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Papillomavirus DNA Replication

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The papillomaviruses constitute a large family of viruses that cause benign tumors (warts) in their hosts. In recent years, these viruses have been found to have substantial clinical importance (Syrjanen et al. 1987; zur Hausen 1991). The replication properties of papillomaviruses have been studied for a number of years; however, many results obtained in the early studies concerning both *trans*-acting factors and *cis*-acting elements required for replication have not been reproducible. Therefore, a clear picture of the viral replicon has emerged only during the last 5 years and the understanding of papillomavirus replication is relatively primitive compared to, for example, the well-studied SV40 system. For technical and historical reasons, the field has been dominated by studies of one particular member of the papillomavirus family, bovine papillomavirus (BPV), which has served as a prototype for the papillomaviruses. More recently, largely because of information obtained from the BPV system, it has become possible to develop systems to study the replication properties of several human papillomaviruses. In this chapter, I describe the BPV system in some detail and, from this point of reference, discuss the variations and differences that have been observed for replication of other papillomaviruses.

The natural life cycle of papillomaviruses is complex and not understood in detail. However, in the basal cells of the papilloma, viral DNA is maintained in a latent form characterized by episomal replication at a relatively low copy number. At this stage, expression of viral capsid proteins cannot be detected. As the cells differentiate and migrate toward the outer layers of the papilloma, substantially higher copy numbers and assembled virions can be detected (Stoler et al. 1990; Dollard et al. 1992; Meyers et al. 1992). The basis for all papillomavirus replication systems originates in the early observation that BPV can transform mouse cells in culture and that in the transformed cells, the viral DNA replicates as a plasmid with a modest copy number (Dvoretsky et al. 1980; Lancaster 1981; Law et al. 1981). Furthermore, the viral DNA is maintained over

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many cell generations such that permanent cell lines with episomal viral DNA can be established. The replication that takes place in these transformed cells is believed to resemble the replication occurring in the basal layers of the papilloma. Early studies indicated that replication of BPV was under the control of chromosomal once-per-cell-cycle replication. These results have not been reproduced, and more recent experiments demonstrate that selection of molecules for replication takes place at random (Gilbert and Cohen 1987). However, replication of BPV is regulated by the cell cycle in that replication of the viral DNA is strictly confined to S phase (Ravnan et al. 1992).

The behavior of BPV in tissue-culture cells illustrates some clear distinctions between the replication properties of papillomaviruses and other papovaviruses. Whereas, for example, SV40 and polyomavirus rapidly replicate to very high copy numbers in permissive cells, resulting in death and lysis of the infected cell, the papillomaviruses appear to limit their DNA replication to a relatively low copy number, and viral DNA replication has no obvious deleterious effect on the cell (Law et al. 1981; Dvoretsky et al. 1980). In addition, despite a high degree of host and cell-type specificity for infection, little host-cell specificity appears to exist for papillomaviruses at the level of DNA replication, in contrast to the strict specificity observed for SV40 and polyomavirus (Tooze 1981; Murakami et al. 1986). When the required viral genes are expressed from heterologous expression vectors, replication of a variety of papillomaviruses has been achieved in a number of cell lines of different mammalian origins (Ustav et al. 1991; Chiang et al. 1992b; Del Vecchio et al. 1992; Lu et al. 1993). A further distinction is that papillomaviruses require a virus-encoded protein in addition to the initiator for viral DNA replication. This protein, the transcription factor E2, appears to be the counterpart of the auxiliary factors involved in replication of SV40 and polyomavirus, as discussed below (see also van der Vliet, this volume).

