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The E2 binding sites determine the efficiency of replication for the origin of human papillomavirus type 18

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ABSTRACT

Human papillomaviruses (HPV-s) have been shown to possess transforming and immortalizing activity for many different, mainly keratinocyte cell lines and they have been detected in 90% of anogenital cancer tissues, which suggests a causative role in the induction of anogenital and other tumours. We have exploited a quantitative assay to identify and characterize the origin of replication of the human papillomavirus type 18 (HPV-18), one of the most prevalent types in the high-risk HPV group. Replication of HPV origin fragments was studied transiently by cotransfection with a protein expression vector providing replication proteins E1 and E2. We have localized the HPV-18 origin to nucleotides 7767 – 119. This region contains three E2 binding sites and an essential A/T rich DNA region (nucleotides 9 – 35) that is partly homologous to the E1 binding site found in bovine papillomavirus type 1 (BPV-1) genome. At least one of the three E2 binding sites was absolutely required for origin function; addition of other E2 sites had cooperative stimulating effect. This is the first quantitative analysis of the E2 binding sites for papillomavirus replication.

INTRODUCTION

During recent years, eukaryotic replication mechanisms have become objects of extensive studies. Replication origins or autonomously replicating sequences from different eukaryotic viruses (1,2), budding yeast (3), fission yeast (4), and from chromosomes of higher eukaryotes (5–9) have been characterized extensively. Although the majority of information about replication origins and mechanisms of initiation has been obtained using simian virus 40 (SV40) as a model system (see 2 for review), the data obtained do not appear to be directly transferable to other eukaryotic systems. Requirements for other replication origins seem to differ widely from that described for SV40.

Papillomaviruses, particularly prototype BPV-1, provide a useful model to study DNA replication. Their structure is relatively simple and the viral DNA undergoes three different stages of replication, thus offering some unique aspects for investigating the regulation of replication.

The knowledge and understanding of *cis*-acting elements and *trans*-acting factors required for BPV-1 replication has largely changed over a period of time and still remains controversial (see ref. 10 for a review). The latest *in vivo* and *in vitro* data suggest that proteins encoded by both E1 and E2 open reading frames (ORFs) are involved in replication (11,12). The viral E1 protein is considered to be the main initiator protein, because it shows high homology with the SV40 large T-antigen (13) and exhibits specific DNA-binding (14,15) and ATP-binding activity (16). Alternatively, the viral E2 protein is a well-characterized regulator of transcription (17–20). One group has demonstrated that the E2 protein is absolutely required for *in vivo* replication of BPV-1 (12); another group (11) has demonstrated that the *in vitro* replication can occur without E2 protein, but addition of E2 dramatically increases the replication efficiency. Earlier works have shown that mutations in the E2 protein can affect copy number and the plasmid maintenance of the virus (21–25). E2 protein can form a specific complex with E1 protein (26,27). These data suggest that the role of the E2 protein is regulating and that the regulation may occur by complex formation with the E1 protein. The origin of BPV-1 has been mapped to the short DNA fragment around nucleotide number 1 (11,14). This region contains an E1 binding site but presence of E2 binding sites were dispensable for replication (14). Some data about HPV replication has appeared from recent experiments of Chiang et al.(28). They have shown that many human and animal papillomavirus long control regions (LCR) can render prokaryotic plasmid replication competent in eukaryotic cells if BPV-1 or HPV-11 E1 and E2 proteins are provided. No precise mapping results from human papillomaviruses have been published so far. Considering the great importance of HPVs (particularly HPV-16 and HPV-18) as causative agents of genital malignancies (29), we performed a series of experiments to evaluate DNA sequences necessary for the function of the HPV-18 origin of replication in a transient assay. We appreciate that the term 'origin' is not perfectly correct for describing these DNA sequences (an origin is considered to be the site of initiation of DNA replication), but as this word has been used similarly before and in many cases DNA sequences necessary for replication have been shown to function as actual origins, we use it throughout this paper for describing DNA sequences necessary for transient plasmid replication.

