cis-Acting Components of Human Papillomavirus (HPV) DNA Replication: Linker Substitution Analysis of the HPV Type 11 Origin

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Papillomavirus DNA replication requires the viral *trans*-acting factors E1 and E2 in addition to the host cell's general replication machinery. The origins of DNA replication in bovine and human papillomavirus genomes have been localized to a specific part of the upstream regulatory region (URR) which includes recognition sites for E1 and E2 proteins. To fine map *cis*-acting elements influencing human papillomavirus type 11 (HPV-11) DNA replication and to determine the relative contributions of such sites, we engineered consecutive linker substitution mutations across a region of 158 bp in the HPV-11 origin and tested mutant origins for replication function in a cell-based transient replication assay. Our results both confirm and extend the findings of others. E2 binding sites are the major *cis* components of HPV-11 DNA replication, and there is evidence for synergy between these sites. Differential capacity of the three E2 binding sites within the origin to affect replication may be attributed, at least in part, to context. At least one E2 binding site is essential for replication. The imperfect AT-rich palindrome of the E1 helicase binding site is not essential since replication occurs even in the absence of this sequence. However, replication is enhanced by the presence of the palindromic sequence in the HPV-11 origin. Sequence components adjacent to the E1 and E2 binding sites, comprising AT-rich and purine-rich elements and the consensus TATA box sequence, probably contribute to the overall efficiency of replication, though they are nonessential. None of the other *cis* elements of the HPV-11 origin region analyzed seems to influence replication significantly in the system described. The HPV-11 origin of DNA replication therefore differs from those of the other papovaviruses, simian virus 40 and polyomavirus, inasmuch as an intact helicase binding site and adjacent AT-rich components, while influential, are not absolutely essential.

DNA replication commences with the association of specific *trans*-acting factors with a *cis*-acting origin of replication to form the preinitiation complex (16, 25, 29). Local unwinding of the DNA facilitates the activity of a helicase to melt the duplex, assisted by a single-stranded-DNA-binding protein, providing a template for the subsequent initiation of DNA synthesis (5, 6, 10, 12, 24, 38, 66, 73, 77). Although little is known about the organization of replication origins in animal cells, the recent identification by genetic and physical means of a single bidirectional origin of replication upstream of the human β-globin gene provides evidence for the existence of specific, discrete origins of replication in metazoan chromosomes (39). The origins of animal viruses and of the yeast *Saccharomyces cerevisiae* have provided much of the information concerning the structure of eukaryotic DNA replication origins, which generally comprise an essential domain, containing binding sites for origin recognition proteins, essential elements predisposed to or facilitating unwinding or distortion of the duplex, and nonessential auxiliary components, which contribute to the efficiency of replication and often include transcription enhancer *cis* elements (6, 7, 21, 23, 33, 53, 64, 78, 86). Common pathways might regulate the assembly of replication and transcription initiation complexes, since cellular and viral proteins which function in both replication and transcription have been identified (22, 28, 33).

Papillomavirus DNA replication provides a model system for the analysis of eukaryotic replication and the regulation thereof. During the course of infection in proliferating basal epithelial cells and in transformed cell systems, papillomaviruses establish their genomes as low-copy-number, autonomously replicating episomes (42). Papillomavirus DNA replicates during S phase in synchrony with the host cell chromosome (30), and the mechanism of initiation of replication is fundamentally similar to that of eukaryotic chromosomes. In addition to the host cell's general replication machinery, initiation of papillomavirus DNA replication requires the virus-encoded *trans*-acting proteins E1 and E2 (41, 78, 86). Much of the information concerning papillomavirus replication has been derived from the analysis of bovine papillomavirus type 1 (BPV-1) DNA replication in both cell-based (78) and cell-free (86) systems. More recently, cell culture systems (15, 20, 49, 61, 65, 74) and cell-free systems (41, 62) for human papillomavirus (HPV) DNA replication, mainly based on the BPV-1 system, have been described. Since different HPV and BPV E1 and E2 proteins can interact and complement each other in cell culture systems (15, 20, 49, 74, 88), it is reasonable to generalize about the functions of these proteins in papillomavirus DNA replication. The E1 protein is a site-specific DNA-binding protein (47, 49, 70, 76, 79, 82, 86, 88) which can bind and hydrolyze ATP (9, 68, 87), has ATP-dependent helicase activity (68, 87), and is essential for replication (78, 86). E1 also interacts with the cellular polymerase α-primase and perhaps thereby recruits this complex to the viral replication origin (62). The E2 protein, originally identified as a transcription enhancer factor (72), is also able to enhance replication (86, 88) and is essential for papillomavirus DNA replication in

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cells (78). E2 interacts with E1 (4, 50, 58) and greatly stimulates binding of the helicase to the origin of DNA replication (58, 67, 70, 76, 86, 88). E2 can also alleviate nucleosome-mediated repression of papillomavirus DNA replication (45), and in addition, E2 may stimulate replication by binding to and sequestering the host cell single-stranded-DNA-binding replication protein A (84) to the replication origin (44).

Papillomavirus origins, localized to the noncoding region upstream of the early open reading frames (upstream regulatory region [URR]) in the circular duplex DNA genome, overlap with transcription regulatory elements (14, 20, 49, 65, 74, 79, 86). The E1 initiator protein binding site (E1BS) which resides in the origin includes an 18-nucleotide (nt) AT-rich imperfect palindrome (35, 79, 86). Bidirectional unwinding from this region mediated by E1 is required for DNA synthesis (48). An additional AT-rich domain lies adjacent to the E1 palindrome. The origins of papillomaviruses also contain two or more copies of a 12-bp palindromic sequence (15, 20, 49, 65, 74, 78, 80, 86) to which E2 proteins bind as dimers (1, 27, 54, 55). Definition of the minimal sequence requirements for replication has relied primarily upon deletion and fragment mapping analyses (14, 49, 65, 74, 79, 80, 86). A systematic mutational analysis to screen the origin region for *cis*-acting constituents which may contribute quantitatively to replication had not been performed for either BPV or HPV. We present here the results of such an analysis of the *cis*-acting components of the HPV type 11 (HPV-11) origin of replication in a transient replication assay. We chose to perform a linker substitution analysis to delineate the nucleotide composition of the HPV-11 origin region while maintaining the spacing and context of the various *cis* elements of replication. The purpose of this study was twofold: first, to fine map components of the HPV-11 origin and in the process, hopefully, to identify as yet undiscovered *cis* elements within the origin region which influence replication, and second, to determine the relative contributions of the E1 and E2 binding domains to the efficiency of replication.

