Epstein–Barr virus nuclear antigen-1 (EBNA-1) binds directly to a Hodgkin cell line (Kube et al., 1999) and has been associated with enhanced expression of CD25 in certain EBV latent genes (Aiyar et al., 2000; Kieff & Rickinson, 2001; Nonkwelo et al., 1999). The EBNA-1 protein is the only EBV-encoded protein that is required for the replication/retention and segregation/partition of oriP-containing plasmids. Here the chromosomal localization of EBNA-1 fused to green fluorescent protein (GFP–EBNA-1) is examined by confocal microscopy combined with a 'premature chromosome condensation' (PCC) procedure. Analyses show that GFP–EBNA-1 expressed in living cells that lack oriP plasmids is associated with cellular chromatin that has been condensed rapidly by the PCC procedure into identifiable forms that are unique to each phase of interphase as well as metaphase chromosomes. Studies of cellular chromosomal DNAs labelled with BrdU or Cy3-dUTP indicate that GFP–EBNA-1 colocalizes highly with the labelled, newly replicated regions of interphase chromatin in cells. These results suggest that EBNA-1 is not only with cellular metaphase chromosomes but also with condensing chromatin/chromosomes and probably with interphase chromatin, especially with its newly replicated regions.

The Epstein–Barr virus (EBV) nuclear antigen-1 (EBNA-1) protein is the only EBV-encoded protein that is required for the replication, partition/segregation and maintenance/retention of EBV DNA containing the plasmid oriP, which includes the sequences for replication (Aiyar et al., 1998; Hung et al., 2001; Yates, 1996; Yates et al., 2000). EBNA-1 is also a transcriptional regulator (Kieff & Rickinson, 2001; Nonkwelo et al., 1996; Rickinson & Kieff, 2001; Sugden & Warren, 1989) and has been associated with enhanced expression of CD25 in a Hodgkin cell line (Kube et al., 1999). The EBNA-1 homodimer binds directly to oriP, linking regions of oriP DNA; oriP has the two EBNA-1-binding elements, the dyad symmetry and a family of repeats elements (reviewed by Yates, 1996; Fujita et al., 2001; Mackey & Sugden, 1999). EBNA-1 also binds to several cellular proteins (Ito et al., 2000; Kusano et al., 2001; Shire et al., 1999), including RPA (hSSB) (Zhang et al., 1998).

EBNA-1, which is present at constant levels throughout the cell cycle, is detected by immunofluorescence analyses as diffused and punctate spots in the nuclei of interphase cells. EBNA-1 binds diffusely to mitotic chromosomes (Grogor et al., 1983; Marchal et al., 1999; Petti et al., 1990). Three separate domains of EBNA-1, aa 8–54, 72–84 and 328–365, are required for its binding to metaphase chromosomes (Marchal et al., 1999). The binding of EBNA-1 to chromosomes is considered to facilitate the partition of a low copy number of latent EBV plasmids, which replicate once per cell cycle, to dividing cells (Marchal et al., 1999).

In this study, we have investigated whether EBNA-1 colocalizes with cellular chromatin in interphase in vivo using a combined approach of confocal laser-scanning microscopy (LSM) of EBNA-1 fused to green fluorescent protein (GFP–EBNA-1), a ‘premature chromosome condensation’ (PCC) procedure, which facilitates the visualization of chromatin in interphase cells (Gotoh et al., 1995; Johnson & Rao, 1970; Rao, 1977), and pulse-labelling of cellular DNAs with BrdU or Cy3-dUTP.

The CHO-K1 cell line (originally from the ATCC) was obtained via the Japanese Cancer Research Resources Bank (Tokyo). To construct the GFP–EBNA-1 fusion protein, a truncated EBNA-1 lacking the Gly–Ala copolymer was cleaved from plasmid p205 (Yates et al., 1985) with BamHI/HindIII and inserted into the pEGFP-C3 vector (Clontech) at its BglII and HindIII sites (Fig. 1A).

