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Interactions of Epstein–Barr Virus Origins of Replication with Nuclear Matrix in the Latent and in the Lytic Phases of Viral Infection

Elena Mattia,*[1] Mara Ceridono,* Silvia Chichiarelli,† and Maria D'Erme†

*Microbiology Institute, Faculty of Medicine and Surgery, and †Department of Biochemical Sciences, University of Roma "La Sapienza," Rome, Italy

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Eukaryotic DNA is organized into domains or loops generated by the attachment of chromatin fibers to the nuclear matrix via specific regions called scaffold or matrix attachment regions. The role of these regions in DNA replication is currently under investigation since they have been found in close association with origins of replication. Also, viral DNA sequences, containing the origins of replication, have been found attached to the nuclear matrix. To investigate the functional role of this binding we have studied, in Raji cells, the interaction between Epstein–Barr virus (EBV) origins of replication and the nuclear matrix in relation to the viral cycle of infection. We report here that both the latent (ori P) and the lytic (ori Lyt) EBV origins of replication are attached to the nuclear matrix, the first during the latent cycle of infection and the second after induction of the lytic cycle. These findings suggest that the binding of the origins of replication with the nuclear matrix modulates viral replication and expression in the two different phases of infection. © 1999 Academic Press

INTRODUCTION

The nuclear matrix is a proteinaceous framework that organizes the DNA into topologically distinct loops (Phi-Van and Strätling, 1990; Roberge and Gasser, 1992) anchored at specific sites named scaffold/matrix attachment regions (S/MARs) (Mirkovitch *et al.,* 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1987).

These evolutionary conserved DNA elements are generally 300–3000 bp long, contain several AT-rich sequence motifs and Topo II cleavage sites (Gasser *et al.,* 1989; Adachi *et al.,* 1989), and have the potential to unwind under conditions of superelical stress (Bode *et al.,* 1992; Kohwi-Shigematsu and Kohwi, 1990). It has been shown that S/MAR elements flank actively transcribed genes and increase the transcriptional activity of a reporter gene in stably transfected cells (Blasquez *et al.,* 1989; Phi-Van and Strätling, 1990; Stief *et al.,* 1989) or in transgenic mice (Xu *et al.,* 1989).

In addition S/MARs may act as boundery sequences by sheltering the chromatin from position effects (Gasser and Laemmli, 1986a,b; Dijkwel and Hamlin, 1988).

It has been shown that S/MARs–nuclear matrix interactions play a role in DNA replication and gene expression. In this respect, while evidence has been provided that origins of replication are close or coincide with S/MARs in several cell systems (Razin *et al.,* 1986; Mah *et al.,* 1993; Amati *et al.,* 1990; Amati and Gasser, 1990; Brun *et al.,* 1990; Umek *et al.,* 1989; Dubey *et al.,* 1991), the contribution of matrix attachments to mammalian origin function is still not clear.

Several studies have reported that viral genomes replicate and express in association with the nuclear matrix (Deppert and Schirmbeck, 1995; Moen *et al.,* 1990; Schirmbeck *et al.,* 1993; Adom and Richard-Foy, 1991) and that this association in some cases involves the origins of viral replication. Because viral DNA replication mimics DNA replication of the host cell with respect to nuclear compartmentalization, viral systems are usefull tools for investigating the function of the interactions between the origins of replication and the nuclear matrix.

To this purpose, we have choosen Epstein–Barr virus (EBV), which replicates in a latent and in a lytic phase via two different origins of replication, to investigate the binding of the latter to the matrix with respect to the viral state of infection. EBV, a human herpesvirus, is the ethiologic agent of infectious mononucleosis and is associated with a number of tumors, such as Burkitt lymphoma, nasopharingeal carcinoma, and Hodgkin desease (Liebowitz and Kieff, 1993; Herbst *et al.,* 1990). EBV latently infects and transforms human B lymphocytes, where the majority of the viral genome is maintained as circular, double-stranded DNA (Lindahl *et al.,* 1976). Of the approximately 100 genes encoded by the virus, about 10 are differentially expressed during latency, depending on the infected host cell. They comprise six nuclear antigens (EBNA1, -2, -3A, -3B, -3C, and -LP) and three integral membrane proteins (LMP-1, -2A, and -2B). In addition, two genes coding for small RNAs (EBER1 and

[1] To whom correspondence and reprint requests should be addressed at Microbiology Institute, Faculty of Medicine and Surgery, University of Roma "La Sapienza," P.le A. Moro, 5—00185 Roma, Italy. Fax: 39-6-49914641. E-mail: Mattia@axrma.uniroma1.it.