MATERIALS AND METHODS

Cells and transient replication assay. The transient replication assay used is based on those of Ustav and Stenlund (78) and Chiang et al. (15). Chinese hamster ovary (CHO) cells were chosen for this analysis since they replicate HPV-11 origin-containing DNAs consistently and to detectable levels. The cells were cultured in Dulbecco modified Eagle medium plus 10% fetal calf serum and split 24 h before harvesting for electroporation. HPV-11 origin-containing DNAs (HPV-11 origin sequences in vector backbone BluescriptIIKS+ [Stratagene]; 1 μg of uncut plasmid) were transfected by electroporation into CHO cells together with expression vectors for HPV-11 E1 (2.5 μg) and E2 (0.5 and 1 μg) (linearized) and 10 μg of autoclaved salmon sperm DNA per sample. Harvested cells were resuspended in Dulbecco modified Eagle medium plus 10% fetal calf serum with 5 mM BES buffer (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethane-sulfonic acid, pH 7.2) at 2×10^7 cells per ml; DNA was transfected into 4×10^6 to 5×10^6 cells per sample in 4-mm gap cuvettes (Bio-Rad). The electroporation apparatus (Bio-Rad Gene Pulser) was set to 960 μF, 250 V. Following electroporation, 25% of each sample was plated per 100-mm-diameter dish. Hirt lysis-extracted DNA from cells harvested at various times posttransfection was digested with *Xba*I and with *Dpn*I. Digested DNA was electrophoresed on 0.8% agarose (1× Tris-borate-EDTA) gels and blotted onto nitrocellulose (Schleicher & Schull) (10× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, ph 7] transfer buffer). Probe DNA (linearized pKH110WT; 25 ng) was labelled with [α-^{32}P]dCTP by random priming (Amersham). Hybridizations were carried out at 65°C in 0.25 M sodium phosphate–7% sodium dodecyl sulfate–1 mM EDTA–1% bovine serum albumin with 50 μg of sheared denatured salmon sperm DNA per ml. Washed blots were autoradiographed and analyzed with a PhosphorImager (Molecular Dynamics).

Plasmids and oligonucleotides. A plasmid containing the HPV-11 genome, subcloned at its *Bam*HI site into pGEM1 (Promega), was a generous gift from Louise Chow, University of Alabama. Expression vectors for HPV-11 E1 and E2 were constructed by subcloning E1 and E2 coding sequences from the HPV-11 genomic DNA into the expression vector pCG(ATG⁻) (75), which contains the major immediate-early promoter from human cytomegalovirus and the poly(A)

sequence from rabbit β-globin intron 7. To construct the E1 expression vector pCGHE1, the *Mst*I (nt 811)-to-*Sty*I (nt 2993) fragment of the HPV-11 genome, blunt ended at the *Sty*I site by filling in, was ligated into the *Sma*I site of the pCG(ATG⁻) vector. To construct pCGHE2, the E2 expression vector, the *Xmn*I (nt 2665)-to-*Ssp*I (nt 3974) fragment of HPV-11 was ligated into the *Sma*I site of pCG(ATG⁻).

A 250-nt sequence (nt 7795 to 109) from the URR of HPV-11 containing the wild-type (WT) origin of DNA replication was amplified from the HPV-11 genomic plasmid DNA template (linearized) by using PCR and primers 5′ TTGGATCCCAACTCTTAAAAGCA 3′ (sense) and 5′ TTGAATTCCCATAAT GCCTCG 3′ (antisense). The PCR product was digested with *Bam*HI and *Eco*RI and cloned into these sites in each of the vectors BluescriptIIKS+ and BluescriptIISK+, to produce plasmids pKH110WT and pSKH110WT, respectively.

Consecutive 7- to 10-bp linker substitution mutations were engineered into a 158-bp region containing the HPV-11 origin (Fig. 1) by using the following oligonucleotides in site-directed mutagenesis (SDM) or PCR procedures (for each oligonucleotide, the mutation is underlined [note that these oligonucleotide sequences correspond to the complementary strand of the nucleotide sequence shown in Fig. 1]): 0, 5′ GCAGGTGTGT<u>TCAGGATCCCG</u>TACTCATTTT 3′; 1, 5′ GAAACCGGTT<u>CACTCGAGCA</u>GACCTTAGGT 3′; 2, 5′ GGTAACCG AA<u>GGATCCAG</u>CAGGTGTG 3′; 3, 5′ GGGTGTGGGT<u>CGGATCCA</u>CCG GTTGCA 3′; 4, 5′ GGAAATATGTA<u>CTCGAGCCCG</u>AACCGAAACC 3′; 5, 5′ TAAGAAGGAAT<u>GGATCC</u>GGGTGTGGGT 3′; 6, 5′ TTAAGTATAA<u>CG GATCC</u>ATATGTAGGG 3′; 7, 5′ AGATTGTTAT<u>GCCTCGAGC</u>GAAG-GAAATA 3′; 8, 5′ TTTAAACTAA<u>CTCGAGGCG</u>TAAGTATAAG 3′; 9, 5′ CCTCTTTTT<u>CTCGAGC</u>GATTGTTATT 3′; 10, 5′ GGTCCCTCCT<u>GCCC GGG</u>AAACTAAGAT 3′; 11, 5′ CGTTTTCGGT<u>AGAGATC</u>TTTTTTTAAAC 3′; 12, 5′ CGGTTGAACC<u>TAAGGATCC</u>CCCTCCTATT 3′; 13, 5′ AACCG TTTTC<u>TCAGATCTA</u>GTTTTCGGTC 3′; 14, 5′ TTATATATA<u>GGATCCAGA</u> GGTTGAACC 3′; 15, 5′ TTGGGCTGGT<u>AGGATCCG</u>TACCGTTTTCG 3′; 16, 5′ GCTAATTTTT<u>CTCTAGACG</u>TTTATATATAAC 3′; 17, 5′ CCTCGTCT GC<u>GCTCGAGG</u>TGGGCTGGTT 3′; and 18, 5′ GGCCCGGGAATGCCC<u>TC GAGTG</u>TAAATTTTAGG 3′.