These differences appear to represent variations on a common theme rather than fundamental differences between papillomaviruses and other papovaviruses. When DNA replication is examined at the molecular level, substantial similarities can be observed. These include similarities between the respective viral factors that are required for replication and extend to the interactions with cellular factors. For example, what was originally observed as a very limited sequence homology between the BPV E1 protein and SV40 and polyomavirus T antigen is now well established as a functional homology (Clertant and Seif 1984); E1 is clearly the counterpart of T antigen, providing recognition of the origin of DNA replication (*ori*) and *ori*-unwinding activity (Ustav et al. 1991;

Wilson and Ludes-Meyers 1991; Yang et al. 1991, 1993; Seo et al. 1993a). E1 also appears to interact directly with the replication machinery through physical interactions with DNA polymerase-α, which is an interaction partner also for T antigen (Park et al. 1994; Bonne-Andrea et al. 1995; see also Borowiec, this volume). The cellular proteins required at the replication fork also appear to be similar, if not identical, to the factors required for SV40 replication; a minimal replication system for SV40 consisting of factors purified from HeLa cells can function to replicate BPV (Muller et al. 1994). However, a purified system consisting of factors purified from 293 cells that is fully functional for SV40 replication fails to replicate BPV, indicating that some difference in the requirement for cellular factors exists (Melendy et al. 1995; see also Brush and Kelly, this volume).

ASSAYS FOR REPLICATION

DNA replication of papillomaviruses has been studied in three different experimental systems. Since the types of questions that have been addressed in these systems are to some degree different and the results obtained also differ to some extent, a brief summary of the different systems and the experimental results is given here.

Stable (Long-term) Replication Assays

The earliest studies of papillomavirus replication were performed by taking advantage of the transforming ability of BPV. After transfection of the viral genome, DNA could be prepared from transformed foci, and replicating viral DNA could be measured by Southern analysis (Lancaster 1981; Law et al. 1981). Using this method, at least five genes were initially thought to be involved in stable replication (Lusky and Botchan 1984, 1985; Sarver et al. 1984; Berg et al. 1986). Two of these, the genes E1 and E2, have withstood the test of time. However, the conclusions that could be drawn about the function of these genes was relatively limited due to the nature of the assay. Although it was obvious that DNA replication was required, it was by no means clear that DNA replication was sufficient to maintain the viral DNA over the extended periods of time that were required for these assays. In addition, since the assay required transfection of the entire viral genome, it was difficult to determine whether a given gene had a direct involvement in replication or was required indirectly, for example, as a regulator of viral gene expression.

Transient (Short-term) Replication Assays

A clear picture of BPV replication was first obtained using a short-term replication system (Ustav and Stenlund 1991). This system was based on the prediction that if a sufficiently large fraction of the cells could be transfected with viral DNA, detection of replication at early times (1–3 days) after transfection should be possible without the need to select for transformed foci. This assay would have the advantage of measuring replication with only a minimal contribution of mechanisms that would affect, for example, partitioning. It would also be more convenient for genetic analysis, since it would allow simple complementation experiments. Using this system for BPV, it was possible to establish unequivocally that two viral proteins, the full-length products from the E1 and E2 open reading frames, were required for viral DNA replication (Ustav and Stenlund 1991). Provision of these two factors from heterologous expression vectors also allowed the identification of viral *cis*-acting elements that were required for replication (Ustav et al. 1991). This type of assay has now become a standard method for analysis of papillomavirus replication and has been applied to a variety of papillomaviruses.

Cell-free Replication

The identification of the two viral proteins required for replication in vivo has allowed the development of cell-free replication for BPV along the lines of the systems previously established for SV40 and polyomavirus (Yang et al. 1991; Seo et al. 1993a; Bonne-Andrea et al. 1995). This in vitro replication system consists of a nuclear extract preparation from a permissive cell line supplemented with the viral E1 and E2 proteins overexpressed and purified from baculovirus-infected insect cells or from *Escherichia coli*. With the use of this system, a number of important properties of BPV replication have been determined, including the mapping of *ori* sequences active for replication in vitro, demonstration of bidirectional replication, and the requirement for cellular factors. Recently, a similar in vitro system has also been established for the human papillomavirus HPV-11 (Kuo et al. 1994).

REQUIREMENTS FOR VIRAL DNA REPLICATION IN VIVO

The requirements for replication for a number of papillomaviruses have been established using the transient replication assay. The results from these assays demonstrate that the requirements for replication are similar for most, if not all, the different virus types that have been tested, con-

sistent with the high degree of overall similarity within the papillomavirus group. Papillomaviruses in general require the well-conserved E1 and E2 proteins and also a small *cis*-acting sequence element that is conserved both in terms of location and DNA sequence. Some variation as to the relative importance of the two viral factors, as well as the different elements of the *ori*, exists between different viruses. These variations are discussed below.

