Enhancement of hepatitis C virus replication by Epstein–Barr virus-encoded nuclear antigen 1

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Based on our recent observation that Epstein–Barr virus (EBV) is detected in 37% of the tissues of hepatocellular carcinoma, and especially frequently in cases with hepatitis C virus (HCV), the effect of EBV infection on the replication of HCV was investigated. EBV-infected cell clones and their EBV-uninfected counterparts in cell lines MT-2 (a human T-lymphotropic virus type I-infected T-cell line), HepG2 (a hepatoblastoma cell line) and Akata (a Burkitt's lymphoma cell line) were compared in terms of their permissiveness for HCV replication following inoculation of HCV derived from patients who were HCV carriers. The results indicated that EBV-infected cell clones, but not their EBV-uninfected counterparts, promoted HCV replication. EBV-encoded nuclear antigen 1 (EBNA1), which is invariably expressed in EBV-infected cells, supported HCV replication. Deletion analysis of the EBNA1 gene showed good correlation between transactivation activity and the activity supporting HCV replication. The present findings suggest that EBV acts as a helper virus for HCV replication.
Keywords: EBNA1/Epstein–Barr virus/hepatitis C virus/ hepatocellular carcinoma/transactivation

Introduction

Hepatitis C virus (HCV) is an enveloped virus belonging to the Flaviviridae family, whose genome consists of a positive-stranded 9.5 kb RNA molecule and encodes a large polyprotein precursor of ~3000 amino acids (Kato *et al.*, 1990; Berns, 1996). The mechanisms of HCV infection and replication in targeted cells are still poorly understood since a cell culture system capable of efficient HCV replication has not been developed.

Epstein–Barr virus (EBV) is a human herpesvirus that infects the majority of the human population. EBV is commonly transmitted by saliva and, following replication in epithelial cells of the oropharynx, infects B lymphocytes where it persists in a latent state for the lifetime of the host. The virus is associated with human malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, opportunistic lymphoma in immunocompromised hosts and some cases of Hodgkin's disease that occur after prolonged persistence and reactivation of latent EBV (Rickinson and Kieff, 1996). Furthermore, EBV can be associated with liver disease during infectious mono-nucleosis and post-transplantation lymphoproliferative disorders (Markin, 1994). Active EBV infection, particularly in adolescents, provokes infectious mononucleosis, in which the liver shows multiple necrotic foci similar to those in acute viral hepatitis infections. We have recently investigated the existence of EBV in liver tissues from patients with hepatocellualr carcinoma (HCC; Sugawara *et al.*, 1999). The results indicated that there was a high EBV load (>1 EBV DNA copy/100 cells) in the hepatocytes of 13 of the 35 HCC tissues. Furthermore, this was especially frequent in cases with HCV (nine of 22 cases). We therefore hypothesized that there might be some interaction between EBV and HCV.

Thus, we examined the effect of EBV infection on HCV replication *in vitro*. The results indicated that EBV enhanced HCV replication and that EBV-encoded nuclear antigen 1 (EBNA1) was responsible for supporting HCV replication.

## Results

Enhancement of HCV replication by EBV

EBV-infected cell clones were isolated from MT-2 (a human T-lymphotropic virus type I-infected T-cell line; Miyoshi *et al.*, 1981) and HepG2 (a hepatoblastoma cell line; Aden *et al.*, 1979) by infecting recombinant EBV with a selectable marker (Shimizu *et al.*, 1996), and from a Burkitt's lymphoma-derived Akata line (Takada, 1984; Takada and Ono, 1989) by cell cloning (Shimizu *et al.*, 1994). EBV-infected cell clones and their EBV-uninfected counterparts were compared in terms of their permissiveness for HCV replication.

Cells (2×10^5) were incubated with 10 µl of undiluted serum (1B-1, containing ~10^5 HCV genomes/ml, determined by RT–PCR) in 1 ml of culture medium for 2 h at 37°C, as described previously (Mizutani *et al.*, 1996). After cells had been washed three times with phosphate-buffered saline (PBS), 2 ml of fresh medium were added and incubation was continued. The cells were collected at 0, 5, 9, 14, 21 and 30 days post-inoculation, and HCV RNA of each cell sample was detected by RT–PCR. Each RT–PCR contained RNAs corresponding to 2×10^3 cells and this assay could detect five copies of HCV RNA per 10^3 cells.