In the two-step PCR procedure, antisense oligonucleotide 1, 6, 10, 13, 15, or 18 and sense oligonucleotide T3 (5′ ATTAACCCTCACTAAAG 3′) were used as primers on linearized template pSKH110WT in the first PCR; gel-purified PCR products from this reaction were then used as sense primers in a second PCR with antisense primer 21 (5′ GCGGAATACTTTCGATAATG 3′) and the HPV-11 genomic DNA plasmid (linearized) as the template. Second-round PCR products containing mutated origin fragments were subsequently digested with *Bam*HI and *Xmn*I and subcloned into the BluescriptIIKS+ backbone between the *Bam*HI and *Eco*RI sites. SDM was carried out with oligonucleotide 0, 2, 3, 4, 5, 7, 8, 9, 11, 12, 14, 16, or 17 and single-stranded template DNA produced from pSKH110WT in *Escherichia coli* CJ236 with M13 helper phage R408 by the method of Kunkel (40). Primary screening for mutant DNAs was by colony hybridization analysis using the appropriate end-labelled oligonucleotides as probes. For mutants made by SDM, origin-containing fragments were subcloned from the BluescriptIISK+ backbone into a BluescriptIIKS+ backbone between the *Bam*HI and *Eco*RI sites. By comparison with WT or mutant origin DNAs made by SDM, mutant origin DNAs made by the PCR method differ by seven nucleotides at the junction between nt 105 of HPV-11 and the vector polylinker. Therefore, an additional HPV-11 origin-containing DNA, WTA, was constructed incorporating these nucleotide changes within the WT origin DNA. A deletion mutant which contained only the HPV-11 URR sequences from nt 7881 to 107, mutant 0Δ, was produced by *Bam*HI digestion of mutant 0 and religation. The double mutant 2+3 was made by replacing the *Bam*HI fragment from the origin region of mutant 3 with that of mutant 2. Use was made of the unique *Bst*EII site at position 7901 of the origin for the construction of double mutants 2+12, 2+13, and 2+14, in which the *Eco*RI-to-*Bst*EII origin fragment of mutant 2 was replaced by the *Bst*EII-to-*Eco*RI origin fragments of mutants 12, 13, and 14, respectively. The double mutant 10+17 was cloned via a two-step PCR procedure in which antisense oligonucleotide 17 and sense oligonucleotide T7 (5′ AATACGACTCACTATAG 3′) were used to amplify the HPV-11 origin region from mutant 10 DNA, and gel-purified PCR products from this reaction were then used as sense primers in a second PCR with antisense primer T3 and mutant 17 DNA as the template; second-round PCR products containing the double-mutated (10+17) origin fragments were subsequently digested with *Bam*HI and *Eco*RI and subcloned into a BluescriptIIKS+ backbone between the *Bam*HI and *Eco*RI sites. The double mutant 23 was made by replacing the *Bam*HI-to-*Xho*I origin fragment of mutant 8 with that of mutant 7. All mutant and WT origin clones (Fig. 1) were checked by both restriction enzyme and dideoxynucleotide sequencing analyses.

RESULTS

Transient replication of HPV-11 DNA. In papillomavirus genomes, the region upstream of the early open reading frames, the URR, contains sequences involved in both replication and transcription regulation. We were interested to define the *cis*-acting components of replication within a 158-bp

HPV-11 ORIGIN

Labels across the figure: E2BS2 (7892–7903), E1BS palindrome (7928–7933|1–12), A6, E2BS3 (35–46), E2BS4 (50–61), A6

Row labels: 7795, 7870, WT, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 2+3, 2+12, 2+13, 2+14, 23, 10+17

Position markers: 7795, 7870, 103
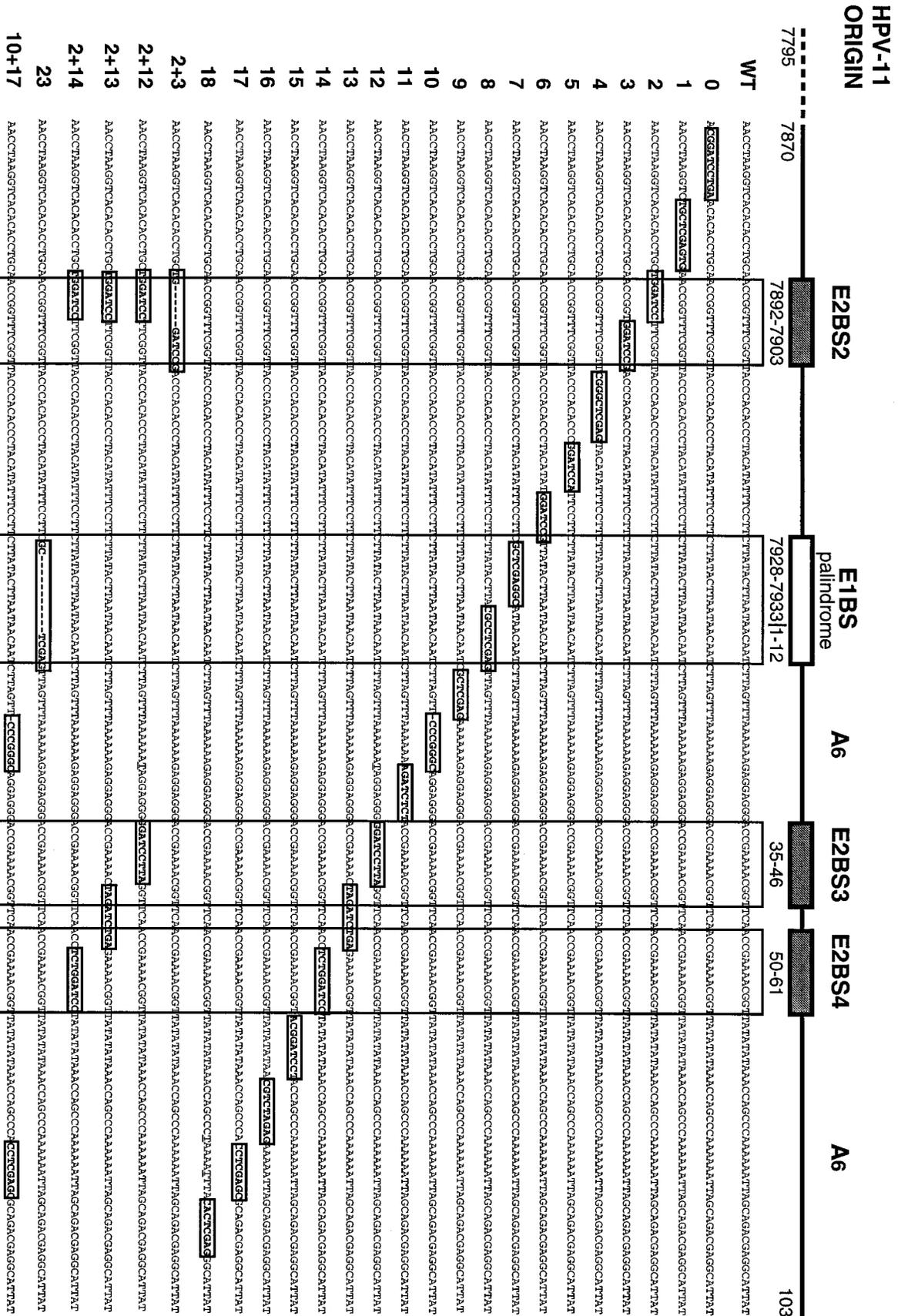
FIG. 1. HPV-11 WT and mutant origin sequences. A portion of the HPV-11 URR (nt 7795 to 109) was subcloned into a vector backbone to produce plasmid WT. Consecutive 7- to 10-bp linker substitution mutations were engineered into the region between nt 7881 and 95. The nucleotide sequence of this region in WT is presented; the positions of the EIBS palindrome, two stretches of six A nucleotides, and E2BS2, -3, and -4 are indicated, as are the positions and sequences of the mutations in origins 0, 1 to 18, 2+3, 2+12, 2+13, 2+14, 23, and 10+17.