Viral Origin of Replication

The *cis*-acting sequences required for replication have been defined for a number of papillomaviruses using transient replication assays where the respective E1 and E2 proteins have been expressed from heterologous expression vectors (Ustav et al. 1991; Chiang et al. 1992b; Del Vecchio et al. 1992; Remm et al. 1992; Lu et al. 1993; Sverdrup and Khan 1995). In all cases, these experiments have resulted in the identification of a small fragment located between the early open reading frames and a region called the long control region (LCR) or upstream regulatory region (URR), which contains regulatory signals for viral transcription. This position appears to be completely conserved between a number of different virus types. The use of a larger fragment containing additional sequences from the URR region commonly results in higher replication activity than for a smaller fragment, but whether these effects are due to specific elements in the URR is unknown. None of these *ori* sequences, including the BPV *ori*, has been examined in great detail, but on the basis of a combination of homology, deletion analysis, and linker substitution analysis (Ustav et al. 1991; Chiang et al. 1992b; Remm et al. 1992; Lu et al. 1993; Zhu et al. 1994; Russell and Botchan 1995), several different elements can be discriminated. The papillomavirus sequences show distinctive similarities to other *ori* sequences that have been described (DePamphilis 1993 and this volume) and can be classified according to two schematic variants (see Fig. 1). Some animal papillomaviruses including BPV-1 are organized as illustrated in Figure 1A, where two E2-binding sites (E2BSs) flank an A+T-rich sequence and the palindromic sequence that constitutes the E1-binding site (E1BS). The majority of the human papillomaviruses have a slightly different organization, as shown in Figure 1B. The E1BS is located between two A+T-rich sequences that in turn are flanked by three E2BSs, one on the upstream (left) side and two on the downstream (right) side. Some *ori*s contain slight variations on this theme, lacking, for example, one of the two

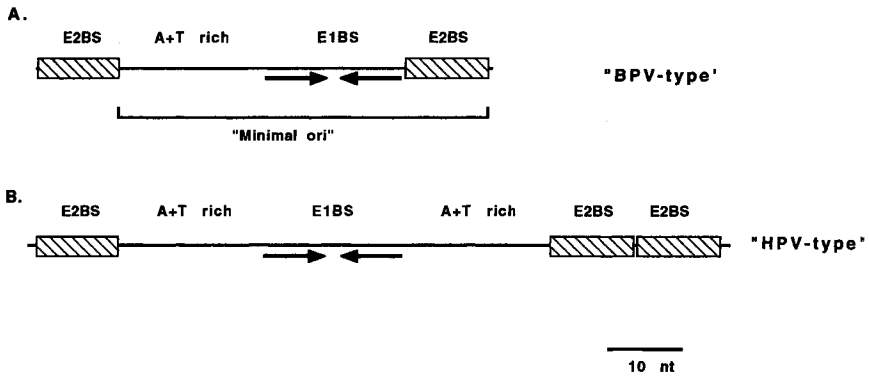

Figure 1 Schematic representation of the origin sequences of papillomaviruses classified in two groups. The "BPV-type" represents the organization found in the small number of animal papillomaviruses that have been examined. The "HPV-type" represents the organization found in virtually all sequenced human papillomaviruses. (E1BS) Binding site for the viral replication initiator E1. (E2BS) Binding site for the viral transcriptional activator E2.

downstream E2BSs. The A+T-rich sequences for both of the groups are of variable length with little sequence conservation.

