Constitutive binding of EBNA1 protein to the Epstein–Barr virus replication origin, oriP, with distortion of DNA structure during latent infection

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Replication of the circular, 170 kb genome of Epstein–Barr virus (EBV) during latent infection is performed by the cellular replication machinery under cell-cycle control. A single viral protein, EBNA1, directs the cellular replication apparatus to initiate replication within the genetically defined replication origin, oriP, at a cluster of four EBNA1 binding sites, referred to here as the physical origin of bidirectional replication, or OBR. A second cluster of EBNA1 binding sites within oriP, the 30 bp repeats, serves an essential role as a replication enhancer and also provides a distinct episome maintenance function that is unrelated to replication. We examined the functional elements of oriP for binding by EBNA1 and possibly other proteins in proliferating Raji cells by generating *in vivo* footprints using two reagents, dimethylsulfate (DMS) and KMnO₄. We also employed deoxyribonuclease I (DNase I) with permeabilized cells. The *in vivo* and permeabilized cell footprints at the EBNA1 binding sites, particularly those obtained using DMS, gave strong evidence that all of these sites are bound by EBNA1 in asynchronously dividing cells. No consistent evidence was found to suggest binding by other proteins at any other sites within the functional regions of oriP. Thymines at symmetrical positions of the OBR within oriP were oxidized when cells were treated with permanganate, suggestive of bends or other distortions of DNA structure at these positions; binding of EBNA1 *in vitro* to total DNA from Raji cells induced reactivity to permanganate at identical positions. The simplest interpretation of the results, which were obtained using asynchronously dividing cells, is that EBNA1 binds to its sites at oriP and holds the OBR in a distorted conformation throughout most of the cell cycle, implying that replication is initiated by a cellular mechanism and is not limited by an availability of EBNA1 for binding to oriP.

Key words: DNA replication origin/Epstein–Barr virus/protein–DNA complexes *in vivo*

Introduction

The first event in the initiation of DNA replication is often recognition of an origin of replication by an initiator protein. The initiator proteins of *Escherichia coli* and of the coliphage lambda destabilize the DNA double helical structure at the replication origins of these organisms and enable a cellular helicase to catalyze extensive DNA unwinding and

subsequent initiation of DNA synthesis (Bramhill and Kornberg, 1988; Schnos *et al.*, 1988). For the mammalian DNA virus, SV40, the initiator protein T-antigen not only binds to the replication origin and unwinds it locally, but also serves as the DNA helicase for subsequent steps in replication (Borowiec and Hurwitz, 1988; Stahl *et al.*, 1986). In the yeast, *Saccharomyces cerevisiae*, a protein complex called origin recognition complex (ORC) was found to bind to yeast replication origins and may cause local distortions of DNA secondary structure (Bell and Stillman, 1992; Diffley and Cocker, 1992). For the replication of nuclear chromosomes of higher eukaryotes, neither the *cis*-acting sequences that constitute replication origins nor the proteins that would recognize them are known.

Epstein–Barr virus (EBV), a human herpes virus, may provide insight into how replication initiates on nuclear chromosomes in humans. EBV can infect cells latently and maintain its genome as a circular plasmid as cells proliferate (Nonoyama and Pagano, 1972; Lindahl *et al.*, 1976; Kieff and Liebowitz, 1990). Latently infecting EBV genomes replicate during S phase of the cell cycle and are controlled by the cellular mechanism that limits each segment of nuclear chromosomes to one round of replication per cell cycle (Hampar *et al.*, 1974; Adams, 1987; Yates and Guan, 1991). A single EBV-encoded protein, EBNA1, directs the cellular DNA replication machinery to initiate replication at the EBV replication origin that is active during latency, oriP (Yates *et al.*, 1984, 1985; Lupton and Levine, 1985). Purified EBNA1 protein lacks helicase activities (Frappier and O'Donnell, 1991; Middleton and Sugden, 1992) and, from a deletion analysis of its gene, does not appear to act by performing any enzymatic function (Yates and Camiolo, 1988). Initiation of replication at oriP is thus under cell-cycle control and, in all likelihood, dependent upon a cellular DNA helicase for the initial unwinding of DNA.

EBNA1 binds to clusters of sites in two regions of oriP which are both essential and, together, sufficient for replication (Lupton and Levine, 1985; Rawlins *et al.*, 1985; Reisman *et al.*, 1985) (see Figure 1). One region spans ~120 bp containing four EBNA1 binding sites and is most commonly referred to as the dyad symmetry region. Experiments to map the origin physically using a two-dimensional gel electrophoretic method showed that the dyad symmetry region coincides with the physical origin of bidirectional replication (Gahn and Schildkraut, 1989). Here we will refer to the dyad symmetry region as the origin of bidirectional replication, or OBR, borrowing the term from DePamphilis who coined it to describe the site from which replication forks emanate, as distinct from the set of *cis*-acting elements necessary for replication, to which the term origin has traditionally applied (DePamphilis, 1988). The other known functional element of oriP is a ~20-member family of 30 bp tandem repeats, with each repeat containing an EBNA1 binding site. The 30 bp repeats act in a highly cooperative manner to activate replication and can do so

rather independently of distance and orientation (Reisman *et al.*, 1985; Wysokenski and Yates, 1989). Since tandem copies of the OBR itself are cooperatively self-activating, the role of the 30 bp repeats in replication appears to be simply to provide multiple molecules of bound EBNA1 (Wysokenski and Yates, 1989). The 30 bp repeat family also can be a potent EBNA1-dependent transcriptional enhancer (Reisman and Sugden, 1986; Sugden and Warren, 1989) and performs a role in DNA maintenance (Reisman *et al.*, 1985; Krysan *et al.*, 1989) that is independent of DNA replication (Yates, Ying and Camiolo, unpublished).

We considered it important to investigate how EBNA1 and possibly other proteins from the cell interact with oriP in living cells. The technique of ligation-mediated PCR (LMPCR), developed by Mueller and Wold (1989), has made it easier to use methods of genomic footprinting to examine protein–DNA interactions *in vivo*. Here we report results of footprinting studies using live or permeabilized cells that indicate that EBNA1 binds to all its sites at oriP *in vivo* and that provide no evidence of binding by any other proteins to the functional elements of oriP. EBNA1 appears to bind to oriP and to hold the OBR in a distorted conformation throughout the cell cycle.