MATERIALS AND METHODS

Plasmids

All inserts for plasmid cloning were obtained by using standard techniques or by amplifying short DNA fragments with polymerase chain reaction. Protein expression vector pCGE1B was constructed by inserting HPV-18 sequences from 119–4734 (BamHI-DpnI) to the eukaryotic expression vector pCG-ATG$^-$ (30). Frameshift mutation in E1 ORF was made by filling in 5′ overhanging ends of the XbaI site at position 1732. Deletions of E2 and E5 ORFs were made by deleting the coding sequences 3′ from DraIII (nt.2999) and NdeI (nt.3918) respectively. All mutations were confirmed by sequencing. Numbering of nucleotides is according to Cole and Danos (31).

All target (replicating) plasmids were made by cloning different fragments from HPV-18 to pUC18 polylinker (Table 1). Correctness of point mutations in E2 binding sites were checked by sequencing of these constructs. Mutations in E2 binding sites in AB71 and AB73 were TCCGAAATACGT, in AB72 and AB73 TCCGTTTTCCGT. Reference plasmid for quantitative assays were made by adding BamHI-XbaI fragment from bacteriophage lambda (nt.22346–24508) to AB.

Cells and transfections

Human cell lines 293 and HeLa were maintained in Dulbeccos modified Eagle medium (GIBCO) supplemented with 10% foetal calf serum, streptomycin, and penicillin. Transfections were performed by using the modified calcium phosphate coprecipitation method (32) Transfection efficiency was determined by adding β-galactosidase expressing plasmid pON260 (33) in 1/20 ratio to replication mixture and assessed as ratio of blue cells to overall amount of cells. The cells were fixed and stained 36 hours after transfection as described previously (12).

Replication assay

Typically, 500 000 cells were transfected with 20 micrograms of DNA on 90mm dishes, washed twice with DMEM 24 hours later and divided equally onto three dishes. A small portion of cells was removed for assessing transfection efficiency. Ateach timepoint the cells were removed from the dish to PBS by brief trypsine treatment, pelleted at low speed, resuspended in 0.6 ml lysis buffer (1/2 mixture of alkaline lysis solutions I and II), and transfered into microfuge tubes. The tubes were left on ice for 5 minutes and then 0.3 ml of the alkaline lysis solution III was added. After 10 minutes on ice the supernatant was recovered by centrifugation and low molecular weight (LMW) DNA was precipitated with 0.6 volume of isopropanol. Plasmid DNA was purified, cut with DpnI and HindIII as described by Ustav and Stenlund (12), and Southern blotted to Hybond-N (Amersham). High specific activity probes were made by random priming of linearized pUC18.

Quantification of replication activity

In quantitative replication assays the test plasmids were cotransfected with an appropriate reference plasmid on a 90 mm dish and transferred onto a 150 mm dish 24 hours after transfection. Small aliquot from input transfection mixture was taken before adding it to cells. This input DNA was cut with HindIII and analyzed by Southern blotting to confirm that all samples have got an equal ratio of test and reference plasmids. LMW DNA was extracted from the transfected cells 72 hours after transfection, purified and analyzed as described above. After the hybridization and exposure to X-ray film, both test and reference plasmid bands from cells as well as those from input DNA were cut out from nylon filter and counted in liquid scintillation counter. Test/reference ratio was calculated, normalized with input test/reference ratio and expressed as a percentage comparing with the best replicating plasmid (100%) and pUC18 (0%). Majority of the test plasmids were assayed twice or more with good repeatability.

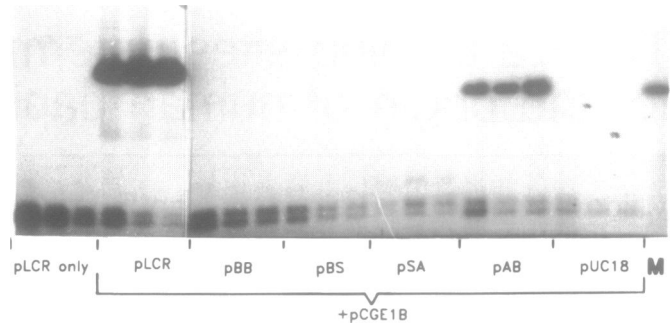

Figure 1. Plasmids containing long control region of HPV-18 (pLCR) or its subfragment AccI-BamHI (AB) replicated in 293 cells if cotransfected with protein expression vector pCGE1B. Plasmids BB and AB contain different subfragments from long control region. At each time point (48,72 and 96 hours post transfection) LMW DNA was purified from cells, linearized and digested with DpnI. Material at lower part of gel are DpnI cleavage products. On slot M is 10 pg of linearized marker DNA (AB).