FIG. 2. Transient replication of HPV-11 plasmids. Plasmid WT (with HPV-11 URR nt 7795 to 109) was tested for its ability to support DNA replication in a cell-based transient replication assay with E1 and E2 provided in *trans* (see Materials and Methods). The titration data and inset graph (replication is given in arbitrary units) show the effects of different concentrations of the E1 and E2 expression vectors upon replication of WT DNA. In control lanes, WT was tested for replication in the absence of E1 and E2 and in the presence of E1 or E2 alone. An originless plasmid (KS) and plasmids WTA (with HPV-11 URR nt 7795 to 109 plus PCR-incorporated changes) and WT0Δ (with HPV-11 URR nt 7881 to 109) were also tested for the ability to replicate when provided with E1 and E2 in *trans*. The tim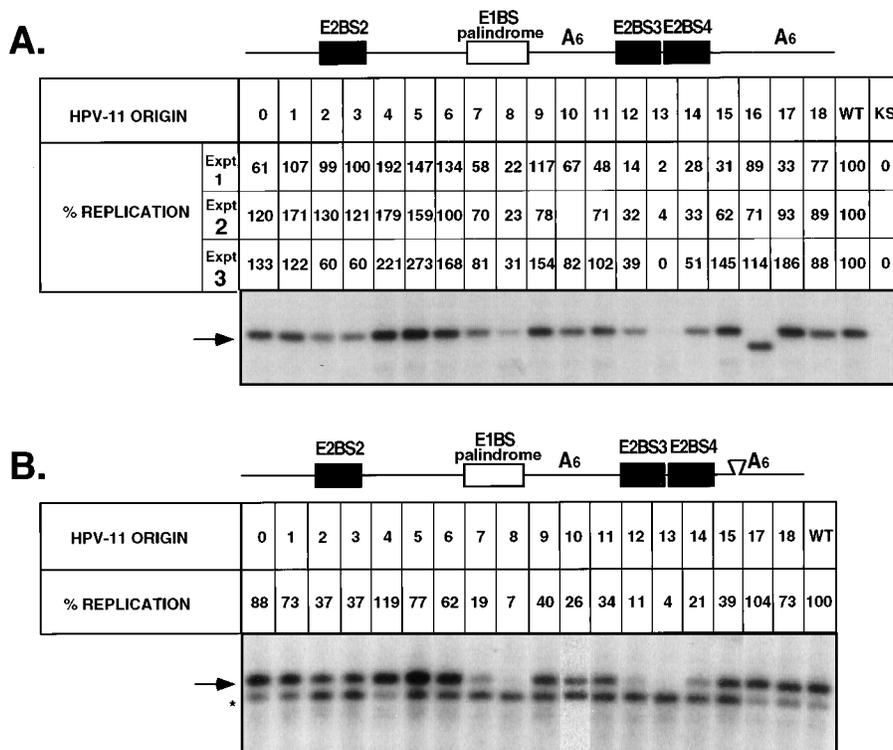e course for WT DNA replication shows 12-, 24-, 48-, and 72-h time points; all other samples were analyzed 24 h posttransfection. Arrows depict bands which represent replicated HPV-11 DNAs; the marker contains 50 pg of linearized WT DNA.

stretch (nt 7871 to 7933 and 1 to 95) of the HPV-11 URR which can support DNA replication in a transient replication system (see below). Some of the notable domains within this nucleotide sequence are indicated in Fig. 1. Three of the four E2 binding sites, E2BS2, -3, and -4, in the HPV-11 URR are situated within the region of interest. The E1BS, which lies between E2BS2 and E2BS3, includes an 18-bp imperfect palindrome rich in A and T nucleotides (9, 47, 49). Other sequences of particular note include a purine-rich tract (nt 27 to 34), a consensus TATA box (nt 66 to 71) which abuts E2BS4, and two A_6 motifs, one between the E1BS palindrome and E2BS3 and the other between the TATA box and the translational start site of E6 at nt 104.

HPV-11 URR nt 7795 to 109 were subcloned into the BluescriptIIKS$^+$ vector to create plasmid WT. Transient replication assays in which this HPV-11 origin-containing plasmid DNA was cotransfected into mammalian cells together with expression vectors for HPV-11 E1 and E2 were performed. Cells were generally harvested 1 and 2 days posttransfection, and episomal DNA isolated from these cells was digested with *Xba*I, to linearize the products of replication, and with *Dpn*I, to digest unreplicated input DNA (methylated) while leaving intact the replicated DNA. The DNA was then subjected to electrophoresis, blotting, and hybridization to a radioactively labelled DNA probe (WT) which recognizes HPV-11 origin-containing DNA.

WT DNA replicates in the transient assay system when HPV-11 E1 and E2 proteins are provided in *trans*, as shown in Fig. 2. An originless vector backbone (KS) was unable to replicate significantly in the presence of E1 and E2 expression vectors in the transient replication assay (<3% of WT levels; Fig. 2). Sufficient *cis* information to direct transient replication is contained within 162 bp of the WT HPV-11 URR, since WT0Δ (HPV-11 nt 7881 to 109 in BluescriptIIKS$^+$) attains at least half the WT level of replication (Fig. 2).

Both E1 and E2 viral proteins are essential for replication from the HPV-11 origin in this system, since no replication above background levels is detectable from the WT HPV-11 origin either in the absence of E1 and E2 expression vectors or in the presence of E1 or E2 alone (Fig. 2), consistent with results previously obtained for BPV-1 (78) and HPV (15, 20,

49, 65, 74). Moreover, the extent of replication from HPV-11 origin sequences correlates with the amount of E1 and E2 expression vectors cotransfected (Fig. 2; titration). Replication products, detectable as early as 12 h posttransfection, accumulate in increasing amounts until around 48 h, reaching a plateau between 48 and 72 h posttransfection (Fig. 2, time course). In the following transient replication experiments, WT or mutant origin-containing DNAs (1 μg) were cotransfected with nonsaturating concentrations of E1 (2.5 μg) and E2 (0.5 or 1 μg) expression vectors, and replication was assessed 24 h posttransfection. We analyzed replication early to determine intrinsic replication rate differences and avoid exaggerating small differences in mutant, compared with WT, replication abilities.

WTA DNA is identical to WT DNA with the exception of seven nucleotide differences, at the border between HPV-11 nt 105 and the vector sequences, which are also a feature of the HPV-11 mutant origins created by PCR for analysis in this replication assay. The extents of replication of WT and WTA DNAs in the assay (Fig. 2) demonstrate that the sequence differences engineered by PCR at this junction themselves have no significant effect on replication efficiency.

Linker substitution analysis and the identification of the viral E1 and E2 binding sites as the major *cis* components of replication in the HPV-11 origin. To dissect the *cis*-acting components of the HPV-11 origin, consecutive 7- to 10-bp linker substitution mutations were engineered across a 158-bp stretch of the HPV-11 URR (nt 7871 to 95) which contains the sequence elements essential for replication (Fig. 1), and these mutants were tested for origin function in the transient replication assay system under the conditions described above (Fig. 3).