Results

Experimental approach

To investigate the binding of EBNA1 and possible cellular proteins to oriP in living or permeabilized cells, we generated footprints of oriP using three different reagents: deoxyribonuclease I (DNase I) and dimethylsulfate (DMS), in order to identify sites bound by proteins, and potassium permanganate, to probe for distortions of DNA secondary structure that could result from binding of proteins. Footprints were visualized with the aid of LMPCR (Mueller and Wold, 1989). With this method, the nicks introduced by a footprinting reagent are identified relative to a reference point defined on each strand by a specific oligonucleotide primer. The products of LMPCR were denatured, separated on sequencing gels, transferred to nylon membranes and detected by hybridization to specific end-labeled oligonucleotide probes. Specific primers were designed to support the generation of footprints on both DNA strands of the three regions of the EBV genome in which EBNA1 binding sites are clustered: the two essential elements of oriP and at a promoter, Fp (Figure 1). For these studies we used the latently infected Burkitt's lymphoma cell line, Raji, which carries ~50 circular EBV genomes per cell.

Binding of EBNA1 to the 30 bp repeats of oriP *in vivo*

Figure 2 shows the DNase I footprints of the 30 bp repeats obtained using Raji cells that had been permeabilized with NP40 (lanes, 'In Vivo'). When compared with the digestion pattern obtained with purified ('Naked') Raji DNA, it is clear that DNase I digestion of permeabilized cells left a footprint in each 30 bp repeat. When viewed from the right (upper strand) and from the left (lower strand), it can be seen that all members of the repeat family (~20 in the Raji EBV strain) contain a clear footprint.

Almost identical DNase I footprints were generated *in vitro* when increasing amounts of GST-EBNA1 fusion protein were added to purified genomic DNA from Raji cells

Fig. 1. EBNA1 binding sites on the EBV genome. The approximate positions of oriP, the EBNA1 coding region, the terminal repeats and the major internal repeat, IR1, are indicated on the circularized EBV genome. The positions of EBNA1 binding, originally called regions 1, 2 and 3 (Rawlins *et al.*, 1985), are indicated in the expanded maps of oriP and the promoter, Fp. Open rectangles represent EBNA1 binding sites at each region. The EBNA1 binding sites at Fp are ~200 bp 3' to the start site of transcription (Schaefer *et al.*, 1991; Sample *et al.*, 1991, 1992). The nucleotide position at the beginning of each region is indicated in accordance with the B95-8 sequence (Baer *et al.*, 1984).

(Figure 2, lanes 'In Vitro'). The GST-EBNA1 fusion protein contains the carboxyl-terminal DNA-binding domain of EBNA1 fused to glutathione-S-transferase and was produced in *E. coli*. We have obtained virtually identical DNase I and DMS footprints *in vitro* whether using GST-EBNA1 or intact EBNA1 expressed from a baculovirus vector in insect cells, so GST-EBNA1, the more conveniently produced protein, was used in these studies for comparisons with the *in vivo* footprints. The nearly identical appearance of the DNase I footprints obtained with permeabilized Raji cells or with purified Raji DNA to which GST-EBNA1 was added implies that every EBNA1 binding site within the 30 bp repeats is occupied by EBNA1 in permeabilized Raji cells and gives no indication that any additional protein joins in binding to this region. Footprinting studies using DMS supported this conclusion.

Studies of EBNA1 binding *in vitro* have shown that EBNA1 inhibits DMS from methylating guanines at positions -7 and +3 of the upper strand and at positions -3 and +7 of the lower strand of each binding site, with base numbering relative to the center of the symmetric EBNA1 binding site (see Figure 8A), whenever guanines are present at these positions (Kimball *et al.*, 1989; Hearing *et al.*, 1992). As shown in Figure 3, in footprints obtained by treating living Raji cells with DMS, these positions were clearly protected from methylation in all repeats where guanines occurred at these positions. Weaker protection from methylation was observed for guanines at -8 and -4 on the upper strand and at +8 and +4 on the lower strand. Very similar DMS footprinting patterns were observed *in vitro* when GST-EBNA1 was added to Raji DNA, as shown for the upper strand in Figure 3 (right panel). Increased reactivity of some G (± 9) and A residues (-11 , $+12$ and ± 5) was observed *in vivo* but was less obvious *in vitro*.

Fig. 2. DNase I footprints of the 30 bp repeats *in vitro* and in permeabilized cells. *In vitro* digestions with DNase I were performed using 10 μ g of Raji genomic DNA alone (naked) or with 100, 400 or 1600 ng of GST-EBNA1 fusion protein (from left to right, indicated by the triangle). For permeabilized cells, DNase I was added to NP40-treated Raji cells for increasing times, as indicated. Maxam–Gilbert chemical-treated purine (A+G) and pyrimidine (T+C) sequencing ladders are shown for each strand. Subsequent analysis, using LMPCR, sequencing gels, transfer to membranes and hybridization, was as described in Materials and methods.

Again, we did not find any evidence that any protein other than EBNA1 binds to the 30 bp repeats *in vivo*.

The pattern of methylation *in vivo* is summarized in Figure 8A. It should be noted that because the 30 bp repeat cluster is close to 600 bp long and the specific primers used in LMPCR were from unique flanking sequences, the upper and lower strands were viewed from different repeat units, at the right and left ends of the repeat cluster, respectively. Figure 8A summarizes the data of the lower strand, obtained from the repeats at the left end of the cluster where the repeats of the Raji strain of EBV match the 30 bp consensus sequence of the prototype strain, B95-8.

