbers 308 and 327, respectively (Bream *et al.*, 1993). Laser desorption mass spectrometry confirmed the predicted molecular weight and homogeneity of the preparation.

Synthetic Oligonucleotides. PAGE-purified fluorescein-labeled and unlabeled oligonucleotides were obtained commercially (Synthecell Corp. and from Genosys Corp.) and used without further purification. Commercially obtained 5′-fluorescein phosphoramidite-labeled oligonucleotide was prepared by the method of Schubert *et al.* (1990). Contaminating unlabeled oligonucleotide was less than 2−3% (manufacturer's quality-control report). Oligonucleotide concentrations were calculated from the measured 260 nm absorbencies. Complimentary oligonucleotides were annealed by combining equimolar amounts of each, and heating the samples to 95 °C for 2 h. The samples were then allowed to cool at 5 °C/h to 45 °C (approximately 25 °C below the calculated melting point of the double-stranded oligonucleotides) and allowed to anneal for 48−72 h. PAGE analysis of samples showed near 100% annealing (data not shown). Annealed oligonucleotides were stored at 0 °C.

Fluorescence Measurements. All fluorescence measurements were made using an SLM 48000S spectrofluorometer assembled in the "T" geometry. Samples were excited at 490 nm, with 4 nm resolution, and emitted light was collected through orange glass filters (OG 515, Schott). Sample temperatures were maintained at 25 ± 0.5 °C. The dissociation constants for the Fl-E2BS−E2C complex were determined in the presence and absence of divalent cations by measurement of the steady-state anisotropy of Fl-E2BS fluorescence as a function of added E2C. The anisotropy limits of the titrations, corresponding to free Fl-E2BS (A_f) and the Fl-E2BS−E2C complex (A_b), as well as the observed anisotropy (A) were used to calculate the fraction of Fl-E2BS associated with E2C as described in eq 1:

$$\text{fraction bound} = (A - A_f)/(A_b - A_f) \qquad (1)$$

where A_f is the fluorescence anisotropy of the free fluoresceinated oligonucleotide and A_b is the fluorescence anisotropy of the E2C-bound fluoresceinated oligonucleotide. No computational corrections for emission intensity were required as the quantum yield of the fluoresceinated oligonucleotide did not change significantly upon E2C binding. Typically, the blank fluorescence was less than 4% of the sample fluorescence intensity.

Oligonucleotide Phosphorylation and Gel Shifts. The 5′ ends of the E2C-BS oligonucleotides were radiolabeled with [γ-^{32}P]ATP prior to annealing; 2×10^{-11} mol of [γ-^{32}P]-ATP was mixed with 1×10^{-11} mol of each single-stranded oligonucleotide in a buffer containing 50 mM TRIS, pH 9.0, 10 mM $MgCl_2$, 1 mM DTT and 30 units of T4 polynucleotide kinase in a total reaction volume of 30 μL. The reaction mixture was maintained at 37 °C for 1 h and then heated to 95 °C for 2 h and allowed to anneal as described above. After annealing was completed, the sample was diluted to an oligonucleotide concentration of 1×10^{-8} M.

Oligonucleotide gel shifts were performed by a method similar to that of Li *et al.* (1989). Briefly, 30 μL reaction mixtures were prepared containing 10 mM TRIS, pH 8.0, 250 mM KCl, 1.1 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 5 mM DTT, 0.05 mg/mL poly(dI-dC)·poly(dI-dC), 0.2 mg/mL BSA, and 1×10^{-9} M phosphorylated E2BS(35,50). Increasing amounts of purified E2C were added to each quadruplicate sample. After 30 min equilibration at 25 °C, 20 μL sample aliquots were subjected to 15% PAGE at 10 V/cm in gel containing TRIS−borate buffer at 25 °C. After electrophoresis was completed, the gels were dried, and the shifted and unshifted oligonucleotide bands were quantified by the phosphorimager.

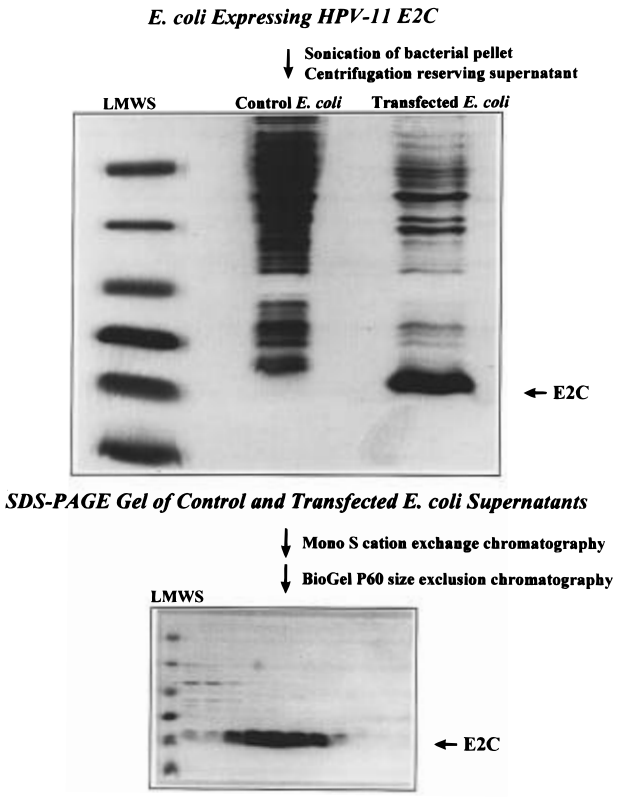

FIGURE 1: Purification of E2C from recombinant E. coli. Recombinant E2C protein was purified to homogeneity using a method similar to that of Hegde *et al.* (1992) as described under Experimental Procedures. Shown are two SDS−PAGE gels demonstrating the composition of crude supernatant from sonicated control and E2C-expressing *E. coli* (top), and column fractions obtained from size exclusion chromatography (bottom). Peak sizing column fractions were pooled, assayed, and used for experimental studies.