We opted for linker substitution mutational analysis, rather than deletion analysis, for fine mapping of *cis* elements of the origin region, in order to conserve the spacing between sequence elements and avoid possible variation caused by introduction of foreign DNA near the origin sequences. Linker substitution mutations were designed so that, as far as possible, all purines of the mutated interval were converted to pyrimidines and vice versa. The only limitation to this was the incorporation of restriction enzyme sites into the mutated regions

A.

HPV-11 ORIGIN		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	WT	KS
% REPLICATION	Expt 1	61	107	99	100	192	147	134	58	22	117	67	48	14	2	28	31	89	33	77	100	0
	Expt 2	120	171	130	121	179	159	100	70	23	78		71	32	4	33	62	71	93	89	100	
	Expt 3	133	122	60	60	221	273	168	81	31	154	82	102	39	0	51	145	114	186	88	100	0

B.

HPV-11 ORIGIN	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	WT
% REPLICATION	88	73	37	37	119	77	62	19	7	40	26	34	11	4	21	39	104	73	100

FIG. 3. In vivo transient replication of consecutive linker substitution mutants of the HPV-11 URR (nt 7871 to 95). Consecutive linker substitution mutant DNAs (1 μg) 0 and 1 to 18 were provided with E1 (2.5 μg) and E2 (0.5 μg [panel B and experiment 2 in panel A] or 1 μg [experiments 1 and 3 in panel A]) in *trans* and assayed for origin function in a transient replication system in the absence (A) and in the presence (B) of 0.5 μg of internal control DNA (mutant origin 16). Arrows indicate bands representing replicated WT or mutant origin DNAs. The asterisk indicates bands representing replicated control DNA. The schematic indicates relative positions of the linker substitution mutations with respect to E1 and E2 binding sites and A$_6$ motifs. (A) Numerical data from three independent transient replication assays, expressed as the percentage replication of each mutant compared with the WT origin (100%). The autoradiograph corresponds to experiment 3. (B) Replication levels were standardized to that of the internal control DNA. The autoradiograph shows one representative replication assay, with the standardized replication for each mutant compared with that of WT (100%).

for the purpose of screening to select for mutant origins and also for the potential to recombine mutant DNAs and produce double or multiple mutants. Mutants 0 and 1 to 18 contain linker substitution mutations incorporated, by PCR or SDM, into HPV-11 origin sequences from nt 7795 to 109 and subcloned into a vector backbone (BluescriptIIKS$^+$) (Fig. 1).

In the absence of E1 and E2 expression vectors, no signal was detectable with any of these DNAs above the background levels of an originless vector DNA (KS) in the cell culture system (data not shown). Figure 3A shows one representative autoradiograph and the numerical data from three separate replication assays (performed with two different plasmid preparations for each mutant and WT origin DNA) in which each mutant, WT, or KS DNA was cotransfected into cells with expression vectors for E1 and E2. The level of replication for each mutant origin or KS DNA is expressed as a percentage of the WT level of replication (100%). Samples are referred to in the text in the format Fig. *X*, *Y*, where *X* is the figure number and *Y* both denotes the lane and corresponds to the name of the mutant, WT or KS DNA.

With E1 and E2 provided in *trans*, almost all of the mutant origins supported replication, indicating that the majority of sequence elements within the origin region, taken individually, are nonessential for replication. Specifically, the only mutations which produced significant and reproducible negative effects on the efficiency of replication compared to WT were those affecting E1 and E2 binding sites (Fig. 3A, 8, 12, 13, and

14). Mutation of the region between E2BS2 and the E1BS, which contains at least two potential transcription factor binding motifs, consistently produced a small (approximately 1.5- to 2.5-fold) increase in replication efficiency compared with WT (Fig. 3A, 4 and 5). These results are summarized in Fig. 5A.

Identification of additional potential *cis*-acting elements of HPV-11 replication. The linker scan analysis was also carried out such that, in addition to the expression vectors and the test origins, an internal control DNA, mutant 16 (Fig. 1), was cotransfected into cells for more accurate quantitation (Fig. 3B). Furthermore, since this origin-containing control plasmid also served as a potential competitor for limiting factors required for replication, it could thereby accentuate or reveal the importance of a *cis* element missed by the assay described above. This particular origin-containing DNA, mutant 16, was chosen as the control for two reasons; it replicates to the same extent as WT DNA, and its replication can be distinguished from replicated WT or test origin DNAs in the transient assay because it exhibits a relatively increased electrophoretic mobility due to the presence of an extra *Xba*I site engineered in the mutated sequence (Fig. 3A, 16). Note that to improve resolution of the replicated WT or mutant and internal control DNAs and for quantitation purposes, the control DNA was transfected at half the concentration of the WT or mutant plasmids; the difference in replication levels of WT compared with control DNA reflects this difference in input plasmid concentrations (Fig. 3B, WT).
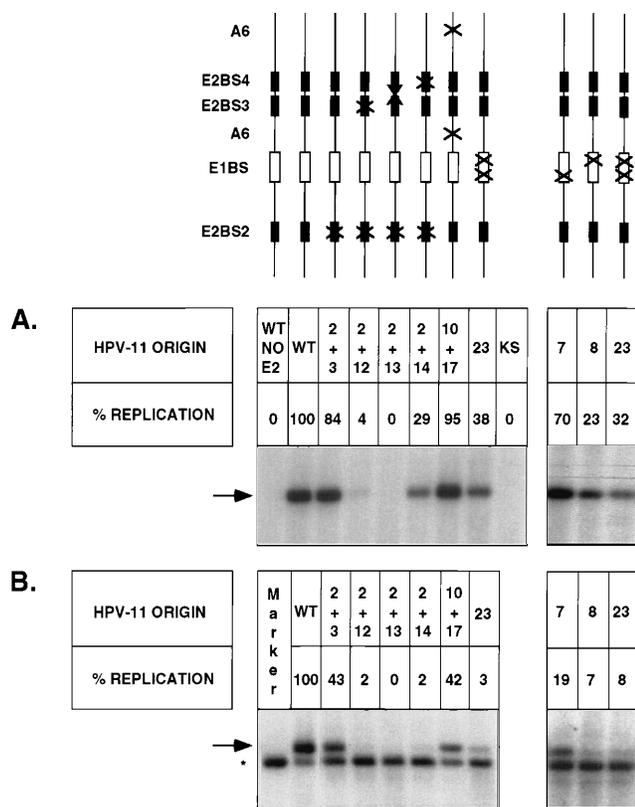
A.

HPV-11 ORIGIN	WT NO E2	WT	2+3	2+12	2+13	2+14	10+17	23	KS	7	8	23
% REPLICATION	0	100	84	4	0	29	95	38	0	70	23	32

B.