Complete binding of EBNA1 to the OBR of oriP *in vivo*

The site of initiation of bidirectional replication, or OBR, of oriP contains two pairs of closely spaced EBNA1 binding sites (Figure 1). Using permeabilized Raji cells to obtain DNase I footprints of this region, footprints spanning each pair of EBNA1 binding sites and DNase I-sensitive sites

between the two pairs were seen (Figure 4). The footprints spanned almost identical nucleotides on both strands as the footprints generated *in vitro* using GST-EBNA1 with purified Raji DNA. Differences between DNase I footprinting patterns obtained *in vitro* and with permeabilized cells were observed beginning a few base pairs to each side of the cluster of four EBNA1 binding sites. This could reflect the presence of nucleosomes bound to these flanking sequences within cells, since experiments using DMS as the footprinting agent, described below, gave no evidence of proteins binding to the major groove of double-stranded DNA in these flanking regions.

DMS footprinting of this region showed a pattern of protection from methylation at the four EBNA1 binding sites *in vivo* that was almost identical to the pattern of protection given by GST-EBNA1 *in vitro* (Figures 5 and 8B). The only differences in protection from methylation were for lower strand guanines at 9119 and 9123, or positions +4 and +8 of site 1. GST-EBNA1 appeared to block methylation of these guanines effectively *in vitro*, but methylation was only

Fig. 3. DMS footprints of the 30 bp repeats *in vitro* and *in vivo*. *In vitro* footprints were generated using 10 μ g of Raji genomic DNA bound by either 0 (naked), 100, 400 or 1600 ng of the GST-EBNA1 fusion protein (indicated by triangle). *In vivo* DMS footprints were generated by treating cells with 0.5% DMS at room temperature for increasing lengths of time, as indicated. Positions of the repeated binding sites are indicated by vertical bars on the sides. The protected guanines are indicated by dots. Chemical sequencing ladders (A+G and C+T) are also indicated.

weakly inhibited *in vivo*. However, the protection of guanines at -3 and $+7$ that is characteristic of EBNA1 binding was clear for site 1 *in vivo*, and for the same site on the opposite strand there was near identity in the extent to which specific guanines were protected from methylation *in vivo* and *in vitro*. We conclude that EBNA1 is bound to site 1 as well as to the three other EBNA1 binding sites at the OBR in Raji cells. No clear evidence to implicate binding by any additional proteins, that is protection of specific guanines from methylation, was found within the DNA sequence spanning the four EBNA1 binding sites or within 100 bp to either side (Figure 5 and other data, not shown).

As was found at the 30 bp repeats, hypersensitivity to methylation was more pronounced *in vivo* than *in vitro* (Figures 5 and 8B). Certain adenines outside the binding sites, especially at 9022, 9080 and 9134, and a guanine at 9078 were hypersensitive *in vivo* but not *in vitro*. These hypersensitive adenines were within one to four bases of DNase I-sensitive sites. In addition, increased, albeit weak, reactivity was observed at certain cytosines *in vivo*. These differences between footprints obtained *in vivo* and *in vitro* could be due to differences in ionic conditions, differences in torsional stress on the DNA, to effects of chromatin proteins bound to nearby sequences or to interactions between EBNA1 and cellular proteins.

The visual clarity of the DMS footprints at the OBR *in vivo* indicates that most of the guanines within the EBNA1

binding sites are protected from methylation as effectively *in vivo* as they are *in vitro* when saturating amounts of GST-EBNA1 are present. This was confirmed by quantifying the signals representing specific bases using a PhosphorImager. Guanines at 9108 and 9109 in site 1 (upper strand) and at 9047 and 9048 in site 4 (lower strand) were chosen for measurements because these were among the guanines most completely protected against methylation *in vitro* and were in the lower portions of the gels where bands were resolved well. Within each gel lane, the values at the protected guanines were normalized to values observed at guanines where methylation rates appeared to be neither reduced nor enhanced both *in vivo* and *in vitro* (at 9070 and 9125 on the upper strand and at 9032 and 9048 on the lower strand).

These data, presented in Table I, show that with 0.1 μ g of GST-EBNA1 added to 10 μ g of Raji DNA *in vitro*, protection from methylation at guanines in sites 1 and 4 was from 16% to 35% and increased to 68–81% when 0.4 μ g of GST-EBNA1 was added. With four times more GST-EBNA1 present, protection increased just slightly, to 76–85%, indicating that GST-EBNA1 was present at saturating levels and that the levels of protection observed were indicative of essentially complete occupancy of these sites by EBNA1. The extent of protection from methylation found at these positions *in vivo* in two independent experiments was very similar, 70–85%, indicating that these sites are fully occupied by EBNA1 in Raji cells. Judging

Fig. 4. DNase I footprints of the OBR of oriP *in vitro* and in permeabilized cells. The four EBNA1 binding sites in this region are indicated by vertical bars and numbers. The symbols at the top are as described for Figure 2.

the error of measurements from the binding curve obtained *in vitro* suggests to us that if 20% of the sites had been unoccupied *in vivo*, then the amount of protection observed *in vivo* would have appeared significantly different quantitatively than the maximal protection observed *in vitro*. Because the populations of Raji cells had not been synchronized with respect to the cell division cycle, this result implies that EBNA1 binds to the OBR throughout most or all of the cell cycle.

Distortion of DNA structure at the OBR of oriP *in vivo*

Binding of EBNA1 to the OBR of oriP *in vitro* causes specific thymines at two localized regions of the OBR to become sensitive to oxidation by permanganate (Figures 6 and 8B; see also Frappier and O'Donnell, 1992; Hearing *et al.*, 1992). One region is located on the upper strand within site 4 (thymines at positions 9045 and 9046). The other region is on the lower strand at a symmetric position within site 1 (thymines at 9110 and 9111) and also includes thymine 9103, which is located between sites 1 and 2. The positions of these hypersensitive thymines suggests that a distortion of DNA secondary structure, such as bending or untwisting (Borowiec *et al.*, 1987; Borowiec and Hurwitz, 1988), results from binding of EBNA1 to each pair of sites. The same thymines were found to be hypersensitive to

oxidation by permanganate in live Raji cells, implying that similar DNA structures result from EBNA1 binding *in vivo* (Figure 6).