RESULTS

HPV-11 E2C Expression and Purification. Using a method similar to that of Hegde *et al.* (1992), several milligrams of homogeneously pure protein could be reproducibly prepared (Figure 1). No affinity purification steps were required, and protein denaturants were not used. Amino acid sequencing and laser desorption mass spectrometry confirmed the predicted primary structure, molecular weight, and homogeneity of the preparation (data not shown).

Affinity of E2C for Fluorescein-Labeled E2BS(35,50). Because the oligonucleotide gel shift method utilizes conditions which are not truly at equilibrium once chromatography has begun, and because conditions within the polyacrylamide gel matrix may not be reflective of solution conditions, fluorescence anisotropy measurement was chosen as a method to determine E2C association with E2BS(35,50) in a solution at equilibrium. For this purpose, a 5′-fluorescein-

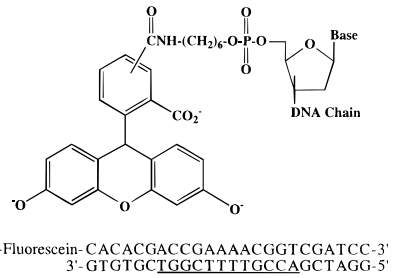

FIGURE 2: Structure of the fluorescein-labeled E2-binding-site oligonucleotide (Fl-E2BS). Illustrated are the structure of the phosphoramidite-linked fluorescein moiety and the sequence of the Fl-E2BS oligonucleotide.

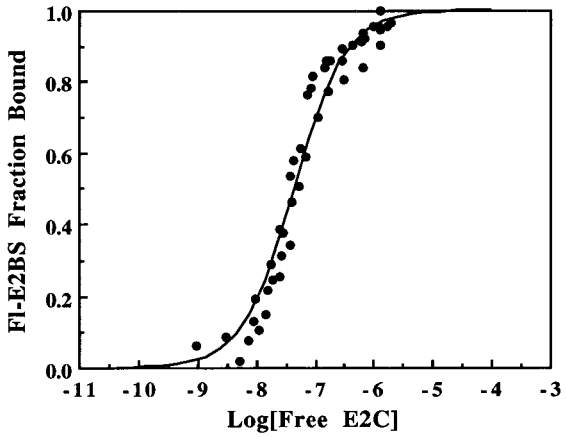

FIGURE 3: Association of E2C with Fl-E2BS in the presence of EDTA. The affinity of E2C for fluorescein-labeled E2-BS oligonucleotide was determined by measuring the increase of the fluorescence anisotropy of Fl-E2BS as a function of added E2C. 1×10^{-7} M Fl-E2BS in a buffer containing 20 mM TRIS, pH 8.0, 150 mM KCl, 5 mM DTT, 0.1 mg/mL salmon testis DNA, and 5 mM EDTA was titrated with a stock solution of E2C. The fluorescence anisotropy of the solution was determined after each addition of E2C, as described under Experimental Procedures. The free anisotropy of the Fl-E2BS oligonucleotide was 0.0616; the anisotropy of the E2C-bound oligonucleotide was 0.0709. Using the observed fluorescence anisotropy and the anisotropies of the free and bound oligonucleotides, the fraction of oligonucleotide bound by E2C could be calculated as described under Experimental Procedures. The data were fit to the Michaelis−Menten equation by Newton−Gauss iterative nonlinear least-squares regression analysis assuming a single class of noninteracting E2C-binding sites. The fraction of Fl-E2BS bound by E2C was then plotted as a function of the log of the free E2C concentration. The binding curve shown corresponds to a K_d of 45 nM.

labeled oligonucleotide was prepared and annealed with a complimentary strand to produce fluorescein-labeled E2BS(35,50) which was designated Fl-E2BS (Figure 2). Because Fl-E2BS fluorescence anisotropy varies as a direct function of E2C binding, the affinity of E2C for Fl-E2BS could be determined (Weber, 1975; Lakowicz, 1983). Furthermore, fluorescein fluorescence is intense and is generally insensitive to solvent conditions and to quenching by DNA (Lakowicz, 1983). Figure 3 shows the combined results of four titrations of Fl-E2BS with E2C. From these data, the affinity of E2C for fluorescein-labeled E2BS(35,50) was calculated to be 45 nM. The mathematical fit shown assumes a single class of noninteracting E2C -S(35,50) oligonucleotide. To confirm the 1:1 stoichiometry experimentally, a concentrated solution of E2BS(35,50) containing a small amount of Fl-E2BS as an indicator was titrated with E2C. Aliquots of E2C were added to a solution of 2×10^{-7} M Fl-E2BS and 9.8×10^{-6} M E2BS(35,50). As predicted, the fluorescence anisotropy of the solution increased with added E2C until the molar ratio of E2BS(35,50) to E2C dimer approached 1:1. Subsequent additions of E2C resulted in minimal fluorescence anisotropy changes (data not shown).

Control experiments were also performed to assess the affects of carrier protein, carrier DNA [poly(dI-dC)·poly-(dI-dC) and salmon sperm], NaCl concentration, divalent cations (Ca^{2+} and Mg^{2+}), and glycerol. Each had little or no effect upon Fl-E2BS−E2C binding (data not shown).