The definition of minimal *ori* fragments has been complicated by apparent redundancies, especially concerning the E2BS. For example, in the BPV *ori*, a fragment comprising the A+T-rich sequence, the E1BS, and the downstream (right) E2BS was originally defined as the minimal *ori* (Ustav et al. 1991). However, another fragment containing the upstream (left) E2BS, the A+T sequence, and the E1BS is also active for replication, albeit at lower levels. Definition of a minimal HPV *ori* is even more complex in this regard; deletion analysis of the HPV-11 and HPV-18 *ori*s demonstrates that even nonoverlapping fragments have replication activity, indicating redundancy (Chiang et al. 1992a; Lu et al. 1993; Sverdrup and Khan 1995). Furthermore, a significant replication activity can be observed with an *ori* containing only the two high-affinity E2BSs (Lu et al. 1993; Sverdrup and Khan 1995). These results indicate that a search for a minimal sequence that can function as an *ori* may be less informative than a more quantitative approach where all elements that affect *ori* activity are identified. Presumably, in the context of the viral genome, conservation of a given element indicates function. In this regard, it is important to note that in the replication assays used for these studies, the viral E1 and E2 proteins are highly expressed from expression vectors. This may mask the requirement for some elements that are important for function at lower levels of E1 and E2.

The E2BS(s)

All papillomavirus origins of replication include one or more binding sites for the virus-encoded transcription factor E2, which binds to the consensus sequence ACCGNNNNCGGT (Androphy et al. 1987; Hawley-Nelson et al. 1988; Hirochika et al. 1988; Li et al. 1989). Results from studies of the BPV *ori* as well as several HPV *ori*s indicate that the requirement for an E2BS is absolute, but that a great deal of flexibility exists in terms of both the position and the affinity of the E2BS (Chiang et al. 1992a; Remm et al. 1992; Lu et al. 1993; Ustav et al. 1993; Sverdrup and Khan 1994). One single exception to this rule has been described: HPV-1 is capable of replication in the absence of E2 and E2BS; however, E2 is stimulatory for replication (Gopalakrishnan and Khan 1994). For BPV it is apparent that a single E2BS of very low affinity is sufficient for an active *ori*, at least under conditions where E1 and E2 are overexpressed (Ustav et al. 1991; Ustav et al. 1993). Moreover, a relationship appears to exist between the affinity of the E2BS and the ability to function at a distance from the binding site for E1 (Ustav et al. 1993). In multimerized form, the E2BSs are capable of exerting their function even when placed at a distance of several kilobases from the rest of the *ori*.

The human papillomaviruses generally show a stricter requirement with regard to the properties of the E2BS. In the natural context, most human papillomaviruses have three high-affinity E2BSs located in the *ori* fragment. Deletion or mutation of any one of these sites results in a small reduction of replication (Chiang et al. 1992a; Remm et al. 1992; Lu et al. 1993; Sverdrup and Khan 1994, 1995; Russell and Botchan 1995). Deletion or mutation of any two of these sites results in a severe drop in replication activity, indicating that at least two E2BSs are required for efficient replication. The requirement for specific spacing has not been tested directly in the HPV system.

The E1BS

The binding site for the E1 replication helicase has been defined in BPV as an imperfect palindromic sequence 18 nucleotides in length with the sequence ATTGTTGTTAACAATAAT (Wilson and Ludes-Meyers 1991; Ustav et al. 1991; Yang et al. 1991; Holt et al. 1993). A related sequence is present in all papillomaviruses examined, but binding of other E1s to these putative E1BSs has not been defined in detail (Bream et al. 1993; Lu et al. 1993; Liu et al. 1995). Therefore, the identity of the E1BS in HPV *ori* is largely based on homology with the BPV E1BS. The dependence on the E1BS for replication varies between different viruses.

Results from BPV demonstrate that single point mutations in the BPV E1BS can reduce binding of E1, as well as replication, more than 20-fold (Sedman and Stenlund 1995). In HPV-11, linker substitution mutations in the putative E1BS have less severe effects, indicating that sequence-specific binding of E1 may be of less importance for HPV replication (Russell and Botchan 1995). However, as stated above, the lack of a significant effect of mutations in the E1BS could be related to the levels of expression of the E1 and E2 proteins. Obviously, the conservation of the E1BS argues that it is likely to play a role in replication of the viral genome.