An obvious discrepancy between our results and the results of two other laboratories (Frappier and O'Donnell, 1992; Hearing *et al.*, 1992) is that the other laboratories did not detect oxidation at the thymine at position 9103, although we have reproducibly observed strong reactivity at this position *in vivo* as well as *in vitro* in the presence of GST-EBNA1. A difference that conceivably might account for the discrepancy is that in the other laboratories oxidized thymines were detected by blocking DNA synthesis in a primer extension assay, while our studies required cleaving the DNA strands at oxidized positions using piperidine. In our studies we also noted weak reactivity at thymines at 9045 and 9111 that was clearly induced by EBNA1 binding, although these thymines reacted to a lesser extent than did the thymines immediately adjacent to them. It is not clear from the published data whether oxidation was induced at these more weakly reactive thymines in the experiments performed in the other laboratories. Only at these five specific thymines, summarized in Figure 8B, was EBNA1 judged to increase reactivity to permanganate *in vitro*; minor lane-to-lane differences seen at other positions (mostly at purines) in Figure 6 were not reproducible and other minor differences between *in vivo* samples and naked DNA treated

Fig. 5. DMS footprints of the OBR of oriP *in vitro* and *in vivo*. The four binding sites are indicated by vertical bars and numbers. Guanines that are clearly protected are shown by dots. Weakly protected guanines were sometimes less obvious for *in vivo* footprints, apparently because of higher levels of background ladders.

in vitro (also mostly at purines) could not be attributed to EBNA1 directly.

Binding of GST-EBNA1 *in vitro* to the 30 bp repeats or to the Fp sites on the EBV genome did not induce noticeable sensitivity to permanganate at specific thymines on either DNA strand. Similarly, *in vivo*, neither of these regions of DNA, including more than 1000 bp of sequences examined, revealed any permanganate-sensitive sites on either strand (data not shown). It is therefore very likely that the sensitivity to oxidation by permanganate of the specific thymines at the OBR results from specific interactions between EBNA1 and DNA of the OBR *in vivo*.

The extent of hypersensitivity to oxidation by permanganate that was observed at these sites *in vivo* tends to corroborate a conclusion drawn from the DMS footprinting studies, namely that EBNA1 binds to the OBR throughout most of the cell cycle. The extent of oxidation at specific thymines can be judged by the intensity of the bands representing these thymines relative to background bands, which are primarily due to cleavage at purines during treatment with piperidine. At a low concentration of GST-EBNA1 *in vitro*, which resulted in partial binding to the OBR sites as judged from the DMS footprints, the gel bands representing the hypersensitive sites were less prominent, relative to background bands in each lane, than when enough GST-EBNA1 was added to bind the OBR sites completely (Figures 5 and 6). *In vivo*, the extent of permanganate hypersensitivity relative to background bands appeared very similar to the extent induced *in vitro* using saturating amounts of GST-EBNA1. It therefore appears that the majority of

Table I. Quantification of protection from methylation by EBNA1 binding at the OBR *in vitro* and *in vivo*

Experimental conditions	Relative methylation (protection)			
	Site 1		Site 4	
	G9108	G9109	G9048	G9047
<i>In vitro</i> ^a , 0 µg GST-EBNA1	100 (0)	100 (0)	100 (0)	100 (0)
<i>In vitro</i> ^a , 0.1 µg GST-EBNA1	84 (16)	73 (27)	65 (35)	77 (23)
<i>In vitro</i> ^a , 0.4 µg GST-EBNA1	32 (68)	20 (80)	29 (71)	19 (81)
<i>In vitro</i> ^a , 1.6 µg GST-EBNA1	19 (81)	15 (85)	24 (76)	18 (82)
<i>In vivo</i> ^a	16 (84)	17 (83)	30 (70)	18 (82)
<i>In vivo</i> ^b	15 (85)	15 (85)	27 (73)	16 (84)

^aThe data are from the experiments shown in Figure 5. Relative methylation is the value for each base expressed as a percent of the value obtained in the absence of added GST-EBNA1 protein (*in vitro*) or of the value obtained with naked DNA (*in vivo*). In each lane values were normalized to the product averages of values from G9070 and G9125 for site 1 and from G9084 and G9032 for site 4.

^bFrom an independent experiment. Values were normalized using signals from G9125 for site 1 and G9032 for site 4.

DNA molecules in asynchronously dividing cells are held in the permanganate-sensitive conformation that results from binding by EBNA1. However, more complex explanations are possible and are considered in the Discussion.

Fig. 6. Permanganate-sensitive sites at the OBR *in vitro* and *in vivo*. The four binding sites are indicated by vertical bars and numbers. The positions of thymines that were made reactive to permanganate by binding of EBNA1 *in vitro* are indicated by arrow heads.

Binding of EBNA1 to sites at the EBV promoter, Fp, *in vivo*

The two EBNA1 binding sites located at the EBV promoter, Fp, are the lowest in affinity for EBNA1 among the specific binding sites on the EBV genome and are presumed to perform a role in regulating transcription. We tested whether these sites were occupied by EBNA1 in Raji cells by performing *in vivo* DMS footprinting. As can be seen in Figure 7, specific guanines on both strands within these EBNA1 binding sites were clearly protected from methylation *in vivo* when compared with the patterns obtained using purified DNA. The same guanines were protected from methylation *in vitro* when GST-EBNA1 was added to Raji DNA (lower strand, Figure 7; upper strand, data not shown). However, the highest concentration of GST-EBNA1 used *in vitro*, 1.6 μg in a volume of 100 μl , was insufficient to give footprints that were as complete as those observed *in vivo*, even though one-quarter of this concentration was adequate for complete binding to the specific sites at both regions of oriP (compare Figure 7 with Figures 3 and 5). This is consistent with the binding sites at Fp having lower affinities for EBNA1 than the sites at oriP. *In vivo*, the extent of protection at certain guanines at both Fp sites was $\sim 80\%$ as determined by radiographic image analysis, similar to the level of protection achieved with saturating amounts of GST-EBNA1 *in vitro* at the oriP sites. The results are thus consistent with the Fp sites being essentially completely bound by EBNA1 in Raji cells.

