Interaction of EBV latent origin of replication with the nuclear matrix: identification of S/MAR sequences and protein components

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Abstract During latency, Epstein Barr virus (EBV) genome, as an episome, is attached to the nuclear matrix (NM) via the latent origin of replication ori P. Within this element, we have found that a region, 580 bp long, encompassing the replicator DS element, shows the strongest affinity for the NM. In addition, by cross-linking with *cis*-diamminedichloroplatinum, we have identified two NM proteins with an apparent molecular weight of 85 and 60 kDa that, with high affinity and specificity, bind ori P. These proteins are not induced by EBV infection, but their interaction with ori P is lost upon induction of EBV lytic cycle. These data strongly suggest that the binding of ori P to specific components of the NM is required for EBV latent replication.

Key words: Epstein Barr virus; Nuclear matrix; S/MAR; Cross-linking

1. Introduction

During latency, Epstein Barr virus (EBV) genome, as an episome, is replicated only once per cell cycle by the cellular enzymes, when the infected cells proliferate [1]. The latent origin of replication ori P and its binding protein EBNA1 are the only two viral components required for the replication. ori P is composed of two *cis*-acting elements, the family of repeats (FR) and the region of dyad symmetry (DS), which are separated by approximately 1 kb of DNA [2,3]. The functional component FR, an array of 20 EBNA1 binding sites, provides the mitotic stability of episomes by tethering them, via EBNA1, to human chromosomes [4,5]. However, replication initiates at or near DS, a 120 bp region that contains four EBNA1 binding sites and therefore represents the replicator of ori P [6,7]. Recent data indicate that replicator DS uses essentially the same mechanisms and factors that operate at the replication origins of cellular chromosomes by recruiting cellular DNA origin recognition complex and replication licensing complex MCM [8,9].

In the eukaryotic nucleus, chromosomal DNA is organized into distinct, topologically independent loops of variable size [10,11] that define non-overlapping territories where DNA and RNA metabolisms are confined. These loops are anchored at their bases to a proteinaceous framework generally referred to as the nuclear matrix (NM) or scaffold [12]. The DNA specialized elements involved in the binding of chromatin loops to the NM, termed scaffold or matrix attachment regions (S/MARs), may be up to a few hundred nucleotides in length, do not share a strictly conserved consensus sequence, but often contain AT-rich stretches and clusters of topoisomerase II cleavage sites [13,14]. S/MARs have been found close to promoters, enhancers and origins of replication, suggesting they may serve to bring various *cis*-responsive elements close to matrix-bound DNA and RNA enzymatic machineries [15–17].

It is generally acknowledged that viral replication and expression occur in close association with the NM [18–23]; this association is different for each virus, involving either viral genome sequences or virus-codified proteins.

Previous studies carried out in Raji cells have shown that the binding activity of EBV genome to the NM resides in a 5.2 kb region of DNA, covering the latent origin of replication ori P and the genes for the small viral RNAs (EBERs) [19]. Moreover, we have reported that also the lytic origin of replication ori Lyt binds the NM, following activation of the EBV lytic cycle [24].

In the present study we set out to investigate the molecular features of ori P binding to the NM of Raji cells. To this end, we aimed at defining which regions within ori P have the highest affinity for the NM and searched for NM proteins involved in ori P binding.

We report here that a 580 bp sequence, encompassing the DS element of ori P, shows the highest S/MAR activity. In addition, by cross-linking with *cis*-diamminedichloroplatinum (*cis*-DDP) on intact nuclei, we identified by Southern–Western blot assay two cross-linked NM proteins, with an apparent molecular weight of 85 and 60 kDa, respectively, that bind ori P with high affinity.

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2. Materials and methods

2.1. Cell culture and lytic cycle induction

Raji and Ramos cells, derived respectively from an EBV-positive and an EBV-negative Burkitt's limphoma, were cultured in RPMI 1640 medium containing 5% fetal calf serum and antibiotics, in a 5% CO_2 atmosphere and maintained at a cell density of 3.5×10^5/ml. To quantitatively induce an EBV lytic cycle (more than 60% of the cells positive for EBV early antigens), Raji cells were treated as previously described [24].

2.2. Preparation of nuclei

Nuclei from Raji and Ramos cells have been isolated as described in [24], except for the addition of protease inhibitors phenylmethylsulphonylfluoride (PMSF), aprotinin and leupeptin (1 mM, 1 μg/ml and 0.5 μg/ml, respectively) to all the buffers used for nuclei isolation.