Table 1. Test and control plasmids used in this study. The HPV18 DNA fragments were cloned into polylinker of the pUC18.

Plasmid	Nucleotides from HPV-18	Comments
pLCR	6929–119	BamHI-BamHI
BB	6929–7109	BamHI-BspMI
BS	7109–7428	BspMI-SfaNI
SA	7428–7767	SfaNI-AccI
AB	7767–119	AccI-BamHI
C1	119–2440	BamHI-EcoRI
C2	2440–6929	EcoRI-BamHI
C3	921–4734	Sau3A-Sau3A
C4	5730–7105	
C5	6929–7856	
AB1	7761–40	1 E2 bs
AB2	7761–58	2 E2 bs
AB3	7857–119	2 E2 bs
AB4	7857–58	1 E2 bs
AB5	7857–40	no E2 bs
AB51	4–119	
AB52	9–119	
AB53	7761–34	
AB55	14–119	
AB56	42–119	
AB57	7857–70	
AB58	42–70	
AB59	26–119	
AB60	7761–20	
AB70	7822–57	2 E2 bs
AB71	7822–57	1 E2 bs
AB72	7822–57	1 E2 bs
AB73	7822–57	no E2 bs
AB-R	7767–119	contains 2 kb fragment from lambda-phage

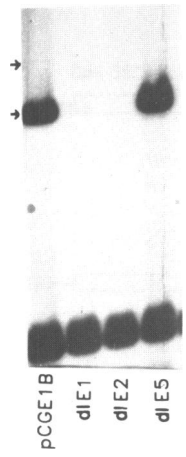

Figure 2. Replication of pLCR plasmid cotransfected with different protein expression vectors. LMW DNA was purified from cells 60 hours post transfection and digested with HindIII and DpnI. Arrow indicate the size of linear pLCR plasmid. Material at lower part of gel are DpnI cleavage products. dlE1, dlE2 and dlE5 contain mutations in E1, E2 and E5 ORFs respectively.

RESULTS

Noncoding region of HPV-18 contains eukaryotic replication origin

Following the earlier findings of Ustav and Stenlund (12), we supposed that for replication of papillomaviruses at least two viral proteins—E1 and E2—are required. To express these proteins in eukaryotic cells we cloned almost the entire early coding region (nt.119−4734) from HPV-18 genome to eukaryotic expression vector pCG under CMV promoter. We expected E1 and E2 proteins to be expressed from the resulting plasmid pCGE1B, because mRNAs with similar initiation site are shown to be prevalent in HPV-infected cells (34).

To localize the replication origin of HPV-18, we exploited a method where fragments from viral genome were cloned to pUC18 polylinker and the resulting plasmids then cotransfected to eukaryotic cells together with protein expression vector pCGE1B. 48−96 hours later LMW DNA was extracted from the cells and DpnI resistant, replicated molecules (35) were identified by Southern blotting. In all experiments equal transfection efficiency of different samples was checked by pON260 cotransfection (see Materials and Methods section). All samples from the same experiment were always processed together. First we tested replication capability of pLCR plasmid containing all noncoding regions from HPV-18 in both 293 and HeLa cell lines. As shown in Figure 1, in 293 cells replication of the pLCR plasmid occurred only if it was transfected together with protein expression vector coding for E1 and E2 proteins. pLCR alone did not replicate, what proves that pCGE1B was able to express factors required for replication. Similarly, pUC18 without HPV sequences was not replicating, even if cotransfected with pCGE1B.This proves the necessity of LCR fragment for initiation of replication.