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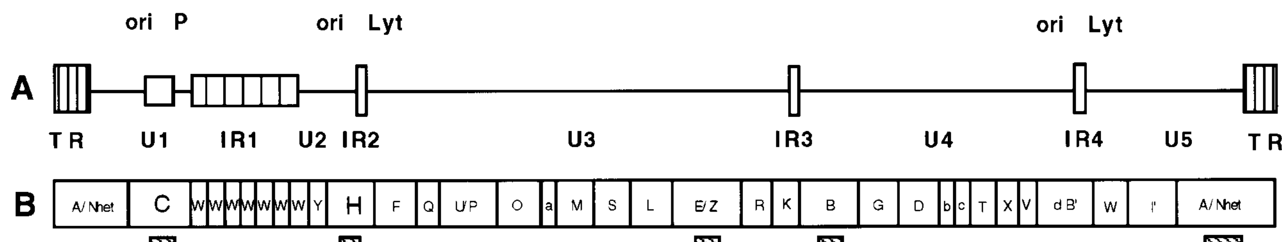

FIG. 1. Linear EBV genome, *Bam*HI restriction map, and position of PCR-generated fragments. (A) Schematic representation of the EBV genome. Indicated are the origins of replication (ori P and ori Lyt), the terminal repeats (TR), the five unique sequences (U1 to U5), and the four internal repeats (IR1 to IR4). (B) *Bam*HI restriction map and underneath the position of PCR-generated fragments used to probe the genome.

EBER2) are actively transcribed in most EBV-infected cells.

Most EBV genes are expressed during the lytic cycle. In the absence of exogenous stimuli, only a small percentage of cells undergo lysis as a result of productive infection, but the latter can be quantitatively triggered *in vitro* by a variety of induction procedures (Bauer *et al.,* 1982; Di Renzo *et al.,* 1994; Ooka *et al.,* 1984). In the latent cycle, EBV replication is mediated by a cis-acting element identified as the origin of DNA replication, ori P (Reisman *et al.,* 1985; Yates *et al.,* 1985), while during the lytic phase, the origin of replication, ori Lyt, is activated (Gruffat *et al.,* 1995).

It has been shown that in latently infected cells, EBV is organized as a single DNA domain, which is anchored on the nuclear matrix by a high-affinity MAR containing the origin of replication ori P (Jankelevich *et al.,* 1992).

To gain insight into the functional role of EBV ori–nuclear matrix interactions, we have studied them with respect to the latent as well as to the lytic cycle of infection.

To this end we have used Raji cells, a Burkitt lymphoma-derived cell line containing multiple copies of the EBV genome in a tightly latent state. These cells can be treated with a number of agents, such as phorbol esters, sodium butyrate, and TGFβ, to induce the EBV lytic cycle, but because of a deletion in the Raji viral genome (Hatfull *et al.,* 1988), only the early events of the lytic cycle take place, making this cell system ideal for our studies.

Here, we report that following induction of the EBV lytic cycle, the ori Lyt origin of replication associates with the nuclear matrix, while the binding of the origin of replication ori P is diminished.

It is proposed that the association of the viral origins of replication to the nuclear matrix is instrumental to viral replication and genome expression in the two different phases of infection.

RESULTS

Induction of EBV lytic cycle

In order to induce the EBV lytic cycle in a large proportion of Raji cells, a combination of $P(BU)_2$, sodium butyrate, and TGF-β2 was used.

Experiments have been carried out to quantitatively induce the EBV lytic cycle in the cell population, while maintaining high values of cell viability. To this purpose, various concentrations of the inducers, different lengths of incubation time, and different cell densities and serum contents in the culture medium had been tested. Under the different conditions, the percentage of cells expressing EBV early antigens (EA) and the number of viable cells, after trypan blue staining, had been evaluated. The results have shown that the combination of $P(BU)_2$ plus sodium butyrate leads about 30% of the cell population to enter the lytic cycle. The addition of TGFβ as third inducing agent allows, under the experimental conditions described under Materials and Methods, to increase the EA-positive cells to about 60%, while cell viability remains higher than 80% (data not shown).

EBV probes

In order to study the specific interactions of EBV origins of replication ori P and ori Lyt with the nuclear matrix in the latent or in the lytic form of infection, probes were generated by PCR that would recognize different regions of EBV genome. Amplified DNA included sequences within the origins of replication ori P and ori Lyt contained, respectively, in fragments C and H of the *Bam*HI-restricted EBV genome (see Fig. 1). Probes were also generated that would recognize sequences within *Bam*HI A, *Bam*HI B, and *Bam*HI Z fragments.

Table 1 reports the nucleotide sequences of the primers used and the genomic locations and the lengths of the amplification products.