The A+T-rich Region

A+T-rich sequences are important elements of both prokaryotic and eukaryotic origins of replication; for SV40 and polyomavirus origins, various activities have been assigned to these elements, including DNA bending and facilitation of the unwinding activity by T antigen (Deb et al. 1987; Bramhill and Kornberg 1988; DePamphilis 1993 and this volume). The A+T-rich regions in papillomaviruses are very poorly characterized, both in terms of the function and the actual sequence requirement. The A+T-rich sequence in the context of the minimal BPV *ori* is clearly important for replication in vivo, since a deletion of this sequence results in a precipitous drop in replication activity (Ustav et al. 1991). The spacing relative to the E1BS also appears to be important, since insertions between the E1BS and the A+T-rich region result in loss of replication activity. In contrast, both deletion analysis and linker substitution analysis in HPV have failed to show significant effects of mutations in these elements (Lu et al. 1993; Russell and Botchan 1995). A possible function for the A+T-rich region in the BPV *ori* has been suggested by the induction of structural changes in this sequence upon binding of E1 to the *ori* in the presence of ATP (Gillette et al. 1994). It has also been suggested that E1 in the presence of ATP and E2 binds in a biphasic manner, first to the E1BS and at higher concentrations to the A+T-rich region. One possibility is therefore that the A+T-rich region serves as a secondary site for binding of E1 that may be involved in the formation of a larger ATP-dependent multimeric E1/*ori* complex.

Viral Proteins

The two viral proteins that are required for BPV replication are encoded from the full-length E1 and E2 open reading frames. E1 is an initiator protein based on a number of criteria and has the expected helicase, ATPase, and unwinding activities (Seo et al. 1993a; Yang et al. 1993;

Gillette et al. 1994; MacPherson et al. 1994; Park et al. 1994). The E2 protein falls in the category of accessory or auxiliary factors simply because this factor is not required for replication under all conditions; *ori*-specific replication in vitro can clearly take place with E1 alone. Both of these factors are discussed in greater detail in the appropriate chapters dealing with general properties of initiator proteins and accessory factors (see Coen; Hübscher et al.; both this volume). However, it should be noted that in contrast to other papovaviruses which show a high degree of specificity for the cognate initiator, for papillomaviruses, E1 and E2 proteins appear to be largely interchangeable for replication of different virus types, suggesting a high degree of functional relatedness and a low degree of specificity (Chiang et al. 1992b; Del Vecchio et al. 1992; Lu et al. 1993; Sverdrup and Khan 1994; Park et al. 1994).

REQUIREMENTS FOR REPLICATION IN VITRO

The requirements for replication of BPV in cell-free replication systems differ in two important respects from the requirements for replication in vivo. The E1 protein is clearly by itself sufficient to generate *ori*-specific replication in a cell-free replication extract (Yang et al. 1991, 1993; Seo et al. 1993a; Lusky et al. 1994; Muller et al. 1994). Consistent with this finding, an E2BS is not a required component for an *ori* functional for in vitro replication (Park et al. 1994). Furthermore, cell-free replication under these conditions shows only limited *ori* specificity; at high levels of E1, efficient *ori*-independent replication can be observed (Seo et al. 1993b; Yang et al. 1993). An interesting difference exists between cell-free replication of BPV and HPV-11 in a similar cell-free replication system. HPV-11 shows a strong dependence on E2 and an E2BS for replication in vitro (Kuo et al. 1994). Possible explanations for this difference are discussed below.

DNA Binding of E1 and E2

Both E1 and E2 are DNA-binding proteins, and in the BPV system it has been demonstrated that both proteins can bind to their respective binding sites at the origin of replication (Ustav et al. 1991; Wilson and Ludes-Meyers 1991; Yang et al. 1991; Seo et al. 1993b; Gillette et al. 1994; Lusky et al. 1994). E2 is very well characterized in this regard, and the X-ray structure of a protein/DNA co-crystal has been solved (Hedge et al. 1992). E2 binds to a palindromic site as a dimer with base contacts in both the major and minor grooves. In contrast, very little is known about