The replicating fragment was further divided to four subfragments and cloned to pUC18. The resulting plasmids—BB, BS, SA, and AB were analyzed for their ability to replicate in 293 cells. As depicted in Figure 1, only one of these plasmids, AB, replicated. However, the replication level of AB is slightly lower compared to the full length LCR containing plasmids, which indicates that other parts of LCR might have some contributing effect to replication of AB. A control experiment was performed to assure that DpnI resistant bands were not due to the incomplete digestion by DpnI. In this experiment 100 ng of different size bacterial DNA was added to purified low molecular weight DNA just before digestion by DpnI. No bands from this control plasmid remained visible after overnight digestion, but DpnI resistant band from plasmids pLCR and AB still persisted. Digestion of purified LMW DNA with MboI (cleaves only unmethylated DNA) gave digestion products of expected size for pLCR and AB, but no digestion products for nonreplicating plasmids pUC18 (data not shown). Results from HeLa cells were identical to those shown in Figure1, which indicates that keratinocyte specific factors do not affect the origin function and do not alter the origin localization. Overlapping parts of other regions from HPV-18 genome were cloned to pUC18 (clones C1−C5 in Table1) and their replication activity was analyzed. None of them showed any noticeable replication (data not shown). Thus, single replication origin of HPV-18 resides between nucleotides 7767−119.

Intactness of both E1 and E2 ORF is necessary for replication

Due to the complexity of our protein expression vector E1B (contains intact E1, E2, E4, E5, E7, and partial E6 and L2 ORFs) we could not be sure that directly E1 and/or E2 are responsible for replication-supporting activity of this plasmid as it was shown previously to be true for other papillomavirus types. To prove the essential role of E1 and E2 in HPV-18 replication, the mutations were made in these ORFs and their ability to support replication was checked. As shown in Figure 2, mutations affecting E1 or E2 ORF but not mutations affecting E5 ORF abolished replication supporting activity of pCGE1B. Thus, both E1 and E2 ORF are necessary for replication of HPV18.

Main determinants of replication efficiency are E2 binding sites

The replicating AccI-BamHI fragment contains well-characterized binding sites for many different transcription factors—Sp1(36), glycocorticoid receptor (37), TATA-box or TFIID binding site(38), transcription initiation site (34), and three binding sites for papillomavirus E2 protein (Fig.3A). To assess the relative contribution of the individual E2 binding sites to HPV-18 replication, series of plasmids with origin fragments containing different combinations of E2 sites (AB1−5) were made and assessed for their replication activity. Surprisingly, replication activity of these plasmids was strictly dependent upon the amount of E2 binding sites. Fragments with two E2 binding sites replicated with similar efficiency as the original AB plasmid but replication of plasmids containing only one E2 site were severely reduced(Fig.3B). Plasmids without E2 sites in origin region did not show any replication. Therefore, it can be concluded that E2 binding sites in the origin region are essential for transient replication of HPV-18. Although pUC18 itself contains two possible E2 binding sites (nt. 181 and 1387), we conclude that they do not have any effect on replication, as disruption of proximal E2 site of the pUC18 at nucleotide 181 (240 bp away from origin fragment in polylinker) did not affect the replication of AB series plasmids (data not shown). Despite the fact that the results shown in Figure 3B were perfectly repeatable, this assay could still give some fluctuations due to the unequal recovery of material during purification or slight differences of transfection