Evaluation of the association of EBV *Bam*HI restriction fragments with the nuclear matrix

Intact nuclei from control and induced Raji cells were digested *in situ* with *Bam*HI restriction enzyme. The nuclear matrix-associated and the soluble DNA fractions were isolated as previously described (Jankelevich *et al.,* 1992). According to this procedure, DNA fragments associated with the nuclear matrix were enriched in the nuclear matrix fraction of the DNA while, by contrast, DNA fragments not anchored to the matrix were found

TABLE 1

Oligomers Used to Amplify EBV Genomic Sequences

	Sequence 5′-3′	EBV genome (nt)	Product (bp)
ori P DP	TTTTTAAACCTCCTGGAATT	7300–9183	1883
ori P RP	CTGTGTAGCTACCGATAAGC		
ori Lyt DP	GCCTTCTTTTATCCTCTTTT	52805–52984	179
ori Lyt RP	GCCCCTCCTCCTCTCGTTAT		
*Bam*HI A DP	GCCTCCGCCGTCCACTCTAT	161574–162926	1372
*Bam*HI A RP	CCGCCTGCCCCATCTTCACC		
*Bam*HI B DP	ATAGACGGTTGGGCAGTAGG	113912–114778	886
*Bam*HI B RP	TATTTCCTTCGTTGCCTCAT		
*Bam*HI Z DP	TTCAAAGAGAGCCGACAGGA	102126–102772	666
*Bam*HI Z RP	TTCAAACAGCAGCAGCAGTG		

Note. DP, direct primer; RP, reverse primer.

equally distributed among the two DNA fractions or more abundant in the soluble one.

After *Bam*HI digestion, the nuclear matrix-bound DNA is about 40–50% of the total, independently of the treatment of the cells with inducers of the EBV lytic cycle. Equal amounts isolated from the insoluble and the soluble fractions were electrophoretically resolved and hybridized with the probes for the different regions of the *Bam*HI-restricted EBV genome.

Figure 2 shows the results of hybridization with the probe for the latent origin of replication ori P. This probe recognizes a 9.5-kb band, corresponding to the *Bam*HI C fragment of the EBV genome.

Untreated, latently infected cells retain about 75% of this fragment in the nuclear matrix DNA fraction.

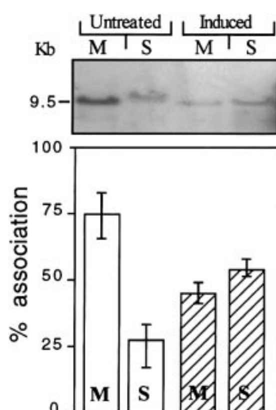

FIG. 2. Association of the *Bam*HI C fragment with the nuclear matrix. Nuclei isolated from latently infected Raji cells (untreated) and from cells treated with the inducers (induced) were *in situ* digested with *Bam*HI. Equal amounts of DNA purified from the nuclear matrix (M) and the soluble (S) fractions were resolved on a 1% agarose gel, blotted, and hybrydized with DIG-labeled ori P probe (top). The bar graph represents the mean values obtained from the densitometric measurement of the hybridization signals of three similar experiments for untreated (empty bar) and induced (filled bars). The values are expressed as the percentage of association of the *Bam*HI C fragment with the nuclear matrix and the soluble fraction.

By contrast, after the cells were treated with the agents that induce the lytic cycle, the percentage of the matrix-associated *Bam*HI C fragment decreases to about 45%, with a parallel increment of the portion released in the soluble DNA fraction.

When the *Bam*HI-restricted EBV genome was hybridized with the probe for the lytic origin of replication ori Lyt, a 6-kb band, corresponding to the *Bam*HI H fragment, was apparent. Figure 3 shows that when the cells harbor the virus in the latent form of infection, the distribution of this fragment is prevalent in the soluble component of the DNA. However, after they are treated with inducers of the lytic cycle, the percentage of the *Bam*HI H fragment that is found nuclear matrix-associated increases from about 35 to 45%.

To evaluate whether regions of the EBV genome other than those containing the origins of replication would also redistribute between the two DNA fractions upon induction of the EBV lytic cycle, we measured the asso-

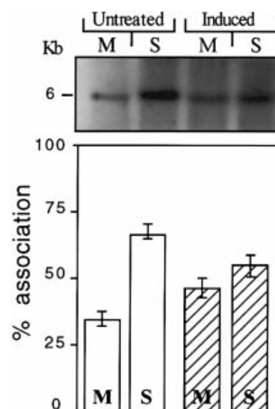

FIG. 3. Association of the *Bam*HI H fragment with the nuclear matrix. Untreated and induced Raji nuclei were *in situ* digested with *Bam*HI. Purified DNA fractions were processed as in Fig. 2 and hybridized with DIG-labeled ori Lyt probe (top). The bar graph, obtained as for Fig. 2, represents the percentage of association of the *Bam*HI H fragment with the two DNA fractions.