DNA binding by E1. Because of the palindromic nature of the E1BS and the large protection that is observed in DNase footprints, it has been assumed that E1 binds in a multimeric form, and an estimate of the stoichiometry of binding has also been made (Lusky et al. 1994). A number of observations have demonstrated that the E1 and E2 proteins can interact specifically with each other in vitro (Mohr et al. 1990; Blitz and Laiminis 1991; Lusky and Fontane 1991; Thorner et al. 1993; Frattini and Laiminis 1994; Benson and Howley 1995). Consistent with these observations, binding of E1 and E2 to the *ori* is cooperative, i.e., binding of either E1 or E2 takes place at substantially lower concentrations in the presence of the other protein, provided that binding sites for both proteins are present (Yang et al. 1991; Seo et al. 1993b; Gillette et al. 1994). It has therefore been suggested that E2 may serve to recruit E1 to the origin of replication.

More detailed analysis of binding of E1 to the *ori*, by itself and in combination with E2, has revealed that the situation is more complicated than was originally believed. The complex formed by E1 alone on the *ori* is substantially larger than the complex that also contains E2, indicating that the E1 protein is capable of binding to the *ori* in one form by itself and in a different form in the presence of E2 (Yang et al. 1991; Lusky et al. 1994; Sedman and Stenlund 1995). The most likely explanation for these results is that the E1 protein can bind to DNA in different multimeric forms; a larger form is capable of binding to *ori* by itself and forms an E1/*ori* complex, whereas binding of the smaller form of E1 takes place in cooperation with E2, forming an E1/E2/*ori* complex. Interestingly, binding of the smaller form of E1 in the absence of E2 has not been detected, indicating that cooperation with E2 is required for DNA-binding activity of this form. A biochemical function has not been assigned to either of these two complexes directly; however, the conditions under which the E1/*ori* complex forms are also the conditions under which replication in vitro can be detected (Lusky et al. 1994).

Role of E2 in Replication

A peculiarity of papillomavirus DNA replication is the absolute requirement in vivo for the viral transcription factor E2. This requirement is highly specific, and various other transcription factors that have been tested are inactive (Ustav et al. 1991; Li and Botchan 1993). This is in contrast to the auxiliary transcription factors required for replication of other papovaviruses. SV40 and polyomavirus are not limited to any one particular factor, but both viruses show some specificity in that not all

transcription factors are capable of providing the auxiliary function (DePamphilis 1993; see also van der Vliet, this volume). A number of activities have been proposed for the function of transcriptional activators in replication in general, and these activities may be involved in the activation of BPV replication. For example, it has been demonstrated that E2 can interact with the cellular single-stranded binding protein RP-A (Li and Botchan 1993), and E2 appears to be capable of counteracting repression of replication by nucleosomes (Li and Botchan 1994). However, these functions do not explain the specific requirement for E2, since other transcription factors have these activities but are not functional for BPV replication in vivo (Li and Botchan 1994).

A possible explanation for the requirement of E2 for DNA replication in vivo could obviously be that the generation of the specific E1/E2/*ori* complex that can be observed in vitro is required for replication. Recent analysis of the BPV *ori* with mutations affecting the binding sites for E1 and E2 and the spacing between the two sites has revealed that a very good correlation exists between the ability to form the E1/E2/*ori* complex and replication activity (Sedman and Stenlund 1995). In contrast, mutations that only affect the formation of the E1 complex have minimal effects on replication. Furthermore, the mutational data also demonstrate that, consistent with a strong interaction between the two proteins on the *ori*, an increase in the affinity of the E2BS can compensate for mutations in the E1BS that reduce the affinity of E1 and vice versa (Spalholz et al. 1993; Sedman and Stenlund 1995). These results indicate that the ability to form the E1/E2/*ori* complex, and hence replication activity, is dependent on at least three different interactions: E1 and E2 with their respective binding sites and a direct interaction between E1 and E2.