HPV-11 ORIGIN	Marker	WT	2+3	2+12	2+13	2+14	10+17	23	7	8	23
% REPLICATION		100	43	2	0	2	42	3	19	7	8

FIG. 4. Replication competence of double mutants 2+3, 2+12, 2+13, 2+14, 10+17, and 23 (1 μg of each) in the absence (A) and presence (B) of internal control plasmid 16 (0.5 μg) in the transient replication system, with E1 (2.5 μg) and E2 (1 or 0.5 [A and B, samples 7, 8, and 23] μg) provided in *trans* (see Materials and Methods). Arrows depict bands which represent replicated WT or mutant origin DNAs. The asterisk indicates bands representing replicated control DNA. The percentage replication of each mutant compared with the WT origin (100%) was calculated, standardized to replication of the internal control DNA where applicable (B). Relative positions of these mutations with respect to E1 and E2 binding sites and A6 motifs are indicated.

Considerable effects upon replication were observed with mutation of either E2BS3 (down 10-fold; Fig. 3B, 12) or E2BS4 (down 5-fold; Fig. 3B, 14). In addition, in the presence of the competitor DNA, mutations affecting E2BS2 now demonstrated a threefold drop in replication (Fig. 3B, 2 and 3). Note that mutation of either half of the E2BS2 palindrome (Fig. 3B, 2 and 3) reduces replication to the same extent as mutation of the whole E2BS2 (Fig. 4B, 2+3), as predicted in view of observations that a half-site is insufficient for E2 binding (14, 45a). Mutation of E2BS3 and E2BS4 together reduced replication to virtually background levels (down 25-fold; Fig. 3B, 13). Apparently, the loss of these two E2BSs cannot be compensated for by E2BS2 or by the consensus E2BS which is present within the vector backbone (BluescriptIIKS⁺, nt 1734 to 1745).

In addition, the presence of the competitor DNA further curtailed the replication ability of E1BS mutants. Replication achieved by mutant origin 8, in which nt 5 to 12 of the E1BS palindrome are altered, was 14-fold lower than that of WT (Fig. 3B, 8). Mutant origin 7, affecting the E1BS palindrome from nt 7929 to 7933 and 1 to 4, which in the absence of the competitor DNA had replicated to 60 to 80% of WT levels (Fig. 3A, 7), now demonstrated a fivefold-lower level of replication than WT (Fig. 3B, 7).

The region between the E1BS palindrome and E2BS3 contains an AT-rich domain, including an A_6 motif, as well as a purine-rich stretch. A significant reduction (approximately threefold) was observed in the replication of DNAs with mutations affecting these sequence elements (Fig. 3B, 9, 10, and 11), suggesting a level of participation in replication. Mutation of the region between E2BS2 and the E1BS palindrome no longer showed the slight increase in replication efficiency seen in the absence of the competitor DNA (Fig. 3B, 4 and 5), perhaps due to dilution of interfering factors. Mutation of the consensus TATA box also had a negative effect on replication (Fig. 3B, 15).

Competition between the mutant and control origins for E1, E2, and/or perhaps other *trans*-acting replication factors probably accounts for the exaggerated effect of the E1 and E2 binding site mutations and has allowed us to identify additional domains in the origin region which, although not essential in this system, have a potential role in replication. These results are summarized in Fig. 5B.

The relative contributions of *cis* elements to HPV-11 replication. We wanted to assess in more detail the contributions to replication efficiency of some of the regions identified as *cis* components of HPV-11 replication, such as the E1 and E2 binding sites.

Mutation of each part of the E1BS palindrome separately has a different effect, nt 5 to 12 apparently being more important for replication than nt 7929 to 7933 and 1 to 4 (Fig. 3 and 4, 8 and 7). In support of this conclusion, a mutation which alters the entire E1BS palindrome (Fig. 4, 23) produces the same effect upon replication as mutation of nts 5 to 13 alone (Fig. 4, 8). Interestingly, although replication is significantly diminished, it is not abolished by mutation of the binding site for the initiator protein E1. In an in vitro system for BPV-1, replication from origins which lack an E1BS can be achieved at elevated concentrations of E1 protein (87). In the transient replication assay described here, the level of E1 might be higher than that which occurs during papillomavirus infection. However, being somewhat close to the lower limits of detection of replicated DNA in this system, it has proven problematic to accurately assess the relative replication ability of origins with mutant E1BSs at reduced levels of E1 protein (data not shown).

Removal of the binding sites for transcription/replication factor E2 from the HPV-11 origin precludes replication (Fig. 4, 2+13). Sequence and relative position are variables which may account for the differential effects of the E2BSs on replication. Two of the E2BSs in the HPV-11 origin region are identical in sequence (ACCGAAAACGGT) and the other, E2BS2, differs by one nucleotide (ACCGAAACCGGT). Mutation of E2BS3 and E2BS4 separately results in similar reductions in the level of replication (Fig. 3, 12 and 14), whereas mutation of E2BS2 appears to have less effect (Fig. 3, 2 and 3; Fig. 4, 2+3), implying the relative importance of E2BS3 and E2BS4 over E2BS2. From this analysis, we are unable to distinguish whether this could be accounted for by nucleotide sequence, which could affect E2 binding affinity (45a), or relative position with respect to other *cis* elements. It seems that E2BS2 and E2BS4 are unable to function alone in replication, since close to background levels of replication are produced upon mutation of both E2BS3 and E2BS4 (Fig. 3, 13) or E2BS2 and E2BS3 (Fig. 4, 2+12). However, E2BS3 still functions to a limited extent in the absence of the other two E2BSs, implying a more crucial role for E2BS3 in replication (Fig. 4A, 2+14). It is possible that proximity of E2BS3 to the most critical domain of the E1 binding motif, or to the AT- and purine-rich elements of the intervening region, may be the determining
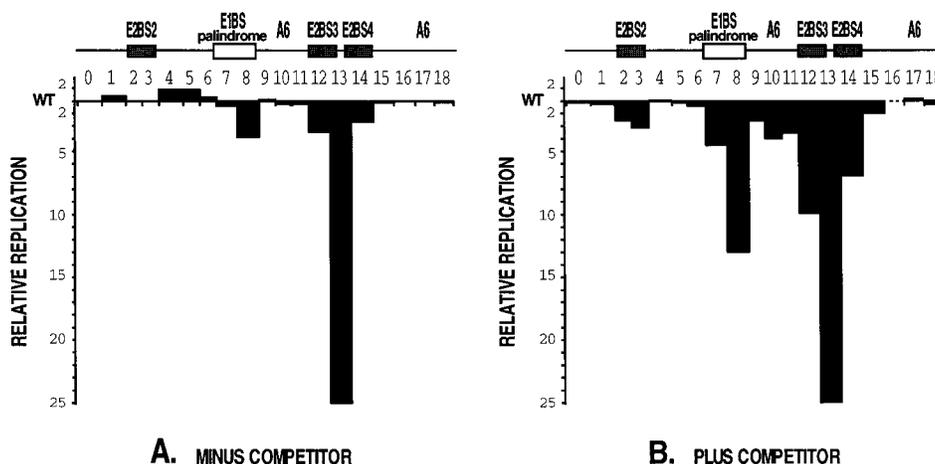
FIG. 5. Summary of transient replication assay data for consecutive linker substitution mutants of the HPV-11 origin in the absence (A) or presence (B) of the competitor origin. The data (averaged from at least two [B] or three [A] assays) are plotted as the fold level of replication of each mutant above or below the WT level.

factor which allows it to function even when it is the sole E2BS present.