Determination of the Native Binding Constant for E2BS(35,50)−E2C. Once the affinity of E2C for the fluorescein-labeled oligonucleotide Fl-E2BS was determined, Fl-E2BS could then be used as an indicator to study the interaction between E2C and any unlabeled oligonucleotide. By measuring the fluorescence anisotropy of a mixture of E2C and Fl-E2BS as a function of added competing oligonucleotide, and knowing the affinity of the Fl-E2BS−E2C complex, the affinity of E2C for the competing unlabeled oligonucleotide could be calculated. Figure 4 shows the data for the association between E2C and unlabeled E2BS(35,50). The nonlinear least-squares fitting of the data in Figure 4 corresponds to a dissociation constant of 4.5 nM. It is noteworthy that, because the affinity of E2C for E2BS(35,50) was 10-fold higher than that of the Fl-E2BS−E2C interaction, high fractions of E2BS(35,50)−E2C association were reflected by only small changes in Fl-E2BS fluorescence. As a result, the data obtained at low E2BS(35,50) concentrations would be predictably less accurate than data obtained at higher E2BS(35,50) concentrations. This trend is reflected in the data within Figure 4, and is a direct consequence of the greater affinity of E2C for unlabeled rather than fluoresceinated E2BS(35,50).

Determination of the Binding Constant for Other Native-Sequence E2-Binding-Site Oligonucleotides. To directly compare the relative affinities of all the potential E2-binding sites in the HPV-11 genome, five additional oligonucleotides were constructed based upon the sequence of E2-binding sites located at bases 585, 2514, 4767, 7592, and 7892. The oligonucleotides were also titrated using Fl-E2BS as an indicator. The titration curves for each oligonucleotide are shown in Figure 4.

The first sequence-modified binding site, E2BS(7592), is structurally similar to the E2BS(35,50) oligonucleotide; however, the strand orientation of the central adenosine and thymidine bases is reversed. Due to its palindromic nature, the nucleotide sequence of the E2-binding site remains unchanged. Thus, this oligonucleotide assesses the effects of the nonpalindromic base sequences flanking the 12 bases of the E2-binding site. Reversal of the central adenosine and thymidine bases relative to the flanking bases had only a modest effect, and decreased the E2C-binding affinity of the oligonucleotide to 10 nM.

E2BS(7892), with an E2C−oligonucleotide affinity of 1.8 nM, demonstrates the minimal effect of altering a single base pair within the nonpalindromic core of the E2-binding-site oligonucleotide. In contrast, alteration of the canonical and central portions of the E2-binding-site palindrome leads to large decreases in the affinity of the oligonucleotide for E2C. E2-binding sites E2BS(585), E2BS(2514), and E2BS(4767) each contain sequences native to HPV-11 in which the palindromic and central structures differ from that in E2BS(35,50). In each case, the binding affinity between E2C and these sites is reduced by 2−3 orders of magnitude.