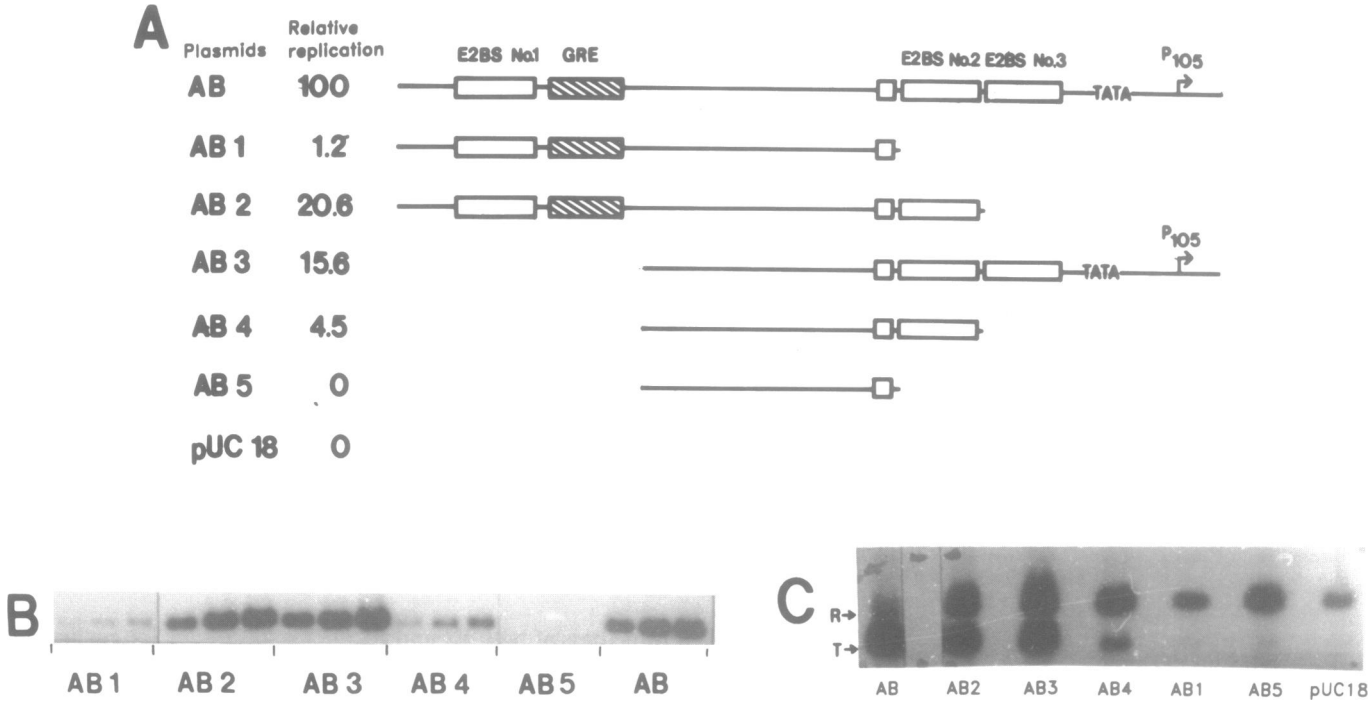

Figure 3.(A) Structure of the replicating AB fragment and its subfragments AB1 – AB5. Numbers left of each fragment show relative replication activity determined by quantitative replication assay. E2BS—E2 binding site; GRE—glycocorticoid response element; P_{105}—HPV-18 main promoter; quadrangle denote the Sp1 binding site **(B)** Replication of plasmids AB1 – AB5. Time points were taken 48, 72, 96 hours post transfection. **(C)** Replication activity of AB1 – AB5 in quantitative replication assay. Test plasmids (T) were cotransfected 1/10 with reference plasmid AB-R (R).

efficiency of different samples. To obtain quantitatively comparable results, new type of assay with internal control described previously by Li and Kelly (39) was taken into use. It utilizes cotransfection of test plasmid with small amount of reference plasmid. The reference plasmid contains similar origin, but has a different size. In these assays, the mutants and reference plasmid compete for the cellular and viral replication proteins. Replication efficiency of each mutant (test) plasmid can be calculated as a ratio of the amount of replication of the mutant template to that of the reference template. Then the test plasmids can be compared with each other in their ability to compete with the reference plasmid. This quantitative assay revealed similar dependence of HPV-18 replication on E2 binding sites around the origin region (Fig.3C). The assay revealed a difference between replication efficiencies of the two E2 binding site containing plasmids AB2, AB3, and of the three E2 binding site containing plasmid AB not observed in usual assay. This may be a result of a strong competition between the reference and test plasmids for limiting replication proteins. Plasmids with weaker origin (without E2 binding sites) could not give any noticeable replication above the pUC18 background level. An interesting feature was that pUC18 without any insert repeatedly gave a faint Dpn-resistant band in this quantitative assay, but never when transfected alone without the reference plasmid (for example, compare bands corresponding to pUC18 in Fig.1 and in Fig.5). The same effect was observed for normally nonreplicating plasmids AB5 and AB73. This phenomenon might be explained by some *trans*-acting stimulating effect of well replicating reference plasmid to normally non-replicating plasmid in the same cell. Similar intermolecular effect, called transvection, has been noticed for transcription transactivators (40).