One consequence of the cooperative binding between E1 and E2 is a substantial increase in the specificity by which E1 can recognize the *ori* (Sedman and Stenlund 1995). Binding of E1 in the form of the E1/*ori* complex can readily be competed by modest quantities of nonspecific DNA, indicating that the sequence specificity of this particular complex is low. The resistance to competitor is significantly increased when E1 binds to the *ori* in combination with E2, demonstrating greater sequence specificity. This effect on sequence specificity by E2 provides a possible mechanism for the function of E2 as an auxiliary factor. This possibility has recently been examined through modification of the in vitro replication conditions by addition of competitor DNA (Sedman and Stenlund 1995). As predicted, E1-dependent replication can be suppressed by addition of competitor DNA. Addition of E2 under these conditions can restore replication completely, in a manner that is dependent on a binding

site for E2 at the *ori*, closely reproducing the characteristics of E2 dependence for replication in vivo. A function for E2 as a specificity factor is also consistent with the observation that E1 alone in vivo, even at very high levels, is incapable of supporting replication (Ustav and Stenlund 1991).

This model for the function of E2 in replication of papillomaviruses, where E2 serves as a specificity factor for binding of the initiator E1, shows striking similarities to the proposed function of the transcription factor NFI in replication of adenovirus (Mul and van der Vliet 1992). The DNA-binding domain of NFI interacts with and promotes binding of the adenovirus preterminal protein/DNA polymerase (pTP/pol) complex to the adenovirus *ori*. The clear distinction between these systems is that for the interaction between E1 and E2, the *trans*-activation domain of E2 is specifically required. Thus, with regard to the requirement for auxiliary transcription factors, papillomaviruses provide an interesting link between SV40 and polyomavirus on the one hand and adenovirus on the other. Both SV40 and polyomavirus require the activation domain of the transcription factor, but the mechanism by which the activation domain exerts its activity is not known. Adenovirus utilizes the DNA-binding domain of a transcription factor that interacts with and affects the binding of the pTP/pol complex. Papillomaviruses utilize the activation domain of the transcription factor E2 for what appears to be the same purpose. This suggests that the auxiliary factors in SV40 and polyomaviruses may also have a function related to facilitation of binding of the initiator to the origin of replication.

A Model for *ori* Recognition

The importance of the E1/E2/*ori* complex in replication of BPV provides a simple explanation for some of the variability concerning the requirement for E1 and E2 and also the *cis*-acting sequences that has been observed for some human papillomaviruses (Fig. 2). It has been demonstrated for HPV-11 and HPV-18 that at least when the E1 and E2 proteins are overexpressed, a duplicated binding site for E2 can serve as an *ori* (Lu et al. 1993; Sverdrup and Khan 1995). HPV-1, on the other hand, appears to be capable of replication using E1 alone, at least when E1 is expressed at high levels (Gopalakrishnan and Khan 1994). Both of these results can be explained by simply allowing for different contributions from the three different interactions "1," "2," and "3" that we have listed. If, for example, interaction 1 between E1 and the E1BS is sufficiently specific, the other two interactions may be of lesser importance,

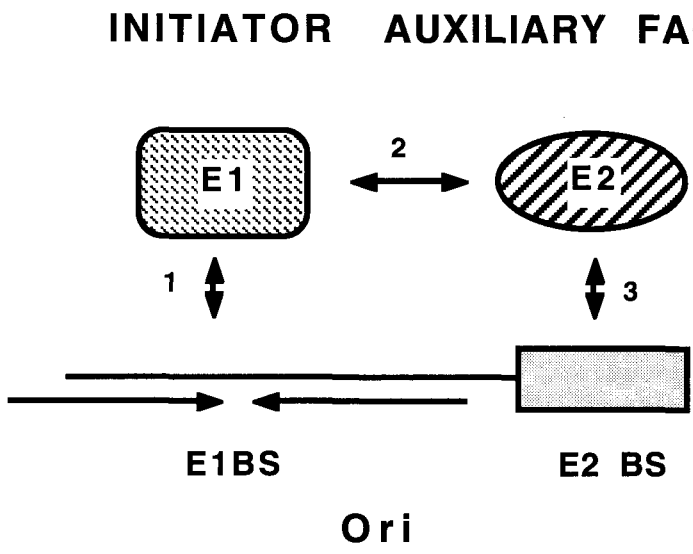

Figure 2 Schematic model illustrating the interactions between E1 and DNA, E2 and DNA, and E1 and E2 that are required for the formation of a replication-competent *ori* complex based on genetic and biochemical analyses. See text for details.