2.3. In vitro binding of end-labeled ori P fragments to nuclear matrices

The origin of replication ori P obtained as described below, was digested with restriction enzymes *Nco*I and *Eco*RV for 3 h at 37°C. The DNA fragments were subsequently digoxigenin (DIG) 3′-end-labeled (Roche Diagnostics, USA) according to the manufacturer's protocol. Nuclei (about 25 μg DNA) were processed for NM preparation by the LIS extraction procedure and the resulting nuclear scaffolds used for in vitro binding assays as previously described [25], in the presence of 50 ng/ml DIG-end-labeled ori P fragments, non-specific competitor DNA (sonicated herring sperm DNA, 1–6 μg/ml) and 30 U each of *Eco*RV, *Eco*RI and *Nco*I restriction enzymes. A centrifugation at $3200\times g$ for 15 min at 4°C was used to separate solubilized DNA from matrix-bound DNA. After protein digestion, purified DNA was subjected to electrophoresis on 1.5% agarose gel and transferred to nylon membrane. DIG-end-labeled ori P fragments were visualized by chemiluminescent detection (Roche Diagnostics).

As a control for the binding assay, pTZ-E20 plasmid [26] was digested with *Dde*I/*Eco*RI. The resulting fragments were DIG-end-labeled and tested for their binding activity to Raji NM under the same conditions used for ori P fragments.

2.4. EBV DNA probes

Amplification of ori P, as well as those of the regions included in the *Bam*HI B and *Bam*HI Z fragments of the EBV genome, was performed by polymerase chain reaction (PCR) with the primers and according to the conditions previously reported [24]. Plasmid pTZ-E20 contains a 2.2 kb *Eco*RI fragment derived from the human INFβ SAR element [27].

For Southern–Western experiments, 10 pmol 3′-OH ends of each DNA probe were DIG-labeled by tailing reaction (Roche Diagnostics). Label efficiency of the different DNA probes was comparable as judged after electrophoresis, blotting and colorimetric detection.

2.5. Nuclei cross-linking by cis-DDP II

Nuclei (200–400 A_{260} units) obtained as described in [24], were sedimented by centrifugation at $3000\times g$ for 20 min at 4°. *cis*-DDP II cross-linking procedure was carried out as described in [28] with 0.5 mM *cis*-DDP for 1 h at 37°C with gentle shaking. NM preparations were performed as described in [25]. The histone-depleted nuclei were digested with *Eco*RI, *Bam*HI and *Hin*dIII restriction enzymes (2 U/μg DNA), for 3 h at 37°C. Nuclear scaffolds were sedimented by centrifugation at $3000\times g$ for 10 min at 4°C.

2.6. Isolation of cross-linked matrix proteins

Isolation of cross-linked matrix proteins was performed as described in [29]. Usually, about 100 μg of cross-linked NM-bound DNA per mg of nuclear DNA were obtained. Based on OD readings, hydroxyhapatite (HTP, DNA grade; Bio-Rad) was added to the cross-linked complexes in the ratio of 1 g/200 A_{260} units. Cross-linked NM proteins were eluted from HTP by thiourea treatment [29], dialyzed against 30 mM Tris, pH 8, 1 mM PMSF and then concentrated by Microcon (Millipore).

2.7. Southern–Western blotting

Cross-linked NM proteins derived from equivalent amounts of DNA (as judged by A_{260} readings performed before binding to HTP) were electrophoresed on a 10% acrylamide gel, transferred onto PVDF membrane in a buffer consisting of 10 mM CAPS and renatured in guanidine hydrocloride [30]. The DNA binding protein blot assay was carried out as previously described [31] with 40 ng/ml of DIG-tailed DNA probes in the presence of different amounts of sonicated herring sperm competitor DNA. DNA-bound proteins were visualized by enzyme-catalyzed color reaction (Roche Diagnostics).

2.8. Western blotting

Membranes used for the Southern–Western blotting experiments were subsequently incubated with the following antibodies: telomeric repeat binding factor 2 (TRF2) goat polyclonal antibodies (Imgenex) was used at a 1:250 dilution; anti-EBNA 1 monoclonal antibodies (Cyto-Bar, The Netherlands), anti SAF-A rabbit serum, anti-lamin B2 monoclonal antibodies (AbCam, UK), anti-lamins D/E antiserum, anti-poly(ADP-ribose)polymerase (PARP) monoclonal antibodies (Alexis), were used each at a 1:1000 dilution. Secondary antibodies, horseradish peroxidase-linked anti-mouse, anti-rabbit and anti-goat/sheep, were used at a 1:5000 dilution. Chemiluminescent detection of the positive bands was performed by ECL Plus Western blotting reagents (Amersham Pharmacia Biotech), according to the manufacturer's protocol.