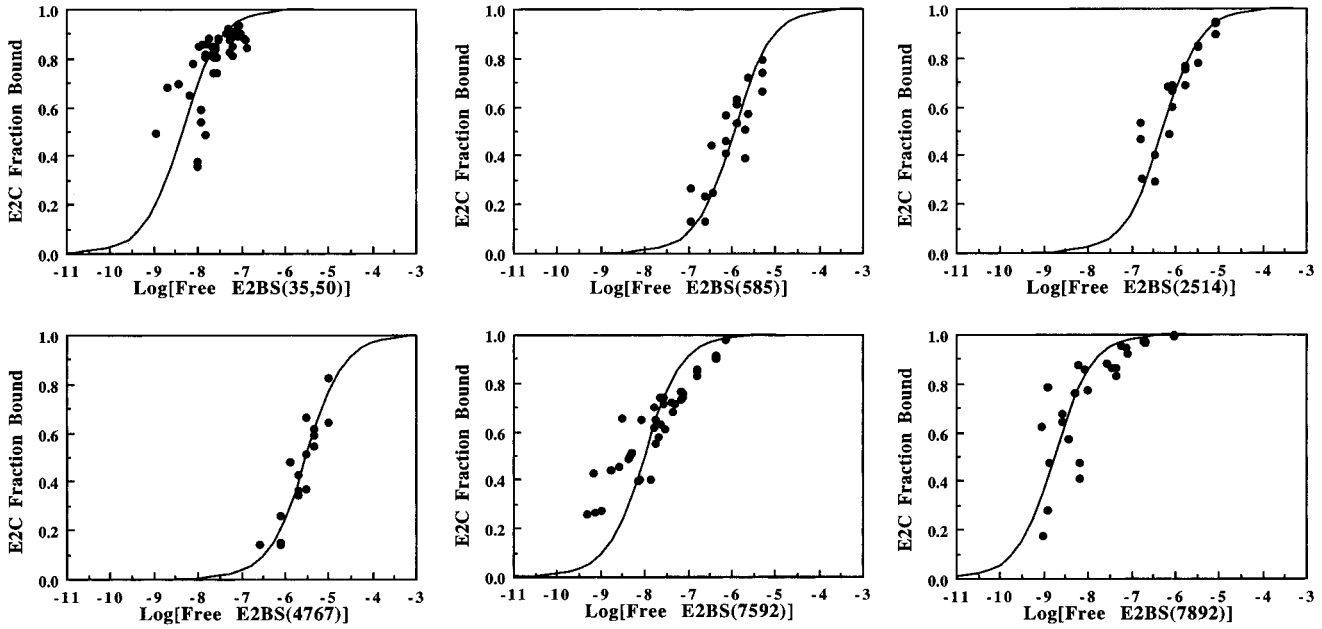

FIGURE 4: Determination of the E2-binding-site−E2C complex affinities by competitive titration with Fl-E2BS. The affinities of E2C for E2BS(35,50), E2BS(585), E2BS(4767), E2BS(7592), and E2BS (7892) were determined in buffer containing 20 mM TRIS, pH 8.0, 150 mM KCl, 5 mM DTT, 0.1 mg/mL salmon testis DNA, 3×10^{-7} M Fl-E2BS, and 1.5×10^{-7} M E2C. Aliquots of E2-binding-site oligonucleotide were then added, and the fluorescence anisotropy of Fl-E2BS was determined with each addition. Samples were allowed a minimum of 15 min for equilibration following each oligonucleotide addition; no systematic drift of anisotropy values (which would be suggestive of incomplete equilibration) was seen. Using the anisotropy data to calculate the fraction of Fl-E2BS bound to E2C and assuming a K_d of 45 nM for the Fl-E2BS−E2C interaction (see Figure 3), the fraction of E2C bound by E2-binding-site oligonucleotide could be calculated. The data were fit to the Michaelis−Menten equation by Newton−Gauss iterative nonlinear least-squares regression analysis assuming a single class of non-interacting E2C-binding sites. The fraction of E2C bound by E2-binding-site oligonucleotide was then plotted as a function of the log of the free E2-binding-site oligonucleotide concentration. The binding curves shown correspond to the dissociation constants indicated in Table 1.

Effects of Inner Nucleotides upon Oligonucleotide−DNA Binding Affinity. To ascertain to what degree the central four base pairs of the E2BS(35,50) oligonucleotide were determinants in the E2BS(35,50)−E2C interaction, discrete changes of these four base pairs were made, and the effect upon E2C−oligonucleotide affinity was measured. As with the E2BS(35,50) oligonucleotide, the affinity of each sequence-modified oligonucleotide for E2C was measured by competition with Fl-E2BS binding to E2C. Figure 5 shows data and a nonlinear least-squares fit for each oligonucleotide binding to E2C. Binding affinities and oligonucleotide structures are summarized in Table 1.

BS(TATA) is an oligonucleotide structurally similar to E2BS(35,50), but with the four central adenosine and thymidine bases alternated (AAAA → TATA). Binding to E2C was of substantially lower affinity than both the E2BS(35,50)−E2C and E2BS(7592)−E2C interactions; the calculated K_d for the BS(TATA)−E2C complex was 94 nM. Substitution of the central adenosine and thymidine bases with alternating cytosine and guanosine (AAAA → CGCG) yielded an oligonucleotide designated BS(CGCG) with an E2C-binding affinity of 71 nM.

Effects of Palindromic Nucleotides upon Oligonucleotide−DNA Binding Affinity. To measure the nonspecific DNA-binding affinity of E2C for an oligonucleotide, an oligonucleotide predicted to have minimal E2-binding affinity was synthesized (Li *et al.*, 1989) and designated BS(nb). Binding of BS(nb) to E2C was of low affinity, and displacement of the Fl-E2BS indicator oligonucleotide from E2C was incomplete, even at BS(nb) concentrations greater than 5 μM. The estimated affinity between E2C and BS(nb) is greater than or equal to 1×10^{-6} M (data not shown). This titration probably reflects the nonspecific DNA-binding affinity of E2C.

Oligonucleotide Gel Shift Analysis. The results of a titration of ^{32}P-labeled E2BS(35,50) with E2C are shown in Figure 6. Using quantitative phosphorimaging, the absolute amounts of shifted and unshifted DNA could be determined as a function of added E2C protein. From these data, the dissociation constant for E2C binding to phosphorylated E2BS(35,50) was calculated to be 32 nM, which is approximately 7-fold higher than the K_d determined by fluorescence (4.5 nM). As with the fluorescence data, the mathematical fit to the gel shift data shown assumes a single class of noninteracting E2C-binding sites and a stoichiometry of 1 E2C dimer to 1 double-stranded E2BS(35,50) oligonucleotide.

DISCUSSION

Transcription and replication of HPV DNA in infected cells are complex processes which require both viral and host cell proteins. The HPV E2 protein is a central control point for both transcription and replication. The E2 protein controls HPV transcription, probably through direct interaction with host cell transcriptional proteins (Li *et al.*, 1991; Thierry & Howley, 1991; Ham *et al.*, 1994). The role of E2 in HPV DNA replication is less clear. E2 appears to function in concert with the HPV E1 protein and is necessary for initiation of origin-dependent replication, although E2 is not required for origin-independent replication (Kuo *et al.*, 1994; Le Moal *et al.*, 1994). In the case of both HPV transcription and origin-dependent replication, the association of E2 with its 12 base palindromic DNA-binding site is a