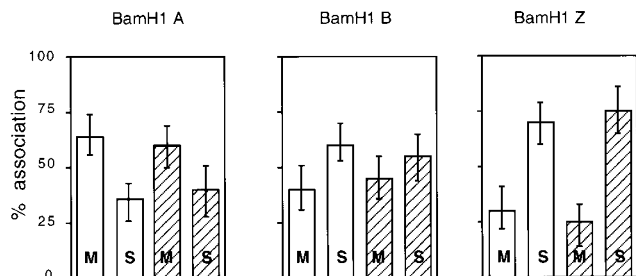

FIG. 4. Distribution of *Bam*HI A, *Bam*HI B, and *Bam*HI Z fragments over the nuclear matrix and the soluble DNA fractions. Untreated (empty bars) and induced (filled bars) Raji nuclei were *in situ* digested as in Fig. 2 and the purified DNA was hybridized with *Bam*HI A, *Bam*HI B, and *Bam*HI Z probes. The hybridization signals from three similar experiments were quantified and expressed in the bar graph as percentages of association with the nuclear matrix (M) and the soluble (S) DNA fraction.

ciation of the *Bam*HI A, *Bam*HI B, and *Bam*HI Z fragments with the nuclear matrix and the soluble DNA component in untreated and induced cells. As shown in Fig. 4, the distribution of these fragments between the two DNA components is not related to the viral phase of replication. The percentage of the *Bam*HI A fragment (10 kb) associated with the nuclear matrix is higher than that measured in the soluble DNA fraction. However, neither the *Bam*HI B (9.6 kb) nor the *Bam*HI Z (8 kb) fragment selectively associates with the nuclear matrix. In particular, a higher percentage of the *Bam*HI Z fragment partitions to the soluble DNA fraction, while *Bam*HI B appears more evenly distributed between the two DNA components.

Interactions of ori P and ori Lyt with the nuclear matrix during the latent or the lytic cycle of EBV infection

We next tested whether the preferential binding of *Bam*HI C and *Bam*HI H fragments to the nuclear matrix, observed, respectively, in the latent and in the lytic phases of EBV infection, was involved the origin of replication ori P and ori Lyt. Nuclei isolated from untreated and induced Raji cells were *in situ* digested with restriction enzymes that delimit ori P and ori Lyt. As shown in Fig. 5, a *Pst*I/*Pvu*II digestion generates a 2.9-kb fragment, which includes the entire ori P consensus sequence and, upstream, the EBER2 gene (Gahn and Schildkraut, 1989). Alternatively, a *Sac*I/*Pst*I digestion was used to generate a 2.3-kb fragment containing the entire minimal ori Lyt consensus sequence (Hammerschmidt and Sudgen, 1988) and part of the auxiliary region which increases ori Lyt efficiency (nt 53581–54713) encompassing the BHRF1 promoter.

The percentage of DNA recovered in the nuclear matrix fraction after each of the two double digestions was about 20% of the total DNA.

Figure 6 shows the results obtained when Raji nuclei

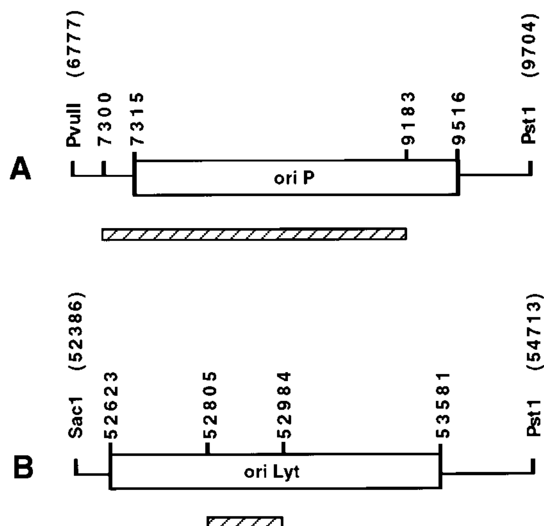

FIG. 5. Schematic representation of *Pst*I/*Pvu*II- and *Pst*I/*Sac*I-restricted regions of EBV genome, containing the origins of replication. Indicated are ori P (A) and ori Lyt (B) coordinates in B95.8 and the positions of the relative probes shown underneath with filled bars.

were digested with *Pst*I/*Pvu*II endonucleases and the purified DNA fractions hybridized with ori P probe. The quantification of the hybridization signals measured for three similar experiments indicates that latently infected cells retain between 2.4- and 4-fold more of the ori P fragment on the nuclear matrix than the amount found in the soluble DNA fraction. Upon induction of the lytic cycle, the percentage of association of the ori P with the nuclear matrix decreases about 20% with respect to the untreated cells.

Figure 7 shows the results obtained after *in situ* digestion with *Sac*I and *Pst*I enzymes and hybridization with the ori Lyt probe.