AT-rich nucleotide sequences, including a poly(A) stretch, are essential components of the DNA replication origins of the papovaviruses simian virus 40 (SV40) and polyomavirus (18, 51, 60). Two AT-rich tracts which include A_6 nucleotide elements are located within the HPV-11 replication origin. Mutation of the A_6 sequence which lies most distal to the putative E1BS (nt 79 to 84) has no significant reproducible effect on the efficiency of replication either in the absence or in the presence of competitor DNA (Fig. 3, 17). The other A_6 motif, between E1BS and E2BS3 (nt 21 to 26), was substituted by G and C residues in mutant 10. Although, this mutant achieves almost WT levels of replication in the absence of the internal control (Fig. 3A, 10), competition with the control origin results in a threefold reduction in replication compared with WT (Fig. 3B, 10). Therefore, this A_6 motif might contribute to optimal replication but is nonessential in this system. To investigate the possibility that the A_6 domains are functionally redundant, we constructed mutant 10+17, in which both A_6 regions (nt 21 to 26 and 79 to 84) were substituted by GC-rich linkers. This mutant behaved in a similar manner to mutant 10 both in the absence and in the presence of competitor DNA, suggesting that there is no functional redundancy between these A_6 domains (compare 10+17 in Fig. 4 with 10 in Fig. 3).

DISCUSSION

Transient replication of DNA containing specific sequences from the HPV URR has been achieved in cell systems with HPV E1 and E2 proteins provided in *trans* (15, 20, 49, 65, 74). A fairly extensive linker scan analysis has enabled us to define the *cis*-acting components of HPV-11 DNA replication in such a transient replication system. The E2 transcription/replication factor binding sites are the major *cis*-acting components of the origin, and there is cooperativity between sites. At least one E2BS is essential for HPV-11 DNA replication, while the context of such sites may dictate a requirement for more than one. Our findings both correlate with and extend related findings of others which indicate that HPV E2BSs have a pivotal role in replication (14, 49, 65). For HPV-11 DNA replication, Chiang and coworkers (14) established the critical role of E2BSs at the origin, and it was demonstrated by Lu et al. (49) that E2BSs are indeed the only components from the HPV-11 origin re-

quired for replication. It was, however, clear from several studies that the region between E2BS2 and E2BS3 has a positive influence on HPV replication (49, 65, 74). In our study, this region of the HPV-11 origin was dissected, and we have defined the relative contributions to replication of the particular elements and subelements within. In brief, the imperfect palindrome within the E1BS is important, but not essential, for replication. The AT-rich domain and purine-rich tract, located between the E1BS palindrome and E2BS3, and the TATA consensus sequence, though nonessential, may act to increase the efficiency of replication. None of the other sequence elements within the 158-bp region analyzed appears to be essential for, or contribute significantly to, replication. *cis* motifs outside the 158-bp region which influence HPV-11 DNA replication remain to be identified; deletion of the adjacent sequences in the URR, nt 7795 to 7881, diminishes replication no more than twofold.

The site for E1 assembly and the E2 replication/transcription factor binding sites. The BPV-1 E1 helicase binds at a specific site in the BPV-1 origin of replication including an AT-rich 18-bp imperfect palindrome, partially conserved between papillomaviruses (35, 79, 86, 88). The equivalent site within the HPV origin was identified on the basis of both homology to the BPV-1 E1 binding motif (79, 86, 88) and its ability to interact with the HPV E1 protein in vitro, using DNA-protein immunoprecipitation analyses (9, 49) and DNase protection assays (47). The importance of the HPV-11 E1BS is demonstrated by the finding that mutation of the palindromic sequence results in a significant decline in the ability of the origin to support DNA replication. The most divergent half of the HPV-11 E1BS palindrome contributes the least to replication, since in the absence of this half of the palindrome, the more conserved half can support an almost WT level of replication. Relative position as well as nucleotide sequence might be a factor, and we note that the more conserved half of the palindrome is proximal to the most influential E2BS and the intervening AT-rich and purine-rich sequences which also provide a selective advantage for replication.

Despite the indispensable role of the E1 helicase itself in replication, an intact HPV-11 E1BS is not an essential *cis*-acting component of replication in this system. This finding correlates with observations in both BPV (56, 70) and HPV (49, 74), in which certain mutations of the E1BS imperfect

palindrome do not abolish replication. The promiscuity of E1 in replication has also been demonstrated in a cell-free system in which origin-independent replication occurs at high concentrations of BPV-1 E1 protein and in the ability of BPV-1 E1 protein to initiate unwinding in an origin-independent manner (87). There is considerable evidence that E1 and E2 interact to form a complex on DNA and that in so doing, E2 can increase the affinity of E1 binding to its recognition site (4, 50, 58, 67, 86, 88). BPV-1 E1 mutant proteins defective for origin binding can bind DNA specifically in the presence of E2 and can support replication from the BPV-1 origin in a cell culture system (76). Furthermore, mutated BPV-1 origins with dramatically reduced capacity for E1 binding can nevertheless bind E1 in the presence of E2 (70). The implication is that the E1 helicase can be directed to DNA via interaction with the E2 protein bound at its recognition sites. Given that the E1 helicase protein must bind DNA to catalyze unwinding, it will be interesting to determine exactly where the HPV-11 E1 protein binds a DNA template with a mutated E1BS in order to initiate replication. It is conceivable that E1 binds to sequences in the origin or plasmid which resemble the consensus E1BS, perhaps interacting with E2 over a distance. Certainly it has been shown that BPV-1 E2BSs can function in replication when placed at a distance from the E1BS, particularly if the E2BSs possess high affinity for E2 protein or are present in multiple copies (80). Alternatively, E1 may bind the DNA in a nonspecific manner, guided by its interaction with E2. It is likely that for HPV-11, as for BPV-1 (70, 86), tethering of E1 or the E1-E2 complex to the origin is necessary but not sufficient for replication and therefore other *cis*-acting determinants, such as the AT domains and purine tracts mentioned, may allow for DNA conformation and unwinding suitability for initiation.

The absolute requirement for E2BSs in the transient replication system for HPV-11 may be partly a consequence of the rather weak interaction of HPV-11 E1 with its putative DNA binding site in the absence of E2 (9, 49), which might also account for the apparent dispensability of the palindromic core of the E1BS in replication. Transient replication of BPV-1 in cells seems to require some specific E2 contacts but not an intact E2BS (80), possibly reflecting a relatively greater affinity of the BPV-1 E1 protein for its binding site compared with that of HPV-11 E1.