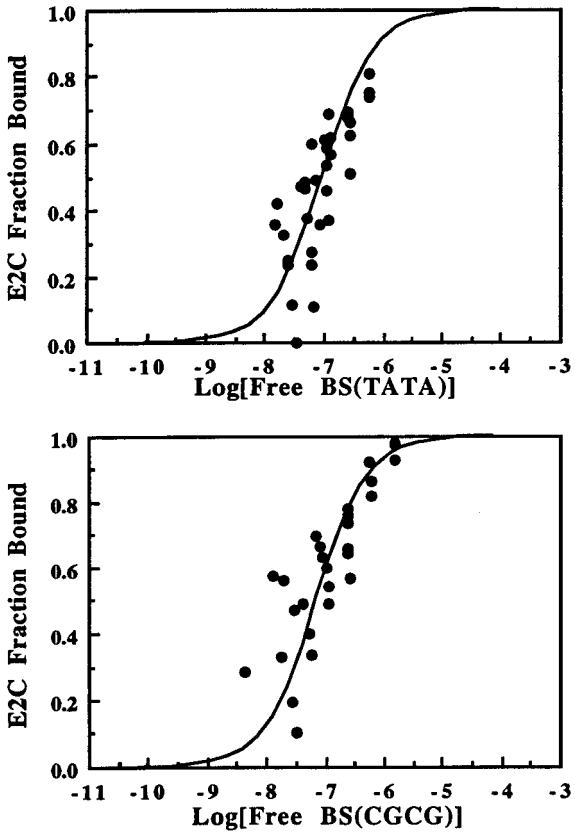

FIGURE 5: Determination of the affinity of E2C for oligonucleotides E2BS(TATA) and E2BS(CGCG) by competitive titration with Fl-E2BS. The affinity of E2C for oligonucleotides E2BS(TATA) and E2BS(CGCG) was determined as described in Figure 4. The binding curves shown correspond to dissociation constants of 94 nM and 71 nM, respectively.

Table 1: E2C-Binding Affinities of E2-Binding-Site Oligonucleotides

Oligonucleotide Designation (HPV-11 Nucleotide #)	*Oligonucleotide Sequence*	*Oligonucleotide:E2C K_d (nM)*	*$\Delta\Delta G$ (kcal/mol)*
FL-E2BS	5'-Ⓟ- C A C A C G A C C G **A A A A** C G G T C G A T C C -3' 3'- G T G T G C T G G C **T T T T** G C C A G C T A G G -5'	45	
E2BS(35,50)	5'- C A C A C G A C C G **A A A A** C G G A C G A A C C -3' 3'- G T G T G C T G G C **T T T T** G C C T G C T T G G -5'	4.5	
E2BS(585)	5'- C A C A C G A C C **C T G T G C** G G T C G A T C C -3' 3'- G T G T G C T G G **G A C A C G** C C A G C T A G G -5'	1200	3.3
E2BS(2514)	5'- C A C A C G A C C G **C T A C T** G G T C G A T C C -3' 3'- G T G T G C T G G C **G A T G A** C C A G C T A G G -5'	520	2.8
E2BS(4767)	5'- C A C A C G A C C **T G A G G T** G G T C G A T C C -3' 3'- G T G T G C T G G **A C T C C A** C C A G C T A G G -5'	3100	3.9
E2BS(7592)	5'- C A C A C G A C C G **T T T T** C G G T C G A T C C -3' 3'- G T G T G C T G G C **A A A A** G C C A G C T A G G -5'	10	0.5
E2BS(7892)	5'- C A C A C G A C C G **G T T T** C G G T C G A T C C -3' 3'- G T G T G C T G G C **C A A A** G C C A G C T A G G -5'	1.8	-0.5
BS(TATA)	5'- C A C A C G A C C G **T A T A** C G G T C G A T C C -3' 3'- G T G T G C T G G C **A T A T** G C C A G C T A G G -5'	94	1.8
BS(CGCG)	5'- C A C A C G A C C G **C G C G** C G G T C G A T C C -3' 3'- G T G T G C T G G C **G C G C** G C C A G C T A G G -5'	71	1.6
BS(nb)	5'- C A C A C G A T **G G A A A A** C T A T T G A T C C -3' 3'- G T G T G C T **A C C T T T T** G **A T A** C T A G G -5'	>1000	>3.2

necessary first step. A detailed understanding of the E2−DNA association is, therefore, central to understanding how papillomavirus transcription and replication are coordinately controlled.

To date, most studies of the E2−DNA interaction have been qualitative, have focused on the BPV or HPV-16 E2 proteins, and have utilized either oligonucleotide gel shift (Bedrosian & Bastia, 1990; Li *et al.*, 1989) or functional analysis (Spalholz *et al.*, 1988; Prakash *et al.*, 1992). A quantitative study by Monini *et al.* (1991) using oligonucleotide gel shift analysis reported protein−DNA dissociation constants of 2×10^{-11} and 4×10^{-10} M for full-length BPV-1 E2 and E2C, respectively. In the same paper, Monini *et al.* also described apparent cooperativity of E2

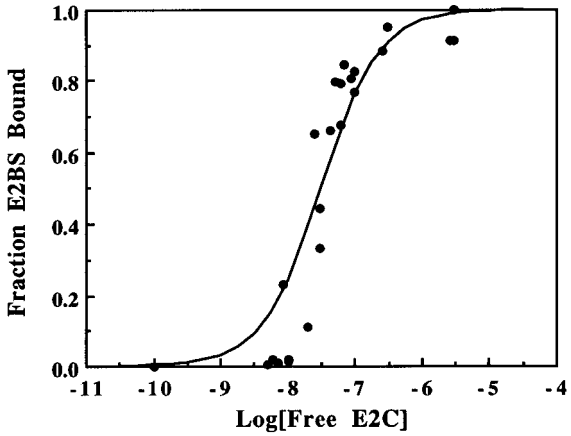

FIGURE 6: Determination of the E2BS(35,50)−E2C affinity by oligonucleotide gel shift. Oligonucleotide gel shift experiments were performed as described under Experimental Procedures. Radioactivity of shifted and unshifted oligonucleotide was quantified using a phosphorimager (Molecular Dynamics). The fraction of shifted oligonucleotide over total oligonucleotide (Fraction Bound) was plotted as a function of the free E2C concentration. The dissociation constant was determined by a Newton−Gauss nonlinear least-squares fit of the data to the Michaelis−Menten equation. The fraction of phosphorylated E2BS(35,50) bound by E2C was then plotted as a function of the log of the free E2C concentration. The binding curve shown corresponds to a K_d of 32 nM.