Differences in how EBNA1 contacts different binding sites

EBNA1 binds to its specific sites at the 30 bp repeats, at the OBR and at Fp with decreasing affinities, and serves different biological roles at each location (Jones *et al.*, 1989). Comparisons of the DMS footprints at these different sites revealed that contacts between EBNA1 and specific guanines often differ among different binding sites; this is summarized in Figure 8. In Figure 8, each binding site is indicated by a line drawn between the DNA strands and indicating a 16 bp site (numbered -8 to $+8$) as was defined by mutational analysis (Ambinder *et al.*, 1990). One clear difference is in the extent of protection of guanines from methylation for upper strand guanines that occur at -8 and lower strand guanines that occur at $+8$. In the 30 bp repeats these bases are always just weakly protected, whereas in the lower affinity sites at Fp and at some sites in the OBR they are strongly protected. When a guanine occurs at $+7$ or -7 it is strongly protected from methylation, with the exception of G9101 at site 2 in the OBR, which is only moderately protected. This base is only two bases away from a permanganate-sensitive thymine. Guanines at positions ± 9 of binding sites in the 30 bp repeats become hyper-reactive to methylation when EBNA1 binds. Although this position is just outside of the mutationally defined binding site, weak protection at this position was seen in two cases, at sites 1 and 4 of the OBR (G9104 and G9049). These bases could be within the regions of altered DNA secondary structure

Fig. 7. DMS footprinting at Fp *in vitro* and *in vivo*. The two binding sites in this region are indicated by vertical bars. The protected guanine residues are shown by dots.

A. 30-bp repeat

C. EBNA1 sites at Fp

B. OBR

Fig. 8. Summary of the *in vivo* footprinting data for the different EBNA1 binding sites. Each EBNA1 binding site is indicated by a horizontal line drawn between the complementary DNA strands. Each binding site is shown to span the 16 bp within which base specificity was shown to contribute to binding by EBNA1 (Ambinder *et al.*, 1990). Nucleotides protected from digestion by DNase I are spanned by thick lines outside of the two strands. Guanine that are strongly protected from being methylated by DMS are indicated by filled circles, moderately protected guanines by crossed circles and weakly protected guanines by open circles. Enhanced reactivity to DMS is indicated by one or two asterisks. (A) The 30 bp consensus sequence of strain B95-8 is shown. The bases are numbered relative to the center of the symmetrical EBNA1 binding site as done by Ambinder *et al.* (1990). The leftmost repeats of Raji EBV match this sequence, as do some of the repeats at the right end of the cluster but in the opposite orientation. In some repeats there were also several sequence differences that would not be expected to affect significantly the binding by EBNA1 (Ambinder *et al.*, 1990). (B) Sequences from 9022 to 9134 within the OBR of oriP, with base numbering according to the B95-8 sequence (Baer *et al.*, 1984). The four EBNA1 binding sites are numbered in accordance with Rawlins *et al.* (1985). Arrows indicate the thymine that are sensitive to oxidation by permanganate and nucleotides that are sensitive to cleavage by DNase I, as labeled. (C) EBNA1 binding sites near the EBV promoter, Fp.

since they are only a few base pairs away from permanganate-sensitive thymines on the opposite strands.

Discussion

Three main conclusions were drawn from these *in vivo* footprinting studies of oriP. First, EBNA1 was found to occupy *in vivo* all sites within oriP to which EBNA1 was known to bind *in vitro*, while no evidence was found to suggest that any other proteins bind specifically within the functional elements of oriP. DNase I-sensitive sites at the 30 bp repeats and at the OBR imply that both of these regions of oriP are free of nucleosomes. Second, binding by EBNA1 to the four sites at the OBR of oriP, where bidirectional replication initiates, causes distortions in the DNA secondary structure *in vitro*, and the same distortions are observed *in vivo*, as revealed by sensitivity to oxidation of specific thymines by permanganate. Third, the clarity of DMS footprints and the degree of sensitivity to KMnO₄ observed *in vivo* suggest that EBNA1 binds to oriP throughout most of the cell cycle while, by the simplest interpretation, constitutively holding the OBR in a distorted conformation.

There have been reports of cellular factors that can bind to oriP *in vitro*, but there is no evidence to suggest that any of these factors does so *in vivo*. Oh *et al.* (1991) identified at least two distinct factors present in HeLa nuclear extracts that could bind to four sites at the OBR which overlapped the EBNA1 binding sites. These investigators showed that factors in the extract competed with EBNA1 for binding to the OBR and raised the possibility that these cellular factors might regulate EBV DNA replication in this manner. Our results argue against such a possibility, at least for Raji cells, by revealing essentially complete binding by EBNA1 to its sites at the OBR over most of the cell cycle and by failing to reveal evidence of binding of any other proteins to the OBR. Although it is conceivable that EBV latent replication might be controlled in cells of epithelial origin, such as HeLa, differently than in the B-lymphoid cell line, Raji, we consider this unlikely. EBV-derived plasmids were found to be governed as strictly in a cell line of epithelial origin, 293, as in Raji cells by the cellular mechanism that allows each DNA molecule to be replicated once, but only once, per cell cycle (Yates and Guan, 1991).

Wen *et al.* (1990) reported that proteins of sizes 40 and 60 kDa that could bind to the EBNA1 binding site of the 30 bp repeats of oriP, as revealed by DNase I footprints, were induced in an EBV-negative Burkitt's lymphoma cell line by treating the cells with the phorbol ester, TPA. These proteins could not be detected in cell extracts when the cells were not treated with TPA. In addition, because it is unlikely that these cellular proteins would give DMS footprints indistinguishable from those of EBNA1 or would distort DNA structure at the OBR in the manner of EBNA1, the *in vivo* footprints that we have observed with Raji cells are very likely to represent binding by EBNA1 rather than by cellular proteins that could recognize the same sites.