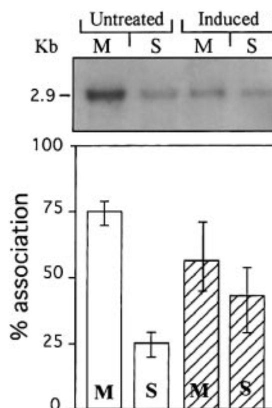

FIG. 6. Identification of ori P binding to the nuclear matrix. Untreated and induced Raji nuclei were *in situ* digested with *Pst*I and *Pvu*II. Equal amounts of DNA purified from the nuclear matrix (M) and the soluble (S) fractions were hybrydized with a DIG-labeled ori P probe (top). The hybridization signals from three similar experiments were quantified and expressed in the bar graph as percentages of association with the nuclear matrix and the soluble DNA fraction for untreated (empty bar) and induced (filled bars) cells.

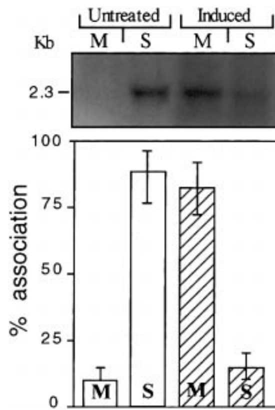

FIG. 7. Identification of ori Lyt binding to the nuclear matrix. Untreated and induced Raji nuclei were *in situ* digested with *Pst*I and *Sac*I. Equal amounts of DNA purified from the nuclear matrix (M) and the soluble (S) fractions were hybrydized with DIG-labeled ori Lyt probes (top). The bar graph was obtained as for Fig. 6.

In latently infected Raji cells, the ori Lyt is almost completely released in the soluble DNA fraction. By contrast, following induction of the EBV lytic cycle, the ori Lyt fragment specifically associates with the nuclear matrix. The quantification of the hybridization signals related to three similar experiments indicate that in induced Raji cells, the ori Lyt fragment is about fivefold more abundant in the nuclear matrix than in the soluble DNA component.

Association of ori Lyt surrounding sequences to the nuclear matrix in the latent and the lytic phases of EBV cycle

To further evaluate the association of the EBV genome to the nuclear matrix in the regions adjacent the ori Lyt, Raji nuclei from control and induced cells were *in situ* digested with the restriction enzyme *Nco*I. After nuclear matrix isolation and protein digestion, insoluble and soluble DNA fractions were subjected to Southern blot analysis with cloned *Bam*HI H (6 kb) and *Bam*HI F (7.4 kb) fragments of the EBV genome randomly labeled with DIG-dUTP.

As shown in Fig. 8a, following *Nco*I digestion, the ori Lyt is contained in a 2.5-Kb fragment, similar in size and position to that obtained by the *Sac*I/*Pst*I digestion. The fragments generated by *Nco*I-restricted EBV DNA, enlighted by the *Bam*HI H or the *Bam*HI F probe, appear in Fig. 8b. The distribution of these fragments in untreated and induced Raji cells, over the matrix and the soluble DNA components, was studied after quantification of the