binding to oligonucleotides containing multiple E2-binding sites. Work by Sanders and Maitland (1994) using oligonucleotide gel shift analysis to measure the affinity between the bacterially-expressed HPV-16 E2 carboxyl terminus and several E2-binding-site oligonucleotides showed very high affinity between the binding sites and E2C; however the affinity of "non-specific" DNA binding was also very high. To study the E2−DNA interaction in detail, to assess the energetic effects of specific base sequences as determinants of the E2C−DNA affinity, and to avoid the problems inherent in the oligonucleotide gel shift technique, fluorescence polarization measurements were used to study the E2C−DNA interaction.

To study the E2−DNA interaction at equilibrium in solution, a fluorescence-based assay was constructed using a fluorescein-labeled 24 base pair oligonucleotide. Fl-E2BS was prepared by covalently attaching a highly fluorescent fluorescein moiety to the 5′-end of one of the oligonucleotide strands (Figure 2). The carboxyl-terminal portion of HPV-11 E2 (E2C) was expressed in *E. coli* and purified to homogeneity without use of an affinity column (Figure 1). E2C association with fluorescein-labeled DNA was determined by measuring the anisotropy of fluorescence. From these measurements, the concentrations of free and bound E2C and oligonucleotide were calculated.

The apparent affinity between E2C and the Fl-E2BS oligonucleotide was 45 nM (Figure 3). The curve for E2C association with Fl-E2BS was consistent with a single class of noninteracting DNA-binding sites on E2C. The stoichiometry of interaction in solution was 1 mol of E2C dimer associating with 1 mol of double-stranded oligonucleotide (data not shown). This finding is consistent with the crystal structure of the C-terminal portion of BPV E2 (Hegde *et al.*, 1992), which showed a single dimer of E2 bound to one double-stranded oligonucleotide. Furthermore, the 1:1 stoichiometry observed indicates that essentially all of the E2C protein was active in binding DNA.

Once the K_d of the Fl-E2BS−E2C complex was established, the labeled oligonucleotide was used as an indicator to measure dissociation constants for E2C association with unlabeled oligonucleotides. Six oligonucleotides containing sequences from each of the putative HPV-11 E2-binding sites were used as competitive antagonists of E2C interaction with Fl-E2BS. Because the affinity of E2C for Fl-E2BS was determined, the affinity of each competing oligonucleotide for E2C could be calculated from experimental data (Figure 4 and Table 1). In addition, three test oligonucleotides were studied to assess the effects of specific base substitutions (Figure 5 and Table 1).

Oligonucleotide E2BS(35,50) was identical in sequence to the Fl-E2BS oligonucleotide; however, it lacked the 5′-fluorescein label on the top strand. Using E2BS(35,50) as a competitive antagonist of the Fl-E2BS−E2C interaction, the K_d for the E2BS(35,50)−E2C interaction was determined to be 4.5 nM, which is 10-fold lower than the K_d for the Fl-E2BS−E2C interaction. This difference in binding affinity is likely attributable to steric effects of the fluorescein label. Why the affinity of the E2BS(35,50)−HPV-11 E2C complex measured using fluorescence anisotropy is lower than those reported by Monini *et al.* (1991) for BPV-1 E2 and by Sanders and Maitland (1994) for HPV-16 E2C is unclear, but may be due to several factors: First, the apparent lower *trans*-activation activity of HPV E2 proteins when compared to that of BPV-1 E2 (Phelps & Howley, 1987; Thierry & Yaniv, 1987) may indeed be a reflection of a lower affinity of the HPV E2 proteins for the E2-binding-site palindrome. Second, E2 polypeptide sequences extrinsic to the previously-defined E2C DNA-binding site may have some role in DNA binding, either directly, or indirectly through the tertiary structure of E2C. Third, the length of the oligonucleotide probe used may be a factor in E2 binding such that longer oligonucleotides bind E2 with higher affinity. The oligonucleotide probe used by Monini *et al.* was 41 base pairs in length, and the oligonucleotides used by Sanders and Maitland were 22 base pairs with 5 base overhangs at both 5′ ends; these are in contrast to the 24 base pairs of the E2BS(35,50) oligonucleotide used in this study. Indeed, a fluorescein-labeled 16 base pair oligonucleotide similar to E2BS(35,50) bound E2C poorly (Alexander and Phelps, data not shown). A fourth possible explanation for differences in measured dissociation constants is variation in the proportion of active E2C protein. It should also be noted that our E2C protein concentrations were determined by quantitative amino acid analysis because routine dye-binding methods (BioRad) yielded erroneously low results. This is probably due to the poor dye-binding properties of small proteins. Finally, the affinity of HPV-11 E2C for DNA falls within the range of DNA-binding affinities of several other transcription factors as determined by fluorescence polarization. These factors include yeast TATA-binding protein (K_d = 5 nM) (Perez-Howard *et al.*, 1995), λ repressor (K_d = 2.3 μM) (Banik *et al.*, 1993), and the *trp* repressor (K_d = 0.1 nM) (LeTilly & Royer, 1993). Thus, our results are consistent with those of other transcription factor−DNA interactions determined using similar methodology.