3. Results

3.1. Binding of ori P restriction fragments to Raji nuclear matrices

In order to assess which sequences within EBV latent origin of replication would show the highest affinity for the NM, we have generated by PCR a probe, 1903 bp long, corresponding to region 7300–9203 of the B95.8 EBV genome [32] that represents the ori P consensus sequence. This DNA region contains both the FR and the DS element of EBV latent origin of replication.

As is shown in Fig. 1A, digestion of the purified amplification product with *Nco*I and *Eco*RV restriction enzymes generates four fragments, two containing the FR and the DS elements respectively, and two covering a stretch of about 960 bp, dispensable for ori P function. Fig. 1B shows the analysis of the distribution of ori P 3′ end-labeled fragments, retained on the NM (M) or released in the supernatant (S). In the absence of competitor DNA all of them are largely associated to the NM. However, with increasing amounts of non-specific competitor DNA, only the 372 and 208 bp fragments are retained on the matrix.

To verify that the partition of ori P fragments between the NM and the supernatant was truly dependent on MAR activity, we analyzed in a similar experiment the distribution of the fragments originating from a *Dde*I/*Eco*RI digestion of pTZ-E20 plasmid. Because the binding potential to the NM decreases below 300 bp [33], fragments of 1091, 655, 540 and 409 bp from the vector and of 527, 414 and 411 bp from the IFNβ SAR insert were considered. Fig. 1C shows that in an in vitro binding assay, the bands retained on the NM even in the presence of the highest concentration of competitor DNA localize with fragments that include IFNβ SAR DNA. In contrast, vector DNA fragments of 1091 and 655 bp are efficiently competed off the NM in the presence of increasing concentrations of non-specific DNA.

3.2. Identification of cross-linked NM proteins bound by ori P

DNA-binding NM proteins were obtained from nuclei of latently infected Raji cells incubated with *cis*-DDP and processed as summarized in Fig. 2. Hybridization of cross-linked NM proteins with DIG-labeled ori P probe showed that two species with a molecular weight of about 85 and 60 kDa, respectively, were strongly recognized by ori P in the presence

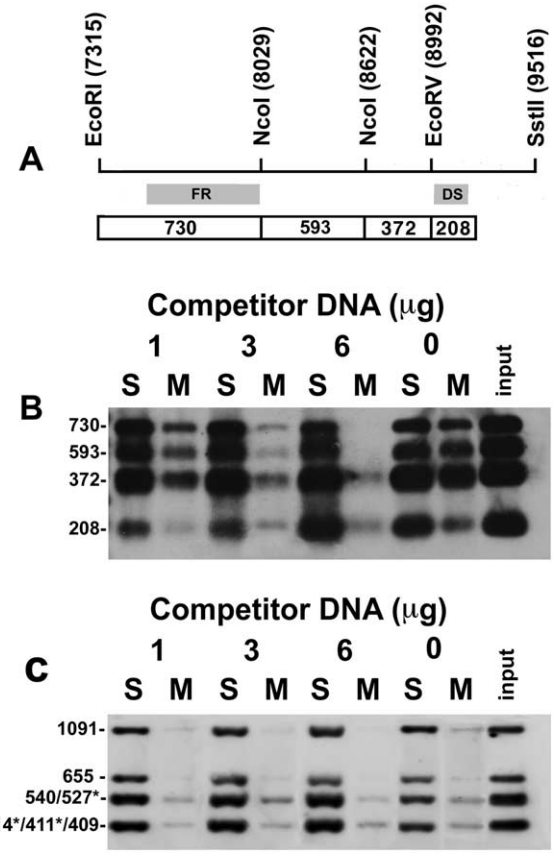

Fig. 1. S/MAR activity of ori P restriction fragments. A: 1.9 kb region of ori P with underneath the sites of the two *cis*-acting components FR and DS. The size and position of the fragments obtained by *Nco*I/*Eco*RV restriction of the PCR-generated ori P amplicon are shown by open boxes. B: In vitro binding of 6 ng of 3′ end-labeled ori P fragments (input) to NM, in the absence or presence of increasing amounts of non-specific competitor DNA (see Section 2). NM-associated (M) and soluble (S) DNA fractions were subjected to electrophoresis, blotting and chemiluminescent detection. C: In vitro binding of *Dde*I/*Eco*RI-digested pTZ-E20 plasmid. Labeled with an asterisk are the fragments derived from the IFNβ SAR insert.

of 10 000-fold excess of non-specific competitor DNA (Fig. 3). The association of ori P to these NM components appears to be highly specific, since sequences of the *Bam*HI Z and *Bam*HI B regions of EBV genome did not bind any NM protein, even in the presence of low concentrations of competitor DNA (data not shown).