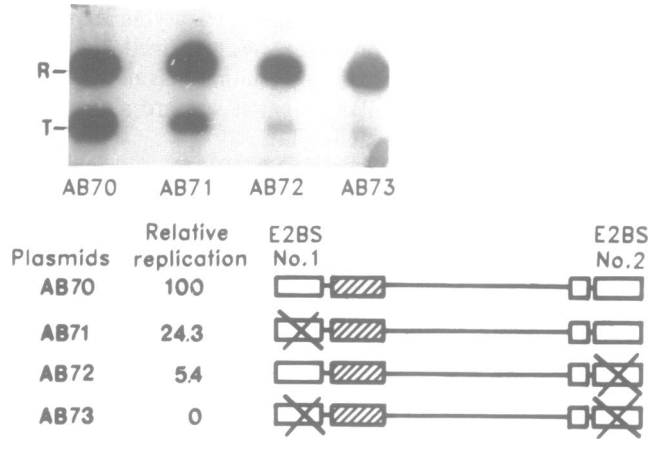

Figure 4. Structure and relative replication activity of plasmids with point mutations in E2 binding sites. Sites with point mutations are crossed; hatched boxes are glycocorticoid response elements, quadrangles are Sp1 binding sites. Test plasmids (T) were cotransfected 1/10 with reference plasmid AB-R (R).

To ascertain that the revealed effect of the E2 binding site deletions was actually caused by the loss of E2 sites and not by the loss of other DNA sequences or by difference in the length of DNA fragments cloned to pUC18, the point mutations were created in E2 binding sites (Fig.4). The point mutations were made in both halves of the binding site palindrome and they were chosen to alter the most conserved nucleotides (see Materials and Methods section). Previously these mutations have been shown to abolish E2 binding to these sites (41). Mutations were made in E2 binding sites no.1 and no.2 (see Figure 3 for numbering)