To directly compare the relative affinities of all the potential E2-binding sites in the HPV-11 genome, oligonucleotides were constructed based upon the sequence of E2-binding sites located at bases 585, 2514, 4767, 7592, and 7892. The titration curves for each oligonucleotide are shown in figure 4. Strand reversal of the A_4·T_4 core of the E2BS(35,50) palindrome yielded oligonucleotide E2BS(7592) which had an E2C-binding K_d of 10 nM. In effect, this oligonucleotide served as a control to assess the contribution of the nonpalindromic base pairs flanking the 12 base E2-binding site. The difference in E2C-binding affinity between E2BS(35,50) and E2BS(7592) suggests that the flanking base pairs outside of the described 12 base palindrome may have a minimal contributory role in the E2−DNA interaction, at least within the limited context of these experiments. Because the flanking sequences are not well conserved among the papillomaviruses or between E2-binding sites within the viral genome, it is possible that any interactions between the flanking bases of the E2-binding site and the E2 protein may be through the phosphate backbone of the DNA strand rather than through specific base−protein interactions. Alternately, the effects of the flanking sequences may be due to the use of a small oligonucleotide, and may be increased or decreased *in vivo.* E2BS(7892), with an E2C−oligonucleotide K_d of 1.8 nM, demonstrates the minimal effect of altering a single base pair within the nonpalindromic core of the E2-binding-site oligonucleotide. E2-binding sites E2BS(585), E2BS(2514), and E2BS(4767) each contain sequences native to HPV-11 in which the palindromic and core structures differ from the optimal sequence, ACCG-(A/T-rich)$_4$-CGGT. In each case, the binding affinity between E2C and sites with altered canonical domains is reduced by 2−3 orders of magnitude relative to E2BS(35,50).

Because E2-binding sites E2BS(585), E2BS(2514), and E2BS(4767) all contain sequences in which both the palindromic and core structures differ from the optimal sequence, it is important to assess the relative contribution of each structural feature to the E2−DNA association. To do this, a nonpalindromic oligonucleotide with an optimal A/T-rich center was constructed and titrated. This titration, in combination with titrations in which the optimal palindromic portions of the oligonucleotide are maintained while the core is modified, measures the contribution of each structural component of the E2-binding-site oligonucleotide to E2 binding. Results are summarized in Table 1. Titrations of an oligonucleotide, designated BS(nb), in which four of the bases within the canonical portions of the palindrome were changed, showed only minimal E2C−oligonucleotide association at free oligonucleotide concentrations greater than 1 μM. Clearly, alteration of the bases within the canonical regions of the E2-binding site lead to large decreases in the E2C−oligonucleotide affinity.

Although the central four base pairs within the E2-binding sites of the HPV-11 LCR lack strong sequence conservation, they maintain a moderately high degree of adenosine/thymidine richness (Giri & Danos, 1986; Giri & Yaniv, 1988). As noted above, Bedrosian and Bastia (1990) asserted that the affinity of HPV-16 binding to DNA was in part determined by the A/T richness of the central four bases of the E2-binding-site palindrome. It was, therefore, proposed that the affinity of E2C for an E2-binding site would vary as a function of the composition of the oligonucleotide center, and that such changes could serve as a regulatory determinant in E2−DNA association. Modification of the four central bases while maintaining the optimal palindromic base sequences led to only moderate decreases in the E2C−DNA

affinity. BS(TATA) is structurally similar to E2BS(35,50), but with the four central adenosine and thymidine bases alternated (AAAA → TATA). Binding to E2C was of lower affinity than the E2BS(35,50)−E2C interaction; the calculated K_d for the BS(TATA)−E2C complex was 94 nM. How bases within the center of the palindrome which ostensibly do not interact with E2C (Hegde *et al.*, 1992) become determinants of E2C−DNA binding affinity is unclear. It is noteworthy that the BPV-1 E2C−DNA cocrystal structure reported by Hegde *et al.* reveals a 14° bend at the middle of the E2-binding palindrome. Oligonucleotides with A_4·T_4 and T_4·A_4 cores such as those within Fl-E2BS, E2BS(35,50), and E2BS(7592) are potentially bent when free in solution, or are easily bent upon association with E2C (Moskaluk & Bastia, 1988; Bedrosian & Bastia, 1990; Crothers *et al.*, 1990; Strauss & Maher, 1994). Thus, we hypothesize that A/T richness is not in itself a promoter of E2−DNA association. Rather, the asymmetrical distribution of adenosine and thymidine bases, combined with the "bendability" of A/T-rich sequences, contributes to high-affinity E2C binding. Substitution of the central adenosine and thymidine bases with alternating cytosine and guanosine (AAAA → CGCG) yielded an oligonucleotide designated BS(CGCG) with an E2C-binding affinity of 71 nM, essentially that of BS(TATA)−E2C association. Because these dissociation constants are essentially the same, these data support the notion that A/T richness is important to E2C−DNA association, not because of the specific locations of adenosine and thymidine bases, but because of their effects on the structure and rigidity of the oligonucleotide as a whole.