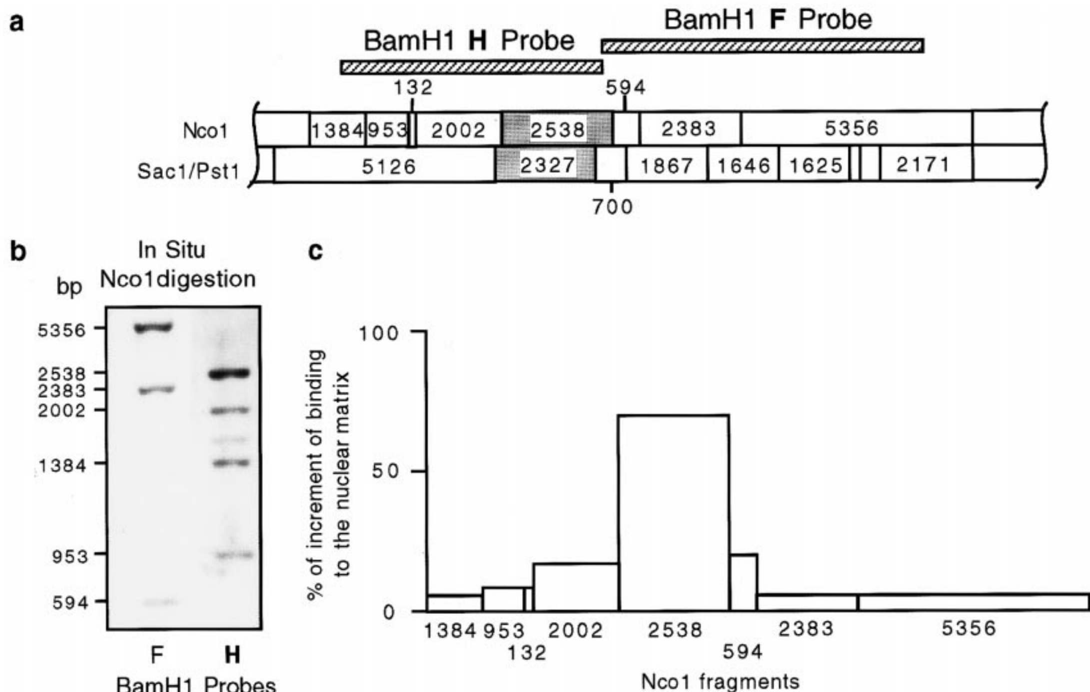

FIG. 8. Association of ori Lyt surrounding sequences to the nuclear matrix. (a) Schematic representation of *Nco*I and *Pst*I/*Sac*I restriction digestion map of EBV genome showing the regions of homology to *Bam*HI H and *Bam*HI F probes. Shaded boxes indicate left ori Lyt containing fragments. (b) Raji nuclei were *in situ* digested with *Nco*I. Soluble and insoluble DNA components, purified as described under Materials and Methods, were hybridized with *Bam*HI H or *Bam*HI F probes. Shown are the fragments enlightened by each probe. (c) The hybridization signals measured in the soluble and insoluble DNA fractions of untreated and induced Raji cells were expressed as percentages of association as reported for Fig. 2. The histogram represents the increment of the binding of *Nco*I-generated fragments to the nuclear matrix in the lytic versus the latent phase of EBV infection.

hybridization signals, corrected for the amounts of total DNA.

The histogram of Fig. 8c shows the increments in the percentages of matrix-associated fragments of induced versus control cells. It appears that in induced cells the association to the nuclear matrix of the 2.5-kb ori Lyt containing fragment is increased about 65%. The binding to the nuclear matrix sharply decreases for the neighboring fragments, the difference between the two phases being about 25% for the fragments of 2002 and 594 bp, lying next to the ori Lyt, and about 5 to 10% for the more upstream and downstream DNA regions.

DISCUSSION

Several reports obtained in different systems suggest a specific association of the origins of replication with the nuclear matrix (Dijkwel and Hamlin, 1995). It has been hypothesized that these interactions play a pivotal role during development, selectively activating, at each step, a fraction of the potential origins of replication, and during cell differentiation promoting the expression of a pattern of selected genes.

Origins of replication, which share sequence motifs with MAR regions, have been found in yeast, viruses, mitochondria, chloroplasts, and mammalian cells (Phi-Van and Strätling, 1990; Aelen *et al.,* 1983). Although many studies have shown that nascent DNA starts at defined sites attached to the nuclear matrix, a complete understanding of the regulatory processes that control the replication pattern within a given cell type or through different stages of development is still lacking. To gain insights on the functional role of oris–nuclear matrix associations, we have used EBV as a model system, to study these interactions with respect to different phases of viral expression. We report here that both the ori P and the ori Lyt function as S/MARs by anchoring the EBV genome to the nuclear matrix, the first during the latent cycle of infection and the second after induction of the lytic cycle.

In order to study the interaction of the EBV origin of replication with the nuclear matrix in the two phases of infection we have used the combination of three different agents known to be inducers of the EBV lytic cycle. Under our experimental conditions, the expression of EBV early antigens was obtained in more than 60% of the Raji cell population, a percentage significantly higher than those previously reported (Bauer *et al.,* 1982; Di Renzo *et al.,* 1994; Ooka *et al.,* 1984).

From the analysis of the distribution of different regions of the *Bam*HI-digested EBV genome over the nuclear matrix and the soluble DNA components, we conclude that only the *Bam*HI C and *Bam*HI H fragments, containing, respectively, the origins of replication ori P and ori Lyt, undergo a redistribution over the nuclear matrix and the soluble DNA components upon induction of the EBV lytic cycle.

Although the Raji EBV genome contains two lytic origins of replication, only the left ori Lyt seems to be involved in binding to the nuclear matrix. In fact, the ori Lyt probe, which shares 76% homology with the right ori Lyt, shows neither a 9.5-kb (Raji coordinates 149,116 to 158,711) nor a 2.1-kb (Raji coordinates 155,512 to 157,696) fragment containing the right lytic origin of replication after restriction, respectively, with *Bam*HI or *Sac*I/*Pst*I enzymes. It is interesting to note that during latency, alternative sites of replication in Raji cells were found in the region upstream of ori P, near the right ori Lyt (Little and Schildkraut, 1995). These findings indirectly support our data suggesting a functional activity of the left ori Lyt when the lytic phase of infection is induced.