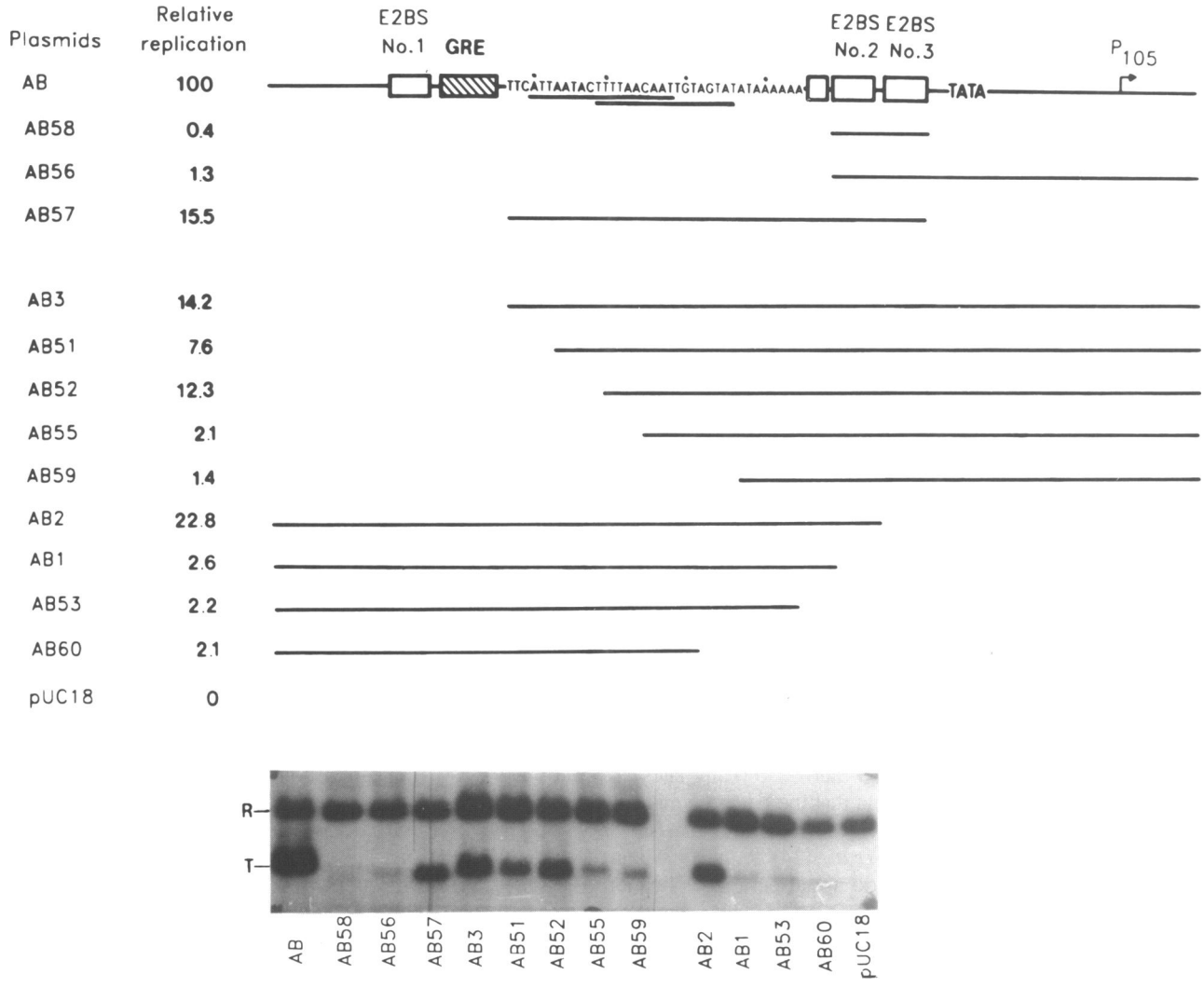

Figure 5. Functional mapping of origin. Structure and relative replication activity of each mutant are shown. Test plasmids (T) were cotransfected 1/5 with reference plasmid AB-R (R). Dots above the nucleotides from left to right show nucleotides number 1, 11, 21 and 31 of the HPV-18 genome. Two regions homologous to BPV-1 E1 binding sites are underlined. Abbreviations are the same as in figure 3A.

and a set of mutation carrying fragments (all fragments have the same length) were assayed for their replication activity by previously described quantitative replication assay. Results, shown on Figure 4, confirm that E2 binding sites are the main determinants of replication activity. Assuming results from these three experiments with E2 binding sites we can rule out that the replication capability of HPV-18 origin depends directly on the amount of neighbouring E2 binding sites. The effect of binding sites is cooperative, rather than additional as plasmids containing two sites have a much greater replication efficiency than the sum of replication efficiencies of two single site carrying plasmids (for example, compare AB/AB1+AB3 and AB2/AB1+AB4 in Figure 3A or AB70/AB71+AB72 in Figure 4). In addition, different sites have different contribution to replication: plasmid AB71 containing single E2 binding site no.2 replicated about 4 times better than plasmid AB72 containing single E2 binding site no.1.

A specific DNA region adjacent to E2 binding site is required for efficient replication

The data described above strongly suggest that E2 binding sites are the main determinant of replication activity of the HPV-18 origin. In one of the previous work (14) the presence of viral E1 protein binding site in BPV-1 origin fragment has been shown. Two DNA regions similar to this binding site exist between E2 binding sites no.1 and no.2 within the replicating AccI-BamHI fragment (Fig.5). Therefore, we questioned whether these adjacent DNA sequences are essential for replication of HPV. Three clones, AB 56−58, were made and examined for their replication activity. One of them, AB58, contained only two E2 binding sites (no.2 and no.3) from HPV-18; another two, AB57 and AB56, contained additional sequences from 5′ (putative E1 binding site) or 3′ (TATA-box and transcription start site) region. Interestingly, only the plasmid with putative E1 site showed noticeable replication activity (Fig.5). These results reveal that although E2 sites determine the replication efficiency, the origin needs additional specific DNA sequences in vicinity to function.

To pinpoint the precise functional limits of origin, series of deletions from both ends of putative E1 binding site were generated and their replication activity tested. As it can be seen in Figure 5, replication activity decreases strongly when 5′ deletions extend further than nucleotide 9 in HPV-18 genome. Surprisingly, among the other well replicating plasmids, the AB51 showed weaker replication. This could be explained by some

hindering protein binding sites or DNA secondary structure forming on the boundary of prokaryotic and viral DNA sequences. Determination of the 3′ end of this important DNA region was hindered by fact that the replication activity of constructs containing only E2 binding site no.1 was not high enough to detect a certain cut-off point of replication activity. There is still the possibility that in these constructs another A/T rich DNA region adjacent to E2 binding site no.1 can function as a weak nonspecific E1 binding site. Taken together these results show that in addition to E2 binding sites no.2 and no.3, a specific DNA region (nt.9−34) is required for high replication activity. Replication activity of this fragment can be further increased by adding additional E2 site no.1.