3.3. ori P-bound NM proteins are not induced by EBV infection

In order to assess whether cross-linked NM proteins specifically recognized by ori P in latently infected Raji cells were related to viral infection, ori P binding was tested on similar protein preparations obtained from induced Raji cells as well as from EBV-negative Ramos cells. Fig. 4A, showing the Coomassie staining of NM proteins recovered from equivalent amounts of nuclear DNA, indicated that the pattern related to cross-linked NM proteins isolated from latently infected Raji cells or from EBV-negative Ramos cells are substantially similar. Conversely, the protein components of induced Raji cells are less represented, with more species migrating at low molecular weight. Fig. 4B clearly shows that in Ramos cells, ori P recognized the 85 and 60 kDa NM components also detected

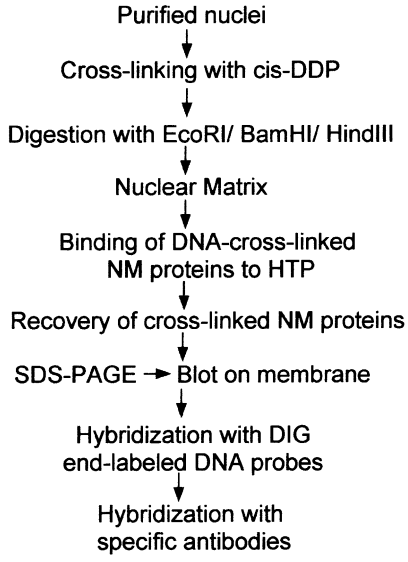

Fig. 2. Scheme of the experimental procedures devised to obtain cross-linked NM proteins.

in latently infected Raji cells, indicating that these protein species are neither codified, nor induced by EBV infection. In contrast, EBV latent origin of replication did not bind the 85 and 60 kDa proteins in similar preparations of induced Raji cells.

3.4. Identification of ori P binding proteins

To identify the NM proteins detected by ori P, blots first used for Southern–Western experiments were re-hybridized with a number of antibodies, either for cellular proteins known to bind EBV latent origin of replication, or for abundant S/MAR binding NM proteins, with molecular weights in the range of 90 and 60 kDa.

Among the cellular proteins recently identified by affinity chromatography to bind the DS element of ori P in an EBNA1-dependent manner [34], TRF2 has an approximate

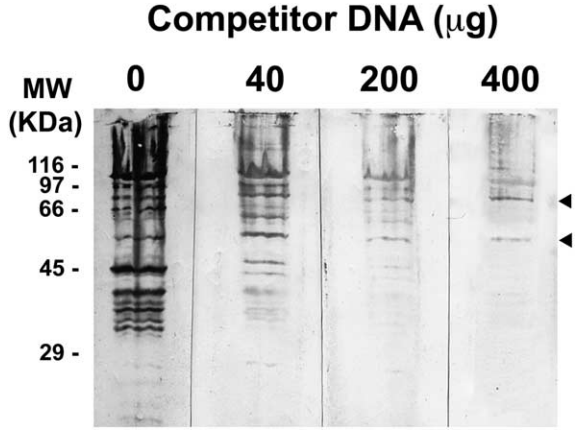

Fig. 3. Southern–Western blot of NM protein with ori P. Cross-linked NM proteins from latently infected Raji cells were hybridized with DIG-labeled ori P probe without or with non-specific competitor DNA. The detection of the positive bands was performed by alkaline phosphatase-catalyzed color reaction. The arrowheads indicate ori P-bound proteins with molecular weights of about 85 and 60 kDa. These results were confirmed by more than three independent experiments.

size of 66 kDa and the apoptotic form of PARP has a molecular weight of about 86 kDa. Among NM DNA-binding proteins, SAF-A, a very abundant component of the NM, presents a cleavage product of about 90 kDa [35] and the lamins of the B-type (B1, B2 and lamins D/E) the only ones expressed in Raji cells [36], have molecular masses ranging between 64 and 68 kDa. Moreover, because virus-encoded ori P binding protein EBNA1 has a molecular weight that varies considerably (from 69 to 94 kDa) depending on the EBV-infected cell line [37], and because it might co-purify with ori P regions bound to the NM, hybridization of the blots with EBNA1-specific antibodies was also evaluated. Fig. 5 shows the results of the Western blots carried out with the different antibodies on Raji cross-linked NM proteins previously tested for ori P binding.