In summary, it appears that specific binding of the E2 enhancer protein at the origin and E2-E1 complex formation are more crucial in replication initiation events than interaction of the E1 helicase with its binding site at the HPV-11 origin.

Intervening and flanking sequences. Apart from the consensus TATA box, none of the elements in the flanking regions (nt 7871 to 7891 and 62 to 109), including an A_6 motif (nt 79 to 84) and a partially conserved GT-1 box (26) (nt 7881 to 7888), appear to contribute significantly to maximal replication in the system described. Likewise, sequences between E2BS2 and the E1BS (nt 7904 to 7927), including a GT-1 box (nt 7907 to 7914) (26) and an AT-rich stretch (nt 7915 to 7927), seem to have little or no effect on replication.

The sequence between the E1BS and E2BS3 in the HPV-11 origin comprises an AT domain (nt 13 to 26) and a polypurine tract (nt 27 to 34), both of which contribute to optimal replication yet appear to be nonessential in this system.

An AT domain is a common feature of both prokaryotic and eukaryotic replication origins (8, 21, 66). In SV40 and polyomavirus origins, it has been identified as a site for DNA bending (18), is required for extension of the primary replication bubble (63), and may facilitate unwinding by enhancing the helicase activity of large T antigen (6, 63, 83). In view of the essentiality of the AT domain in initiation of replication from

the SV40 and polyomavirus origins (point mutations are enough to abolish replication from these origins) (18, 51, 60), the modest effect upon replication of mutation at the A_6 motif proximal to the E1BS in the HPV-11 origin was not anticipated. We considered the possibility that this A_6 stretch was functionally redundant because of the presence of another A_6 motif close to E2BS4. While this did not appear to be so (Fig. 4), it is conceivable that other AT domains, such as those actually within the E1 or E2 binding sites or those of intervening regions, perhaps even the TATA box (nt 66 to 71), can functionally substitute for the A_6 motif. In some replication origins, such as the adenovirus origin, an AT-rich sequence is part of the DNA binding site for proteins involved in initiation of replication (21). If there is redundancy, AT content rather than specific nucleotide sequence would determine function, in contrast to the specific sequence requirement of the AT-rich domain of SV40 (18). Maybe the interaction between E1, E2, and other replication factors at the origin can somehow circumvent the requirement for a specific sequence to perform equivalent functions in papillomavirus replication.

Intriguingly, the polypurine tract (nt 27 to 34) adjacent to the AT domain (nt 13 to 26) demonstrates some homology to purine-rich elements located adjacent to AT stretches upstream of the P1 transcription start site of the human c-*myc* gene, near the center of a reported zone of initiation of DNA replication. This sequence motif is a major site of DNA bending (3) and a common element of other eukaryotic origins, including that of the *dhfr* locus (11) and the recently identified origin upstream of the β-globin gene (39), in which it usually occurs as a repeated element. A 28-kDa single-stranded-DNA-binding protein from HeLa cell extracts shows affinity for the purine-rich elements of these cellular origins (3), while two cellular single-strand-specific DNA-binding proteins, of 42 and 39 kDa, interact with sequences derived from an equivalent purine-rich element previously identified in the BPV-1 origin (32). The importance of these proteins in replication has yet to be determined.

The polypurine tract also contains a nonconsensus SP1 transcription factor binding site (26). The ability of cellular transcription factors to stimulate viral DNA replication is well documented (22, 33). Therefore, it is feasible that the host transcription factor SP1 participates in HPV-11 replication, potentially by sequestering replication factors to the origin region or disrupting nucleosomes. It is known that E2 and SP1 interact directly to mediate synergistic activation of transcription from BPV-1 promoters (46, 71), and a similar role for SP1 in replication could be envisaged. However, Dong et al. (26) have recently shown that repression of transcription from the HPV-11 E6 promoter, exerted by E2 proteins at E2BS3, is due to preclusion of binding of SP-1 or SP-1-like proteins to the aforementioned nonconsensus SP1 binding site, which may imply that E2 and SP1 cannot occupy these adjacent binding sites simultaneously.

Perspective. The SV40 replicon was the first eukaryotic replication system which relies upon the host DNA polymerase to be dissected in detail (12, 73). Lessons from this papovavirus seem to be intricately conserved in the description of the polyomavirus replicon. In these systems, the protein that targets the specific locus for replication initiation contains both the activity that opens up the template (melting) and the helicase activity (5, 81). The SV40 origin of bidirectional replication contains three essential functional domains, precisely positioned in the minimal origin sequences, including a recognition site for this initiator protein (T antigen), a site where T antigen induces localized DNA melting, and an AT-rich region involved in DNA distortion and facilitating unwinding (63). The

sequence requirements for these elements in SV40 and poly-omavirus origin functions are very inflexible (17–19, 51, 60). Transcription factor binding motifs serve as auxiliary flexible components of replication (13, 31, 43).

The HPV-11 origin of replication provides a distinct solution to the way in which a site-specific initiation mediated by cellular replication enzymes can be achieved, inasmuch as an intact binding site for the helicase protein (E1) and AT-rich stretches are nonessential, and transcription factor (E2) binding sites are essential, rather than auxiliary, components of replication. Transcription factor E2 probably provides a major targeting function for the helicase activity of E1 and therefore may be designated an origin recognition protein in HPV-11. In this particular aspect, organization of the HPV-11 origin is reminiscent of that of the Epstein-Barr virus plasmid origin, since the EBNA-1 transcription protein bound to its recognition site presumably targets the cellular helicase to this origin (57). Similarly, in the adenovirus origin, the cellular transcription factor NFI facilitates binding of the preinitiation complex (59). The essential *cis*-acting components of the HPV-11 origin appear to be redundant in that multiple E2BSs are present. Furthermore, although other *cis* elements contribute to maximal replication, additional essential components of replication either do not exist or are functionally redundant within the HPV-11 origin. Therefore, the HPV-11 replicon does not have as stringent a sequence requirement as the more classical paradigm for papovaviruses.

Although there is a precedence for origin sequence-independent replication of nonviral DNA in a variety of cellular systems (34, 36, 52, 69), which may reflect the ability of helicases to bind nonspecifically to DNA and sequester other replication factors to that site, the weight of evidence suggests that specific sequences direct cellular chromosomal initiation of DNA replication (21, 39). For the well-defined autonomous replication sequence elements of *S. cerevisiae* (53), it is believed that targeting of the melting and helicase functions which initiate replication is achieved by a dedicated complex of polypeptides (origin recognition complex) which do not themselves appear to have ATPase and helicase activities (2). The apparent diversity in the way in which known origins can specifically recruit replication initiation proteins to a site makes it hard to predict the organization of metazoan origins of DNA replication.

ACKNOWLEDGMENTS

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