Other researchers have reported that binding of EBNA1 to the OBR of oriP *in vitro* causes specific thymines to become sensitive to oxidation by permanganate, suggestive of distortions of DNA secondary structure (Hearing *et al.*, 1992; Frappier and O'Donnell, 1992). The finding of this study, that the same thymines that become sensitive to oxidation when EBNA1 binds to the OBR *in vitro* are sensitive to oxidation *in vivo*, and apparently to a comparable

extent, adds validity to the observations made *in vitro*. No such distortions of DNA secondary structure were found to be induced by binding of EBNA1 to other regions of the EBV genome, the 30 bp repeats and the F promoter, either *in vitro* or *in vivo*. It is therefore tempting to speculate that the localized DNA structural changes that result from EBNA1 binding to the OBR of oriP are involved in initiating DNA unwinding to allow replication to begin.

The nature of the distortions of DNA structure induced by binding of EBNA1 to the OBR has not been determined. Thymines are sensitive to oxidation by permanganate in single-stranded DNA or in double-stranded DNA that is structurally distorted by a bend or, apparently, by reduced helical pitch (Borowiec *et al.*, 1987; Borowiec and Hurwitz, 1988). The conformational changes at the OBR appear to be quite localized since nearby thymines do not become reactive. It will be important to determine precisely what sequences within the OBR are required for replication, what sequences are required for the DNA conformational changes that result from binding of EBNA1, and the relationship between the two. An obvious factor could be the spacing between the EBNA1 binding sites. The EBNA1 binding sites at the OBR can be viewed as two pairs of binding sites, each pair with a center-to-center spacing of 21 bp (two full turns of the B form double helix), and each pair associated with a conformational change caused by binding of EBNA1 (Figure 8B). The spacing between binding sites at the 30 bp repeats is, of course, 30 bp, and it is 26 bp for the Fp sites. Interestingly, when comparing the OBR of EBV with the homologous region of the closely related virus, herpes virus Papio, the only features that are obviously shared are the presence of EBNA1 binding sites and the 21 bp spacings between sites (Loeb *et al.*, 1990).

EBNA1 protein lacks detectable helicase activity, so a cellular helicase must perform the strand separation reaction to permit initiation of replication (Frappier and O'Donnell, 1991; Middleton and Sugden, 1992). The structural distortions at the OBR apparently result directly from binding of EBNA1 to the OBR. Our experiments using the GST-EBNA1 fusion protein showed that little more than the DNA binding domain of EBNA1 is required to distort the OBR structure. From the results of a deletion analysis of the EBNA1 gene it appeared that a host-specific factor that has been postulated to be required for initiation of replication at oriP interacts with the DNA-binding domain of EBNA1 (Yates and Camiolo, 1988; Wysokenski and Yates, 1989). This host-specific factor could involve a DNA helicase that might initiate DNA unwinding at the positions where EBNA1 distorts DNA secondary structure.

The conclusion, that all EBNA1 binding sites within oriP are occupied by EBNA1 for apparently all EBV genomes throughout most of the cell cycle, implies that initiation of replication at oriP is not limited by an availability of EBNA1 for binding to oriP. This is consistent with the previous observation that over-expression of EBNA1 in cells did not increase the rate of replication of plasmids carrying oriP (Yates and Guan, 1991). It should be pointed out that a small fraction of EBV genomes lacking bound EBNA1, less than 20%, might have gone undetected in the *in vivo* footprints. However, the results clearly demonstrate that binding of EBNA1 to oriP could not be limited to the S phase of the cell cycle.

The implication of the permanganate sensitivity studies that the OBR of oriP is held constitutively by EBNA1 in a

distorted conformation deserves some caution. Unlike the DMS footprinting studies in which the absence of binding is detected as reactive bases in the sequence, the absence of induced sensitivity of thymine bases to permanganate in a significant fraction of the DNA molecules cannot be observed directly. For this reason it was necessary to judge the extent of reactivity of specific thymines *in vivo* against a background of cleavages that occurred mainly at purines as a result of the piperidine treatment. If, for unknown reasons, DNA from cells treated with permanganate gave a higher or lower rate of nonspecific cleavage during piperidine treatment than occurred with DNA treated with permanganate *in vitro*, then the comparison of reactivities *in vivo* with those *in vitro* at the hypersensitive thymines could be inapplicable. However, there is no reason to suspect that this is the case. In addition, since the distortion of DNA structure at the OBR results directly from binding by EBNA1 *in vitro*, it is reasonable to expect that the same structural alteration might result from EBNA1 binding *in vivo*, and this is the simplest interpretation of the results.

All of these observations are in keeping with the conclusion that initiation of replication at oriP is controlled by the cellular mechanism that limits each region of chromosomal DNA to a single round of replication per S phase (Adams, 1987; Yates and Guan, 1991). In the yeast, *S.cerevisiae*, as with latent EBV replication in human cells, the step that confers origin specificity, binding by specific proteins required for replication, appears not to be under cell-cycle control, though the event of initiation is. A multi-protein origin recognition complex, or ORC, was identified based on its ability to bind *in vitro* to a consensus sequence thought to be essential for origin activity (Bell and Stillman, 1992). A recent footprinting study using permeabilized yeast cells revealed a replication origin in an apparent complex with the ORC and exhibiting some local distortions of DNA secondary structure throughout most of the cell cycle (Diffley and Cocker, 1992).

It remains possible that proteins other than EBNA1 bind to specific sequences at the OBR, but do so only briefly during S phase. Attempts to view initiation events by synchronizing cells using a double thymidine block did not reveal any significant changes in the DMS or KMnO₄ footprinting patterns at the OBR when examined at different times in S phase after release of the block, in comparison with results with asynchronous populations (data not shown). Potential interactions between replication proteins and the OBR as well as the separation of DNA strands during initiation may occur over a time interval that is too brief to be observed even in synchronized populations.