The data obtained clearly indicate that none of the proteins revealed by the specific antibodies co-migrates with either the 85 or the 60 kDa ori P binding proteins detected by Southern–Western assays. In particular, SAF-A and PARP were both present with the full forms of the proteins (120 and 116 kDa, respectively) and EBNA1 antibodies highlighted in these cells, a band with an apparent molecular weight of 70 kDa.

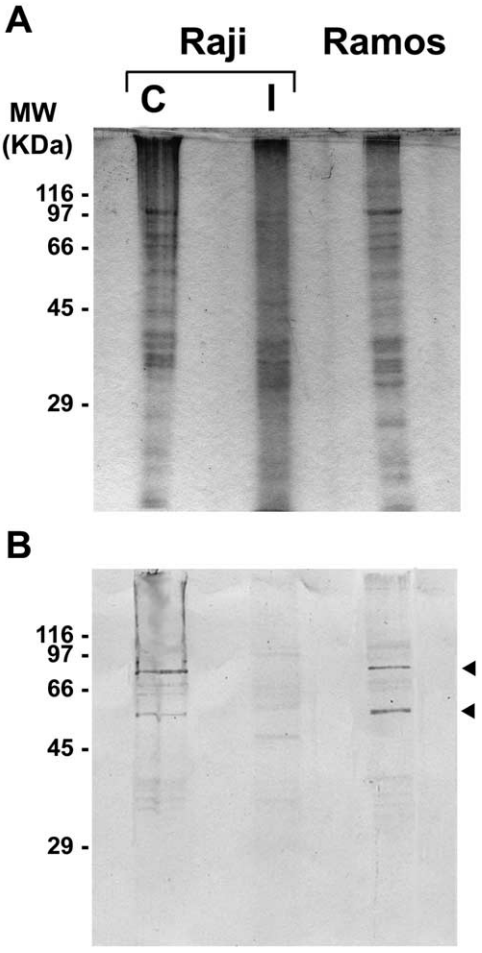

Fig. 4. ori P binding to cross-linked NM proteins from Raji and Ramos cells. A: Cross-linked NM proteins related to equivalent amounts of DNA (see Section 2), obtained from latently infected (C), induced (I) Raji cells and from EBV-negative Ramos cells, were electrophoretically separated and stained by Coomassie R 250. B: Southern–Western blot carried out with DIG-labeled ori P probe in the presence of 400 μg/ml of unlabeled non-specific competitor DNA. Positive bands were detected as in Fig. 3. Arrowheads indicate the 85 and 60 kDa proteins.

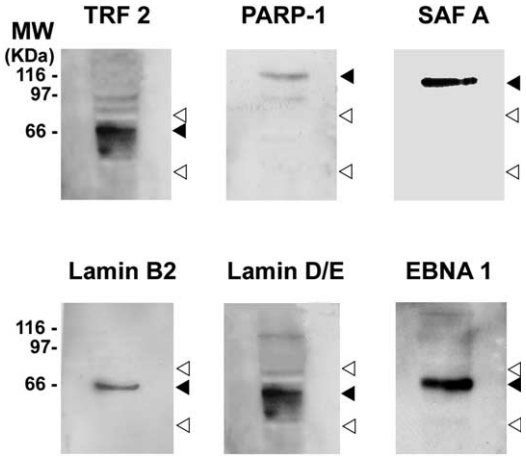

Fig. 5. Western blots of cross-linked NM proteins with specific antibodies. Cross-linked NM proteins isolated from latently-infected Raji cells previously hybridized with ori P were incubated with specific antibodies according to the conditions described in Section 2. Detection of the positive bands was performed by chemiluminescent assay. Full arrowheads indicate the position of the related proteins; empty arrowheads indicate the position of the 85 and 60 kDa proteins highlighted by ori P binding.