Knowing the ratios in dissociation constants between the E2BS(35,50)−E2C complex and other E2C−oligonucleotide complexes, the energetic contributions of each oligonucleotide structural modification were assessed. The changes in the Gibbs free energy of binding ($\Delta\Delta G$) relative to E2C−E2BS(35,50) association were calculated using eq 2:

$$\Delta\Delta G = RT\, ln[K_{d(2)}/K_{d(1)}] \qquad (2)$$

where $K_{d(1)}$ is the dissociation constant for the E2C−E2BS(35,50) complex and $K_{d(2)}$ is the dissociation constant for the E2C−modified oligonucleotide complex. These results are summarized in Table 1, and reiterate that although the dominating oligonucleotide structural determinants of the E2C−DNA interaction are the palindromic bases, the non-palindromic core bases are also determinants of the DNA-binding affinity of E2C. The data also emphasize the remarkable specificity of DNA binding by E2C; the dissociation constants of the highest and lowest affinity oligonucleotides differ by almost 2000-fold and suggest that only four of seven potential HPV-11 E2-binding sites bind E2 with high affinity. All of the E2-binding sites near or within the *ORI* have high affinity for E2. In contrast, all of the E2-binding sites distant from the *ORI* have low affinity for DNA. Significant occupancy of the lower affinity sites would require free E2 concentrations in the micromolar range. Thus, the high-affinity E2-binding sites located in or near the *ORI* could be bound with a high degree of occupancy while the low-affinity sites remain essentially unoccupied. Significant occupancy of the lower affinity sites by E2 would require either very high concentrations of free E2 or the addition of another factor which would promote E2−DNA binding. Alternately, clustering of binding sites

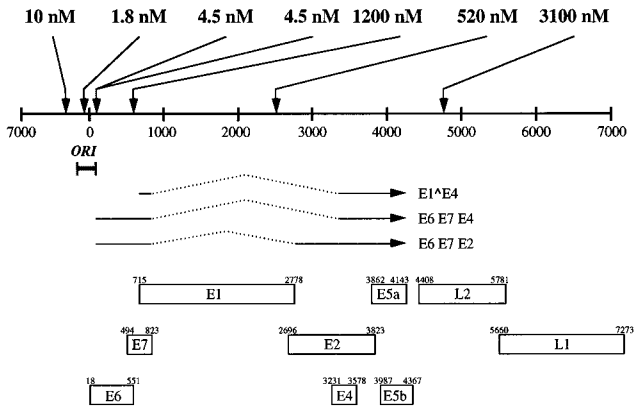

FIGURE 7: Graphic representation of the HPV-11 genome with E2-binding-site affinities and major RNA transcripts. Binding sites from E2C−oligonucleotide titrations (Figure 4) are indicated. Major HPV-11 RNA transcripts are as described by Chow *et al.* (1987). The location of the *ORI* is as proposed by Chiang et al. (1992a,b).

or DNA looping (Knight *et al.*, 1991) could provide a mechanism for utilization of lower affinity E2-binding sites. When the affinities of each E2-binding site are plotted on a map of the HPV-11 genome, it is apparent that the major RNA transcripts are reflective of the relative affinities of each specific E2-binding site (Figure 7). Major transcripts derive from two RNA start sites located near the *ORI* (Chow *et al.*, 1987). These RNA start sites are within the E7 ORF and upstream from the E6 ORF.

It is reasonable to assert that titrations of small oligonucleotides may not provide an accurate measure of E2−DNA affinities in *vivo*. Nonetheless, these data do establish that the potential for a hierarchy of E2-binding sites exists based upon their relative affinities for E2. Furthermore, because these data allow calculation of the change in Gibbs free energy ($\Delta\Delta G$) as a function of oligonucleotide sequence, the absolute dissociation constants are of lesser importance than the relative affinities between sites.

In summary, HPV-11 E2C may be expressed in *E. coli* and purified to homogeneity without the use of affinity chromatography. When the affinity of a single oligonucleotide for E2C was compared using both fluorescence anisotropy measurement and oligonucleotide gel shift, the fluorescence assay used yielded a significantly lower K_d. The affinities of E2C for several single-site E2-binding oligonucleotides were measured using fluorescence anisotropy. Oligonucleotide sequences tested included those of all the putative E2-binding sites with HPV-11, as well as several non-native sequences. Titrations of these oligonucleotides confirmed that the four central base pairs within the E2-binding site palindrome are important determinants in the E2C−oligonucleotide affinity. Substitution of bases within the canonical portions of the E2-binding-site oligonucleotides dramatically decreased E2C−oligonucleotide affinity. In addition, base pairs flanking the 12 palindromic base pairs may also play a minor role in determining E2C−oligonucleotide complex affinity. When the relative affinities of each E2-binding site are plotted on a map of the HPV-11 genome, it is apparent that the major RNA transcripts produced reflect the affinities of each specific E2-binding-site affinity. All of the E2-binding sites near or within the *ORI* have high affinity for E2. E2-binding sites distant from the *ORI* were of such low affinity that their occupancy *in vivo* would be very low without other transcription-related factors impinging upon the E2−DNA interaction.

This paper describes the first use of steady-state fluorescence to study a eucaryotic viral transcription factor. HPV-mediated transcription control is a particularly attractive system for study given the relative simplicity of papillomaviruses and their importance both as commonly transmitted sexual diseases and as clear examples of virus-induced oncogenesis (zur Hausen & de Villiers, 1994). Ultimately, fluorescence anisotropy measurements will be used to construct a quantitative thermodynamic model of HPV transcription and replication control. Fluorescence anisotropy measurements are now being used to study more complicated model systems, including oligonucleotides with multiple E2-binding sites, the interaction of full-length E2 with DNA and with transcription-related factors, and E1−E2−DNA complex formation. Using the data derived from this analysis of the interaction between the isolated DNA-binding domain of E2 with a single E2-binding site, the coordinate regulation of HPV transcription and replication can be systematically dissected.

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