The distribution of *Bam*HI-restricted fragments of the EBV genome has been thoroughly studied in latently infected Raji cells (Jankelevich *et al.,* 1992). We confirm that in this phase of infection about 70% of the *Bam*HI A fragment associates with the nuclear matrix. However, the fractions of the *Bam*HI B and *Bam*HI Z fragments that partition with the nuclear matrix are higher than those reported in the previous study. Our explanation for this apparent discrepancy might reside in the length of the probe used in the two studies. It is conceivable that large *Eco*RI fragments are less easily retained on the matrix than the probes used in our experiments, only a few hundred basepairs long.

The *Pvu*II/*Pst*I and the *Sac*I/*Pst*I digestions of Raji cell DNA has allowed us to establish that both EBV origins of replication, depending on the viral phase of infection, bind to the nuclear matrix. In particular, the association of ori Lyt sequences with the matrix is different when residing in the 6-kb or in the 2.3-kb fragment. In fact, it has been previously observed by other authors that regions of DNA larger than 3–4 kb bind to the nuclear matrix with reduced specificity (Izaurralde *et al.,* 1988). The survey of the DNA restricted regions flanking the left origin of replication has shown that, upon induction of EBV lytic cycle, the fragments immediately next to the ori Lyt are somewhat retained on the nuclear matrix, while the degree of association of more distant fragments to the matrix is much lower. It is conceivable that sequences neighboring the origin of replication would concur in ori Lyt activity and are therefore found, in the lytic cycle, in the insoluble DNA fraction. A similar gradation in affinity for the nuclear matrix, observed for the fragments neighboring the ori P in latently infected Raji cells, was related to the presence of partial digestion products occurring during *in situ* restriction of the nuclei (Jankelevich *et al.,* 1992). More detailed mapping and gel retardation experiments will be necessary to characterize which sequences within the origins of replication show the highest affinity for the nuclear matrix and whether they

share features common to endogenous MARs identified in different cell systems (Boulikas, 1994).

The decrement of ori P and the parallel increment of ori Lyt binding to the matrix at the onset of the lytic phase indicates that these origins of replication are not permanently attached and that the association of EBV oris to the karyoskeleton is strictly related to the type of viral cycle.

The redistribution of EBV oris between the nuclear matrix and the soluble DNA components is evident despite the fact that 40% of the cells, still harboring the virus in the latent phase of infection after treatment with the inducers, cause an underestimation of the event.

Because nuclear matrix proteins vary quantitatively and qualitatively during differentiation (Stuurman *et al.,* 1989), it is conceivable that specific proteins present in the nuclear matrix of either untreated or induced Raji cells modulate the interaction with EBV origins of replication. In this respect, the incomplete release of ori P from the matrix of induced Raji cells could also depend on the kinetics of degradation or inactivation of such specific nuclear matrix binding protein(s). Examples of cellular proteins, specifically expressed after treating a Burkitt lymphoma cell line with phorbol ester and that bind EBV origin of replication ori P, have been reported (Zhang and Nonoyama, 1994). In addition, cellular proteins have been identified which bind ori P at the same sites recognized by EBNA1 (Sang-jin *et al.,* 1991) or that bind different sites in the downstream element of ori Lyt (Gruffat *et al.,* 1995). At present, the function of the binding of these cellular proteins to the viral DNA is not known, nor if any of them belong to the nuclear matrix.

In conclusion, the results here reported represent, to our knowledge, the first example of dynamic interactions between the DNA and the nuclear matrix. It is suggested that the binding of specific nuclear matrix proteins with the two EBV origins of replication might be instrumental in the regulation of viral genes expression and contribute to determine, *in vivo,* the type of infection that the virus establishes in the host cell.

MATERIALS AND METHODS

Cell culture

Latently infected Raji cells were maintained at a density of 5×10^5 cells ml^{-1} in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) (Sigma).

B95.8 cells were cultured in serum-free conditions at 2.5×10^5 cells ml^{-1} in RPMI 1640 with antibiotics and 2% (v/v) Biogro 1 concentrated supplement (Biological Industries).

EBV lytic cycle-inducing treatments

Raji cells were exposed to a combination of agents, each previously reported, which individually induce the lytic cycle in a considerable proportion of cells (Bauer *et al.,* 1982; Di Renzo *et al.,* 1994; Ooka *et al.,* 1984). Therefore, in order to induce the EBV lytic cycle, Raji cells grown at a density of 10^6 ml^{-1} were diluted to 5×10^5 ml^{-1} in RPMI 1640 with 2.5% FCS. Phorbol-12, 13-dibutyrate ($P(BU)_2$) (Sigma), sodium butyrate (Sigma), and recombinant TGF-β2 (Genzyme, sp. act. 5×10^7 units/mg) were added to the cell culture at a final concentration of 20 ng ml^{-1}, 2 mM, and 0.04 ng ml^{-1}, respectively. The viability of the cells during the treatment, estimated by trypan blue exclusion, was never below 80%.