For LSM of the PCC process in living cells, cells were grown in a glass-bottomed dish (Iwaki). Calyculin A (Wako) was added to 50 nM after incubation in the presence of 20 µM Hoechst dye 33258 and the cells were kept under microscopy to observe the PCC process continually. For spreading the individual premature condensed chromosomes (PCCs), calyculin A-treated cells were swollen in 0·075 M KCl, fixed using...
Fig. 1. Construction and expression of GFP–EBNA-1. (A) The GFP–EBNA-1 fusion protein construct lacking the Gly–Ala copolymer. The regions of EBNA-1 that are essential for its binding to cellular metaphase chromosomes (Marechal et al., 1999) are indicated between the maps of EBNA-1 and GFP–EBNA-1. (B) Immunoblots of EBNA-1 from whole cell extracts of CHO-K1 cells expressing full-length B95-8 EBNA-1 or CEW21N4 cells (lane 1) and GFP–EBNA-1 (lane 2) using the anti-EBNA-1 mouse monoclonal antibody OT1x and alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody. GFP–EBNA-1 (84 kDa) and B95-8 EBNA-1 (78 kDa) proteins were produced in CHO-K1 cells. (C) LSM images of GFP–EBNA-1-expressing CHO-K1 cells in mitotic phase and interphase. Shown are profiles of GFP–EBNA-1 (a1) and DNA (a2, stained with Hoechst dye 33258); their merged (a3) and Nomarski (a4) images are also shown in a living, round GFP–EBNA-1-expressing CHO-K1 cell in mitotic phase. The profiles of the GFP–EBNA-1 protein in living GFP–EBNA-1-expressing CHO-K1 cells in interphase (b1) and its Nomarski image (b2) are shown alongside profiles of immunostained non-fused full-length EBNA-1 in CEW21N4 cells. (c) Fixed CEW21N4 cells were incubated with the anti-EBNA-1 rat monoclonal antibody 2B4-1 and FITC-conjugated secondary antibody; immunostaining (c1) and Nomarski (c2) images are shown.

Carnoy’s fixative or methanol–acetic acid (3:1) and then dropped onto glass slides. GFP fluorescence lost through using the acidic Carnoy’s fixative was restored by incubating PCCs in 0.1 M borate buffer (pH 8.5) at room temperature for 30 min followed by incubation in PBS at 4 °C overnight, according to Ward & Bokman (1982). Fixed PCCs were stained with 1 µg/ml Hoechst dye 33258 in PBS. Giemsa staining has been described previously (Gotoh et al., 1995).

Digital images of the fluorescent profiles were acquired on an Axiovert 100M LSM equipped with argon and helium-neon laser light sources using the LSM510 system software, which included the palette option (Zeiss). The peak UV excitation wavelengths were 351 and 364 nm. An objective lens of C-Apochromat 63 x/1.2w corr for optical correction was used for water immersion examination.

For BrdU labelling, cells were incubated in medium containing 10 µM BrdU (Sigma) for 30 min at 37 °C and then in BrdU-free medium for 3 h at 37 °C. These BrdU pulse-labelled cells were treated with calyculin A, swollen under hypotonic conditions, fixed with methanol–acetic acid (3:1) and spread onto glass slides. The fluorescence of GFP was observed as described above. The glass slides were treated with 100 units/ml DNase I (Takara) in 100 mM sodium acetate (pH 5.3) and 5 mM magnesium sulfate at 37 °C for 60 min, according to Carayon & Bord (1992). The PCCs were then incubated with an anti-BrdU mouse monoclonal antibody (MBL) followed by Texas red-conjugated donkey anti-mouse IgG antibodies (Jackson Laboratories).

Cy3-dUTP (Pharmacia) was incorporated into monolayer cells according to the methods of McNeil & Warder (1987) and Manders et al. (1999). Briefly, Cy3-dUTP was added at 10 µM to cells and 425–600 µm diameter glass beads (Sigma) were immediately sprinkled onto the cells. The cell culture covered with the beads was tapped and the beads were washed out immediately. The cells were then incubated in fresh medium.

The GFP–EBNA-1 fusion protein colocalizes with cellular chromatin that is ‘prematurely’ condensed during interphase. The GFP–EBNA-1 fusion protein lacking the Gly–Ala copolymer, which is not required for the replication and maintenance of EBV DNA (Yates et al., 1985) or for metaphase chromosome binding (Marechal et al., 1999), was expressed in CHO-K1 cells; chromosomes of CHO-K1 cells are more clearly visible owing to their small number (20 per cell) and correspondingly large size. The 84 kDa GFP–EBNA-1 protein was detected in...
EBNA-1 colocalizes with interphase chromatin

![GFP–EBNA-1 colocalization with interphase chromatin](image)