The 30 bp repeats of oriP act as a replication enhancer *in vivo*. *In vitro*, EBNA1 molecules bound to the 30 bp repeats will form a complex with EBNA1 molecules bound to the OBR, creating a DNA loop that can be observed by electron microscopy (Frappier and O'Donnell, 1991; Su *et al.*, 1991; Middleton and Sugden, 1992). It has been suggested that this interaction could activate replication in one of three ways. It might stabilize the binding of EBNA1 to the OBR, which was demonstrated *in vitro*, it might affect DNA structure to aid unwinding, or it could help to attract the cellular replication machinery to the OBR (Frappier and O'Donnell, 1991; Su *et al.*, 1991; Middleton and Sugden, 1992). Our results also could have relevance to the possibility that the essential role of 30 bp repeats in replication is to stabilize the binding of EBNA1 to the OBR. The fact that

the Fp sites, which have lower affinities for EBNA1 than the OBR sites, are fully occupied in Raji cells could be interpreted in two ways. One possibility is that binding of EBNA1 to these sites is also stabilized, perhaps by cooperative interactions with other proteins bound at the F promoter or conceivably through interactions with the EBNA1 30 bp repeat complex forming a 50 kb DNA loop. However, it is also a possibility that EBNA1 is present at sufficient levels in Raji cells to bind stably to these lower affinity sites without such cooperative interactions.

Regarding the possibility that the EBNA1 molecules bound to the 30 bp repeats might aid in distorting DNA structure at the OBR to aid DNA unwinding, the 30 bp repeats do not contribute *in vitro* to the structural distortions at the OBR that are revealed by permanganate (Frappier and O'Donnell, 1992; Hearing *et al.*, 1992; our unpublished data). The primary role of the 30 bp repeats might be to attract the replication machinery to the OBR. As the OBR is destabilized and unwound during initiation of replication, EBNA1 would be displaced from these sites, but EBNA1 bound to the 30 bp repeats in the same complex could stabilize interactions between replication proteins and the OBR during this reaction.

Materials and methods

GST-EBNA1

This fusion protein contains EBNA1 amino acids 8–40 followed by 422–641, the carboxy-terminal DNA-binding domain, fused to the carboxy-terminus of glutathione-S-transferase. The GST-EBNA1 expression plasmid, p670, was made by inserting an EBNA1 gene carrying an internal, in-frame deletion (*NcoI*–*Bst*XI) into the *Sma*I site of the bacterial expression vector, pGEX-3X (Pharmacia). To obtain GST-EBNA1, an overnight culture of *E.coli* carrying p670 was diluted 20-fold in Luria broth containing ampicillin and 0.2 mM IPTG and shaken for 6 h at 37°C. Bacteria from 4 liters of culture were pelleted, washed with phosphate buffered saline (PBS) containing 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF), 6 µM leupeptin, 6 µM pepstatin and 0.8 µM aprotinin, and then ground with 2 g alumina per 1 g of wet bacteria. Soluble proteins were washed from the alumina in two 10 ml washes with the same buffer containing 1% Triton X-100 and passed over a pre-packed glutathione–Sephacrose column (Pharmacia), from which GST-EBNA1 was eluted with glutathione according to the manufacturer's instructions into tubes containing enough glycerol to give a final concentration of 20%. To remove degradation products containing GST and other contaminants, fractions containing GST-EBNA1 were pooled and passed on to a 3 ml column of heparin–agarose (type I, Sigma), equilibrated with PBS containing 1 mM EDTA, 1 mM DTT and 10% glycerol. The column was washed with the same buffer, then with buffer in which NaCl had been increased to 0.6 M, and finally with buffer containing 0.65 M NaCl to elute GST-EBNA1. Approximately 1.6 mg of GST-EBNA1 was recovered in 2 ml.

DNase I footprinting *in vitro*

The *in vitro* DNase I protection assays were performed mainly as described by Gralla (1985). In 100 µl reaction volumes, 10 µg of Raji genomic DNA was combined with increasing amounts of GST-EBNA1 fusion protein in a buffer containing 15 mM sodium phosphate (pH 7.5), 2.5% glycerol, 1 mM EDTA, 1 mM DTT and 150 mM NaCl. Binding was performed at 37°C for 30 min. 10 µl of 10 × DNase I buffer (500 mM Tris pH 7.4, 100 mM MgCl₂, 100 mM DTT) was then added, along with DNase I to achieve a final concentration of 0.2 µg/ml. Incubation was continued at 37°C for another 2 min, and digestion was stopped by adding an equal volume of buffer containing 1% SDS, 10 mM Tris pH 7.4 and 100 mM EDTA. The reaction was deproteinized by mixing twice with phenol–chloroform (1:1), then twice with chloroform, and then precipitated by adding 2 vols of ethanol. The DNA was resuspended in deionized, filtered water to a concentration of 0.1 µg/µl and subjected to LMP-PCR.

DMS footprinting *in vitro*

DMS was added to the binding reactions to a concentration of 0.2% by volume. After 2 min at room temperature, the reactions were stopped by adding 10 µl of 3 M sodium acetate (pH 5.2), and DNA was precipitated

by addition of ethanol. The pellets were washed with 70% ethanol and resuspended in 100 μ l of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). DNA was deproteinized and precipitated with ethanol as described above. Piperidine cleavage of the DMS-treated DNA was as described by Maxam and Gilbert (1977). After the samples had been dried under vacuum, the DNA was resuspended in water to 0.1 μ g/ μ l and subjected to LMPCR.

KMnO₄ footprinting *in vitro*

KMnO₄ was added to the binding reactions to a final concentration of 10 mM. Incubation was continued at room temperature for 2 min and stopped by adding 200 μ l of 'stopping solution' (1 M 2-mercaptoethanol, 150 mM NaCl, 5 mM EDTA) and precipitated with ethanol. Pellets were washed with 70% ethanol, resuspended in 100 μ l of TE buffer, deproteinized and precipitated with ethanol. The DNA was subjected to piperidine cleavage, resuspended in water to 0.1 μ g/ μ l and analyzed by LMPCR.