The proportion of EA-positive cells in the cultures was assessed by direct immunofluorescence. Samples of cells were harvested 48 h after additions, briefly centrifuged, washed with PBS, fixed on slides in methanol:acetone (2:1) for 10 min at −20°C, and treated for 1 h with the FITC-conjugated direct F_6-Esther reagent, diluted 1:60 in PBS, 1% BSA (Klein *et al.,* 1972).

Evaluation of the samples was performed by a Leitz Orthoplan immunofluorescence microscope and photographed using 400 ASA Kodak Ektacrome Elite film.

Nuclei isolation

Nuclei were isolated (Jankelevich *et al.,* 1992) from untreated or induced Raji cells.

Briefly, 1.5×10^8 cells were washed once with PBS and twice with 40 ml of buffer I (10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.25 M sucrose). After centrifugation the cell pellet was resuspended in 40 ml of buffer I containing 0.1% NP-40 and 1 mM PMSF. The nuclear suspension, kept on ice for 10 min was layered onto a 40-ml sucrose cushion (10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.32 M sucrose) and centrifuged for 10 min at 700 *g* at 4°C. Nuclei were resuspended in 20 ml of buffer I, containing 0.1% NP-40 and 1 mM PMSF, kept on ice for 10 min, and centrifuged at 450 *g* for 25 min at 4°C. Nuclei were stored at a density of 5×10^7 ml^{-1} in a mixture containing 50% (v/v) buffer I, 50% (v/v) glycerol, and stored at −20°C.

Nuclear matrix isolation by *in situ* restriction endonuclease digestion of nuclei

For each digestion, 2×10^7 nuclei isolated from Raji cells treated or not with the agents that induce the EBV lytic cycle were washed briefly in buffer I and resuspended in the appropriate restriction enzyme buffer. Digestion was carried out for 2 h at 37°C with 50 U of each restriction enzyme/mg of nuclear DNA. Digested nuclei were then centrifuged at 450 *g* for 25 min. Nuclear matrices were prepared from in situ-digested nuclei according to the high-salt method, as previously described (Jankelevich *et al.,* 1992). The soluble fractions obtained from *in situ* digestion and salt extraction of the nuclei were combined.

Analysis of EBV DNA

In order to analyze matrix-associated DNA and soluble DNA fractions, each of them was subjected to deproteination in the presence of 1% SDS and 300 μg ml^{-1} proteinase K for 12 h at 42°C. Following phenol extractions and ethanol precipitation, the DNA, resuspended in the appropriate restriction enzyme buffer, was redigested for 2 h at 37°C with the same endonuclease(s) used for the *in situ* digestion of the nuclei. After ethanol precipitation, equal amounts of DNA from each fraction (4 μg) were subjected to electrophoresis on a 1% agarose gel.

The separated DNA fragments were transferred onto a nylon membrane (Hybond N, Amersham) and probed with PCR-generated EBV fragments labeled by random priming with digoxigenin–dUTP (Boehringer Mannheim).

PCR-generated EBV probes

Viral DNA was isolated from B95.8 cells essentially as described (Hirt, 1967). Purified EBV DNA was digested with *Bam*HI, ethanol precipitated, and resuspended at a concentration of 0.5 mg/ml in 10 mM Tris, pH 7.5, 1 mM EDTA. The choice of the PCR primers was based on the complete sequence of EBV B95.8 strain (Baer *et al.,* 1984). Primers (20-mer) were selected by the use of a computerized program and synthesized on a automated solid-phase synthesizer (ABI394, Applied Biosystem Inc), by standard cyanoethylphosphoramidite chemistry (M-Medical, Fi, Italy). All oligomers were ethanol precipitated, washed several times with 70% ethanol, and redissolved in sterile water at a concentration of 125 pmol ml^{-1}. PCR amplifications were performed in a total volume of 50 μl containing 1 μg template DNA, 200 mM dNTP, 1 mM each primer, 3 mM $MgCl_2$, and 0.125 U *Taq* Termoprime Plus in 1× PCR buffer (Advanced Biotecnologies).

The genomic probes, all generated during 35 cycles on a Perkin–Elmer GeneAmp 2400 PCR System, are shown in Table 1. Amplification conditions for ori P and ori Lyt probes were denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and elongation at 72°C for 1 min; for probes to *Bam*HI regions A, B, and Z of the viral DNA thus were denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and elongation at 72°C for 2.5 min.

PCR amplification products were resolved on a 1% low-melting agarose gel and the corresponding bands excised and purified by the QUIAquick gel extraction kit (Quiagen). DNA probes were labeled by random priming with digoxigenin–dUTP (Boehringer) according to the protocol supplied with the kit. Hybridization of filters with the labeled probes was carried out at 42°C according to the protocol supplied with the DIG luminescent detection kit (Boehringer). Specific signals were quantified by densitometric scanning after exposing the filters to Kodak X-Omat AR films for times ranging from 1 to 24 h.

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