4. Discussion

A growing number of studies have been investigating the functional role that specific EBV DNA sequences, within ori P, play in the replication and segregation of the viral genomes. It is becoming clear that at least three viral components, the ori P maintenance element FR, the replicator element DS, and the viral protein EBNA1 interacting with them, are involved in these processes. However, because the properties of EBV plasmid suggest that viral DNA synthesis and expression is linked to the cellular mechanisms that govern genomic DNA replication and transcription, much effort is currently addressed to identify the cellular factors possibly interacting with ori P. In this respect, a major role seems to be played by the NM, since it has been demonstrated to be responsible for the anchorage of EBV origins of replication [19,24].

In latently infected Raji cells a 5.2 kb region of EBV genome, containing ori P and the genes for the small viral RNAs (EBERs), has previously been shown to be associated with the NM [19]. The fairly large size of this region required a more detailed analysis of the DNA sequences involved in ori P interaction with the NM. Therefore, we set up to determine the core of the viral S/MAR element by subjecting different functional regions of ori P to in vitro re-association with isolated scaffold.

By in vitro binding experiments, we report here that two ori P fragments, one 208 bp long, encompassing the DS element, and the one upstream, with a size of 372 bp, containing sequences dispensable for replicative function, show the strongest S/MAR activity. The selective association of IFNβ SAR fragments to Raji NM, in a similar binding assay, validates the results obtained with the different ori P regions. It is well known that the interactions of S/MARs with the nuclear scaffold are substantially different from well characterized protein–DNA interactions that involve a specific DNA consensus

sequence [13,27,38]. However, most S/MARs of cellular DNA share different properties such as A–T-rich regions with predominance of the sequence ATATTT [33], topoisomerase II sites [39], unusual DNA structures such as tracts with a narrow minor groove [40], base unpairing DNA regions [41,42] or DNA bends [43]. Some of these features seem also to characterize the S/MAR of EBV latent origin of replication. A computer program (MAR-Wiz, Futuresoft Corporation) that considers a number of properties for predicting MAR activity has revealed the presence of two kinked DNA stretches and one topoisomerase II site in the 372 and one topoisomerase II site in the 208 bp fragment. In addition, the ATATTT motif occurs five times in the 372 bp fragment with 83% identity, suggesting that a cooperative effect of multiple sites might contribute to determine the higher affinity for the NM.

cis-DDP has scarce propensity to form protein–protein complexes while it very efficiently induces the formation of DNA–protein cross-linkages with low reactivity towards histones [29]. For these features, it represents a highly suitable cross-linking agent to select those NM proteins that most likely bind the DNA 'in vivo'.

We report here the identification of two NM proteins, of about 85 and 60 kDa respectively, that by Southern–Western technique reproducibly bind ori P with high affinity and specificity. Similar results were obtained when the NM isolation procedure was performed by high salt extraction of the nuclei [44] (data not shown). Because ori P recognizes the 85 and 60 kDa proteins also in NM preparations derived from EBV-negative Ramos cells, these proteins appear to be constituents of the NM, independently of the viral infection.

It is intriguing to observe that ori P did not bind these two protein species when cross-linked NM proteins were isolated from induced Raji cells (see Fig. 4B). It has been reported that EBV lytic cycle gene expression protects the infected cells from apoptosis induced by lytic cycle activators [45]. Our results confirm this finding, since a substantial decrement in the concentration of SAF-A and poly(ADP-ribose)polymerase 1 (PARP1) was measured after induction of the EBV lytic cycle, in the absence of the related apoptotic forms (unpublished data). Because also under our experimental conditions induced Raji cells appear to elude apoptosis, the lack of ori P binding to the 85 and 60 kDa species in the NM of these cells cannot depend on cell death-related degradation of nuclear proteins. A likely possibility could be that profound changes in NM composition/architecture take place during the early phases of EBV productive infection. Ongoing studies are addressed to verify this hypothesis.

In an attempt to characterize the 85 and 60 kDa proteins, we have used antibodies directed to main components of the NM, telomere-associated proteins and proteins possessing PARP activity that have been recently found to bind the DS element in an EBNA1-dependent manner [34]. Because the 85 or the 60 kDa proteins are not identifiable with previously characterized ori P binding proteins, nor with the major constituents of the NM, they might represent specific NM components involved in EBV anchorage and replication during the latent cycle of infection.

Ongoing studies, aimed to purify and characterize these proteins, will provide the tools to investigate the molecular features and the functional role of ori P interaction with the NM.

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