DNase I footprinting in permeabilized cells

Permeabilized cells were used as described previously (Rigaud *et al.*, 1991). Briefly, a 50 ml culture of exponentially growing Raji cells ($3-5 \times 10^5$ cells/ml), grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, was centrifuged at 500 g for 5 min in a table-top centrifuge. The cell pellet was washed once with 10 ml of PBS and once with 5 ml of PBS containing 0.1 mM PMSF. The cell pellet was then resuspended in 0.5 ml physiological buffer (PB) (11 mM potassium phosphate, pH 7.4, 108 mM KCl, 22 mM NaCl, 1 mM MgCl₂, 1 mM DTT and 1 mM ATP) containing 0.4% NP40 and held for 1 min at room temperature. 0.5 ml of PB containing 100 μ g DNase I per ml was added and samples were incubated at room temperature for various lengths of time. Reactions were stopped by adding 2 ml of lysing buffer (1% SDS, 10 mM Tris pH 7.4, 100 mM EDTA), and proteinase K to 600 μ g/ml and genomic DNA was purified as described by Sambrook *et al.* (1989). DNA was diluted to 0.1 μ g/ μ l and subjected to LMPCR.

DMS footprinting *in vivo*

Raji cells were pelleted as described above and washed once with ice-cold PBS. The cell pellet was resuspended in 1 ml PBS and DMS was added to a final concentration of 0.5%. Reactions were performed at room temperature for various lengths of time and stopped by adding 2 ml stopping solution and 10 ml ice-cold PBS. Cells were immediately pelleted and lysed, and genomic DNA was purified as described above. DNA was treated with piperidine as described by Maxam and Gilbert (1977) and finally resuspended in H₂O to 0.1 μ g/ μ l for analysis by LMPCR.

KMnO₄ footprinting *in vivo*

Treatment of viable cells with KMnO₄ was as described by Pfeifer and Riggs (1991). Cells were pelleted, washed with ice-cold PBS and resuspended in 1 ml PBS. KMnO₄ was added to a final concentration of 20 mM. Reactions were carried out at room temperature for different lengths of time and stopped by adding 2 ml stopping solution and 10 ml ice-cold PBS. Subsequent DNA purification and piperidine cleavage were as described above for DMS footprinting *in vivo*.

Ligation-mediated PCR

One microgram of Raji genomic DNA that had been treated with DNase I or with chemical reagents was analyzed using LMPCR, essentially as described by Mueller and Wold (1989) except that reverse transcriptase (Superscript RNaseH⁻ RT, Bethesda Research Laboratories) was substituted for Sequenase for synthesis of the first strand, which was conducted at 45°C to minimize secondary structures. The same double-stranded oligonucleotide linker as used by Mueller and Wold was ligated to the blunt ends resulting from this initial primer extension reaction. Nested oligonucleotides were used in each series of reactions: one (i) for the initial primer extension, a second (ii), located 3' to the first, for PCR (the other primer for PCR is one strand of the ligated linker), and a third (iii), located 3' to the second, for detection by hybridization. The following sets of oligonucleotides i, ii and iii for each LMPCR assay are given, 5' to 3', followed by the calculated melting temperatures: 30 bp repeats, for analysis of the upper strand, (i) 8087-CTCATATTCACGAGGTCGC-8059, 50°C, (ii) 8067-CGAGGTCGCTGAGAGCAGCGTGGG-8044, 71°C, (iii) 8052-CACGGTGGGCTAATGTTGCCATGGG-8028, 70°C; 30 bp repeats, for analysis of lower strand, (i) 7287-CCAATTCCAACCATTTTAA-7306, 49°C, (ii) 7330-ACGGCATCGAGGAAAAGGACCAAGCC-7354, 68°C, (iii) 7354-AGCGAAAATTCACGCCCTTGGGA-7378, 71°C; OBR, analysis of upper strand, (i) 9220-AACGTCAATCAGAGG-GGC-9203, 52°C, (ii) 9192-ACCGATAAGCGGACCCCTCAAGAGGG-C-9167, 70°C, (iii) 9184-GCGGACCTTCAGAGGCGGCTTAGCAAT-A-9157, 69°C; OBR, analysis of lower strand, (i) 8943-GGAATCCT-GACCCCATGT-8962, 51°C, (ii) 8966-AAACCGTGACAGCTCATGG-

GGTGGG-8990, 69°C, (iii) 8987-TGGGAGATATCGCTGTTCCCTTAG-GACCC-9014, 67°C; Fp, analysis of upper strand, (i) 62538-ACACCG-TGCGAAAAGAAG-62521, 51°C, (ii) 62513-ATCCGCCGCTCCCA-GCTGC-62494, 70°C, (iii) 62500-CAGCTGCCCAAAATGCCAAAAT-GTAAGG-62473, 68°C; Fp, analysis of lower strand, (i) 62370-TGGT-CGCTAGATGGCG-62385, 50°C, (ii) 62391-GAGGCCACGCTTTGC-GAAAACGAAA-62409, 69°C, (iii) 62411-CGAAAGTGCTTGAAGAAG-GCGCGGA-62435, 71°C.

LMPCR reactions were 'multiplexed' in the following way to minimize the number of sequencing gels required. At each reaction step, upper strand primers for the three regions were mixed, as was done with the lower strand primers, so that only two rather than six reactions had to be performed for each treated DNA sample. Blots of the sequencing gels were then hybridized sequentially with oligonucleotide probes of the three regions. One-twentieth to one-fifth of each PCR product was electrophoresed on an 8% denaturing polyacrylamide gel and transferred to Genescreen nylon membranes (Dupont) electrophoretically using a Church-Gilbert transfer apparatus from Polytech Products (Cambridge, MA) and crosslinked with UV irradiation of 1200 J/m². Oligonucleotides were end-labeled using polynucleotide kinase and hybridized to membranes using a Hoefer hybridization oven. Hybridization was carried out in a solution containing 5 $\times$ SSC (1 $\times$ SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 20 mM sodium phosphate pH 7.0, 10 $\times$ Denhardt's solution [Ficoll, polyvinylpyrrolidone and bovine serum albumin each at 0.2% (w/v)] and 7% SDS at 60°C for 20 h. Membranes were washed once with a solution containing 3 $\times$ SSC, 10 mM sodium phosphate pH 7.0, 10 $\times$ Denhardt's solution and 5% SDS at 60°C for 1 h, then once with a solution containing 1 $\times$ SSC and 1% SDS at 60°C for 1 h, and then exposed to X-ray film without intensifying screens for 2–24 h. To quantify band intensities, radiographic images were generated using a PhosphorImager (Molecular Dynamics).

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