DISCUSSION

The results presented in this paper describe for the first time the functional mapping of the human papillomavirus type 18 origin. At least one E2 binding site together with a specific DNA region was required for HPV-18 origin function. An increasing number of E2 binding sites near origin have a cooperative stimulating effect on replication. These results show several discrepancies with previous work (11,14) characterizing the BPV-1 origin of replication. Although our frameshift mutations in protein expression vector pCGE1B suggest that, similarly to earlier findings, both E1 and E2 ORFs are required for HPV-18 replication, differences appear to be in requirements for E2 binding sites. Yang *et al.* (11) argue that E2 binding sites are dispensable for *in vitro* replication and that the E1 protein is able to bring the E2 protein in contact with DNA in the absence of E2 binding sites. Similarly, Ustav *et al.* (14) found that point mutations in E2 binding site no.12 do not affect the replication activity of a minimal origin fragment of BPV-1 *in vivo*. However, their mutations were made in nonconserved nucleotides and were not proven correctly to eliminate binding of E2 protein. Thus, these sites may retain some affinity at the high concentrations of E2 protein used in their experiments. Moreover, in both works the minimal replicating fragment still retained an intact half of the palindromic E2 binding site. The differences between these results compared to the absolute requirement for E2 sites revealed in our experiments can be explained in two possible ways. First, the replication machinery of HPV-18 may be regulated in a completely different way from BPV-1, as well as their transcriptional regulation (42,43). Another possibility is that the differences are due to different conditions in the replication assay. In previous studies, comparatively high concentrations of E1 and E2 protein were used. In our experiments we exploited a polycistronic protein expression vector with all early ORFs in their native configuration. We speculate that the mRNAs and proteins produced from this plasmid, by splicing and/or internal initiation of translation, have a similar structure and relative ratio as in normal HPV-infected cells, although the concentration might be higher due to the higher activity of CMV promoter. Under these conditions, E2 protein concentrations may be relatively low and limiting for replication, but its concentration can be raised locally by E2 binding sites. Higher local E2 concentration may stimulate the binding of E1 to DNA by forming E1/E2 complexes. Thus, the main function for E2 sites near origin might be saturation of the nearby E1 binding site with E1/E2 complexes. At the high concentrations of both E1 and E2 used in previous studies their complex may saturate the E1 binding site even in absence of the E2 binding sites and the importance of E2 sites not be revealed. The DNA sequence near the E2 sites that was shown by us to be necessary for efficient replication has not proved to be an E1 binding site, although the high level of homology with the BPV-1 E1 binding site, conservation of this sequence in all papillomaviruses and its similar location between E2 binding sites strongly suggest it.

Comparison of the HPV-18 replication with the replication strategy of other well-studied DNA viruses reveals many similarities as well as differences. Most of them use transcription factors for regulation of replication (44), but the way they are used seems to be different for each virus. A classical subject for replication studies, SV40, recruits Sp1 binding sites in the early promoter and the complex enhancer to disturb nucleosomes and thus increase the replication activity of the origin (39,45−48). Similar regulation of replication by transcription factors is described for polyomavirus (44) and even for the yeast chromosomal origin, ARS1 (49). In all replication systems described above, the transcription factors activating replication are interchangeable with other types of transcription factors and need their activation domains to initiate replication. Thus, the activation domain seems to be nonspecifically required to activate replication in these systems. We tried to replace the E2 binding site no.2 with other transcription activator binding sites that were previously shown to activate SV40 replication (Sp1, NF-I, SV40 enhancer), but none of them could restore the replication activity of the HPV-18 origin (data not shown). Therefore, E2 binding sites seem to be essential and specific requirements for activation of HPV-18 replication. The mechanism of recruitment of transcription factors for regulation of replication by HPV-18 might resemble the adenovirus origin activation where cellular transcription factor NF-I activates replication up to fifty-fold, even in the absence of nucleosomes. This occurs by formation of a specific complex between NF-I and adenovirus encoded replication proteins and stabilization of the initiation complex attachment to DNA (50).

Note added in proof

During the completion of this manuscript, Chiang *et al.* (51) and Del Vecchio *et al.* (52) reported the localization of origin sequences of HPV-6b, HPV-11, HPV-16, and HPV-18.

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