**Fig. 2.** Localization of GFP–EBNA-1 in relation to chromatin being condensed in interphase GFP–EBNA-1-expressing CHO-K1 cells by the PCC reagent calyculin A. (A) Photomicrographs of a living cell taken immediately before the addition of calyculin A show GFP–EBNA-1 (a1) and chromosomal DNA (a2, stained using Hoechst dye 33258 but shown in pseudo-colour red); the merged image is shown in (a3). Also shown are GFP–EBNA-1 and chromatin undergoing PCC with their merged images taken, respectively, 20 (b1–3) and 60 (c1–3) min after the addition of calyculin A. Cells shown in the photograph sets (a), (b) and (c) are different ones in the same culture dish. Photomicrographs of control GFP in CHO-K1 cells taken 0 and 40–60 min after the addition of calyculin A are shown in (d) and (e, f), respectively. (B) Localization of GFP–EBNA-1 in relation to methanol–acetic acid-fixed chromatin prematurely condensed by calyculin A. Calyculin A-induced PCCs were fixed with Carnoy’s fixative and spread onto glass slides. GFP fluorescence was restored by renaturation with borate buffer (pH 8.5) and PBS. Merged images of GFP–EBNA-1 and DNA of PCCs (stained using Hoechst dye 33258 but shown in pseudo-colour red) are shown in (a–f); the observed profiles are typical of G1/S phase (a), S phase (b, c) and G2 phase (d–f) PCCs. A Nomarski image of calyculin A-induced PCCs that were Giemsa-stained is shown in (g). This Nomarski image taken from the same glass slide that was examined by confocal microscopy in (a–f) and shows various forms of PCCs, indicating interphase stages of the cell cycle; the solid arrowhead indicates PCCs typical of early S phase, the arrow indicates PCCs typical of middle S phase and the open arrowhead indicates PCCs typical of G2 phase (g). Similarly, merged LSM photomicrographs of CHO-K1 cells expressing GFP as a negative control are shown in (h); GFP was not detected on the fixed and spread PCCs, which indicates that GFP does not bind to PCCs.

Extracts from GFP–EBNA-1-expressing CHO-K1 cells by Western blotting (Fig. 1B). GFP–EBNA-1 was localized in intranuclear granules or spots, similar to those of full-length B95-8 EBNA-1 expressed in CHO-K1 cells (Fig. 1C). LSM confocal images of GFP–EBNA-1 and DNA showed that GFP–EBNA-1 colocalized with mitotic chromosomes in living cells, which is in agreement with the results of Marechal et al. (1999) (Fig. 1C, a).

To visualize chromatin in interphase cells, we took advantage of the PCC procedure, which was discovered following the cell fusion of interphase cells with mitotic ones (Johnson & Rao, 1970; Rao, 1977). Recently, chemical procedures have been developed to induce PCC using the phosphatase inhibitors calyculin A, foscirecin and okadaic acid (Coco-Martin & Begg, 1997; Gotoh et al., 1995; Guo et al., 1995). These reagents induce PCC at a given stage of cell division, bringing about various forms of PCCs that are characteristic of each phase or stage of the cell cycle (Alsbeih & Raaphorst, 1999; Coco-Martin & Begg, 1997; Gotoh et al., 1995).

We analysed the distribution pattern of EBNA-1 throughout the process of calyculin A-induced PCC in living GFP–EBNA-1-expressing CHO-K1 cells by maintaining a dish of cultured cells under the microscope. The GFP–EBNA-1 protein was concentrated together with chromosomal DNAs onto PCCs throughout the PCC process, as shown in the early
Fig. 3. Localization of GFP–EBNA-1 in relation to pulse-labelled or replicated regions of chromatin that was prematurely condensed. (A) BrdU-labelled PCCs in GFP–EBNA-1-expressing CHO-K1 cells were prepared as described in the text. Images of GFP–EBNA-1 and BrdU-labelled chromatin are shown in (a1) and (a2), respectively; the merged image is shown in (a4). The image in (a2) is merged with an image stained for DNA in (a3). Arrow indicates PCCs typical of S phase. Images of
EBNA-1 colocalizes with interphase chromatin

(Fig. 2A, b) and final (Fig. 2A, c) stages of PCC [note that cells shown in Fig. 2(A, a–c) are different cells from the same dish]. In contrast, control GFP remained diffuse after treatment with calyculin A (Fig. 2A, e and f).