especially at high concentrations of E1(HPV-1). If, on the other hand, the interaction between E1 and DNA is of low specificity, a strong interaction between E2 and E2BSs (2) and between E1 and E2 (3), would be required to bring E1 to the *ori* (HPV-11). A low-specificity interaction between E1 and DNA in HPV-11 is consistent with the recent observation that an in vitro replication system utilizing E1 and *ori* from HPV-11 requires E2 for full activity (Kuo et al. 1994; Liu et al. 1995). This model indicates that the deposition of the viral initiator onto the origin of replication, to form an active initiation complex, can take place via different routes. Origin recognition can be performed by the initiator (E1) itself, or by E2, or by a combination of the two, indicating that E1 is likely to be capable of providing the enzymatic activities required for initiation of replication on DNA sequences lacking an apparent E1BS, consistent with the low *ori* specificity observed for replication in vitro (Yang et al. 1993).

Maintenance

An unusual and interesting feature of papillomavirus DNA replication is the ability of the virus to maintain the viral DNA as a nuclear plasmid in transformed cells over long periods of time (Lancaster 1981; Law et al.

1981). This property, which has been termed maintenance, is one of the clear distinctions between papillomaviruses and other members of the papovavirus group and resembles the latent replication of Epstein-Barr virus (see Yates, this volume). For BPV and some HPVs, this behavior can be observed in transformed cells in culture and is believed to constitute a part of the normal viral life cycle, perhaps corresponding to replication in the basal layer of the wart. This aspect of viral DNA replication has been difficult to study in the context of the viral genome for technical reasons. To circumvent these problems, a cell line that constitutively expresses E1 and E2 proteins has been constructed. *ori*-containing plasmids can be replicated in this cell line, which essentially is a BPV counterpart of the COS cells used for SV40 replication. The use of this cell line has established that long-term maintenance can be achieved in this artificial system and that the only viral gene products that are required are the E1 and E2 proteins (Piirsoo et al. 1996). However, replication of a plasmid per se is not sufficient for stable maintenance; the minimal *ori*, despite being fully replication-competent in transient replication assays, is not maintained in long-term assays. A sequence element located in the E2-dependent enhancer can provide the maintenance activity, and maintenance activity can also be supplied by multimerized E2BSs linked to the *ori*. The mechanism of action of this element is unclear, but a likely possibility is that, as has been proposed for the family of repeats of *oriP* (Yates et al. 1984; Krysan et al. 1989; Wysokenski and Yates 1989), nuclear retention or partitioning at cell division is affected by the presence of this element.

Interestingly, density-labeling experiments using these cell lines with stably replicating plasmids demonstrate that replication of BPV, unlike *oriP* (Yates and Guan 1991), is not controlled by once-per-cell-cycle replication, and consequently, in mammalian cells different strategies can be utilized to achieve stable maintenance of extrachromosomal elements (Piirsoo et al. 1996).

CONCLUSIONS

The early studies addressing the replication properties of papillomaviruses seemed to indicate that DNA replication of papillomaviruses was very different from DNA replication of the well-studied SV40 and polyomaviruses. Both the basic mechanisms and the regulatory circuit by which replication was regulated appeared different, and fairly elaborate models were constructed inspired by bacterial plasmid copy number control systems. However, as the understanding of papillomavirus replica-

tion progressively has improved, it has become clear that the similarities between papillomaviruses and other papovaviruses clearly outweigh the differences, and that at the molecular level these systems are in fact very similar. These similarities include the utilization of an identical or near identical set of cellular factors, as well as viral initiator proteins that are clearly homologous. These factors act on viral *cis*-acting sequences that show clear similarities to other papovaviruses. At present, the regulation of papillomavirus DNA replication has not been studied extensively; however, it seems likely that control of papillomavirus replication is achieved at the level of expression of the viral replication factors E1 and E2, since no indication exists that other viral gene products are required for either transient or long-term replication, at least not in the currently used tissue-culture systems.

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