To analyse more precisely the association of GFP–EBNA-1 with PCCs, we spread the PCCs onto glass; spread PCCs from G1, S and G2 phases are clearly distinguishable from each other. The fluorescence profiles of GFP–EBNA-1 associated with the spread PCCs showed that GFP–EBNA-1 was present in fibres and strands (Fig. 2B, a–f). We processed the same glass slides for Giemsa staining and re-examined their Nomarski images to obtain more distinct profiles (Fig. 2B, g). These images exhibited fibrous PCCs typical of early S phase (Fig. 3B, a, solid arrowheads), partly thin and partly thick PCCs typical of middle S phase (Fig. 2B, g, arrow) and bivalent thick PCCs typical of G2 phase (Fig. 2B, g, open arrowhead), as described previously (Gotoh et al., 1995; Johnson & Rao, 1970; Rao, 1977). Together, these photomicrographs show that GFP–EBNA-1 colocalized with PCCs that were most probably formed from G1/S phase chromatin (Fig. 2B, a), S phase chromatin (Fig. 2B, b and c) and G2 phase chromatin (Fig. 2B, d–f); in contrast, control GFP was not detected on the spread PCCs, indicating that GFP does not associate with PCCs and thus was taken off during the fixation and spreading process (Fig. 2B, h). The intensity of GFP–EBNA-1 relative to that of the Hoechst dye 33258 varied greatly across the spread PCCs (Fig. 2B, d–f), suggesting that EBNA-1 is not associated uniformly with interphase chromatin. In addition, there was no difference in the profiles of Giemsa-stained and spread PCCs between control CHO-K1 and GFP–EBNA-1-expressing CHO-K1 cells, suggesting that GFP–EBNA-1 does not affect the chromosome condensation process. These results show that GFP–EBNA-1 colocalized not only with cellular metaphase chromosomes but also with prematurely condensing chromatin and/or chromosomes in these calyculin A-treated interphase cells.

To analyse further the colocalization of EBNA-1 with chromatin in S phase, we pulse-labelled GFP–EBNA-1-expressing CHO-K1 cells with BrdU, treated them with calyculin A and fixed and spread the PCCs onto glass slides. GFP–EBNA-1 was highly colocalized with the fibrous BrdU-labelled and immunostained PCCs that were typical of S phase (Fig. 3A, a, arrow) and G2 phase (Fig. 3A, b), suggesting that EBNA-1 is associated with replicated regions of chromatin.

Next, we pulse-labelled cellular DNAs with Cy3-dUTP to examine the localization of GFP–EBNA-1 on PCCs in living cells. The Cy3-labelled patterns were similar to those reported by Sadoni et al. (1999). Confocal LSM of Cy3-labelled GFP–EBNA-1-expressing CHO-K1 cells, which were maintained in culture medium throughout the microscopic observations, demonstrated that EBNA-1 colocalized well with the Cy3-labelled regions of cellular chromatin in living cells (Fig. 3B, a and b, top rows); the same cells were treated with calyculin A, observed continually under the microscope and sequential photographs of the PCCs of the same cells were taken. The photographs taken at the indicated time after the addition of calyculin A indicated that GFP–EBNA-1 remained associated with the Cy3-labelled, recently replicated regions of chromatin throughout the PCC process (Fig. 3B).

The binding of EBNA-1 to metaphase chromosomes is considered to be involved in the segregation of a low copy number of EBV plasmids in dividing cells (Hung et al., 2001; Marechal et al., 1999). EBNA-1 of herpesvirus papio and LANa of Kaposi’s sarcoma-associated herpesvirus, proteins that are necessary for the persistence of these viral episomes, also bind to mitotic chromosomes (Ballestras et al., 1999; Piolot et al., 2001).

In the studies presented here, we have shown that EBNA-1 colocalizes with cellular chromatin that is condensed during interphase in the absence of oriP DNA by using a combined approach of confocal microscopy of a GFP–EBNA-1 fusion protein, PCC and pulse-labelling of cellular replicating DNAs. The results of our studies in living cells expand previous biochemical data showing that EBNA-1 is found in chromatin fractions from both EBV latently infected cells (Petti et al., 1990) and cells containing no oriP plasmids (Kanda et al., 2001) because isolation or purification of intact chromatin is difficult (reviewed by Cook, 2001). Moreover, we have shown that GFP–EBNA-1 is highly colocalized with recently replicated regions of cellular chromatin in S phase in particular. EBNA-1 might bind to a cellular chromatin/chromosome protein during normal condensation in the mitotic prophase but it seems less likely because our observations in this study showed that GFP–EBNA-1 highly colocalized with not only the PCCs that
were formed during G₂ phase but also those formed during G₁ and S phases. Another possibility that GFP–EBNA-1 bound to PCCs only after the induction of premature condensation is also less likely because GFP–EBNA-1 was as highly colocalized with Cy3-labelled regions of cellular DNA before the induction of PCC as during and after PCC (Fig. 3B). Thus, we conclude that EBNA-1 is likely to be associated with interphase chromatin in living cells and, in particular, with its newly replicated regions. Further studies on the interaction of EBNA-1 with chromatin/chromosomes and their components during the course of the cell division cycle should provide better understanding of the molecular mechanism of the multiple functions of this key protein in EBV latency.

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