As a result, HCV RNA could not be detected on day 5 post-inoculation in any EBV-negative MT-2 cell clone, but was detected on days 5, 9 and 14 post-inoculation in
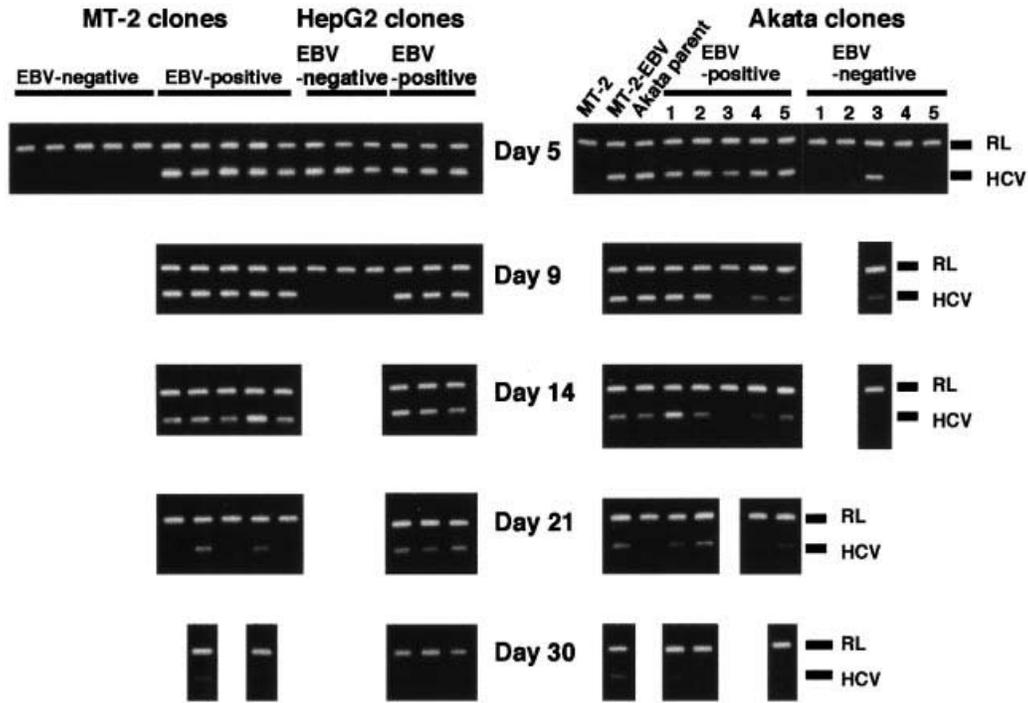
Fig. 1. Detection of HCV RNA by RT–PCR in samples from EBV-infected MT-2, HepG2 and Akata cell clones. Cells were incubated with the HCV suspension for 2 h, washed, and resuspended in fresh medium. After the designated incubation time, a portion of the culture was harvested and assayed for HCV RNA using RT–PCR. As an internal control of RT and PCR reactions, RL RNA was added to each RT–PCR reaction.
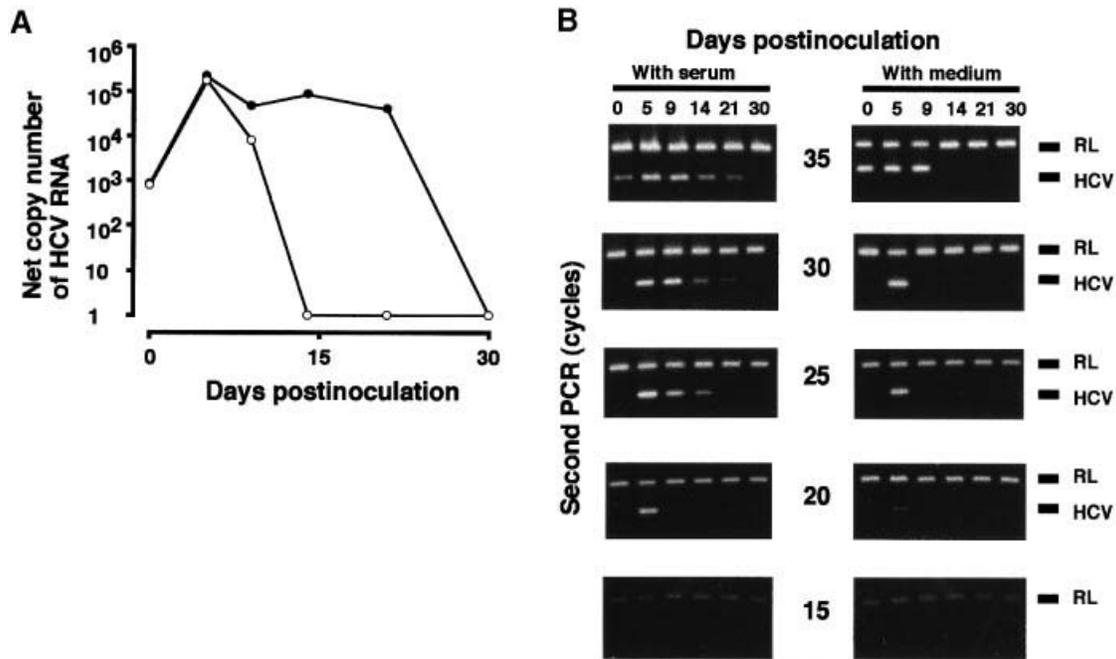
Fig. 2. (**A** and **B**) Semi-quantitative estimation of the net copy number of HCV RNA in an EBV-infected MT-2 cell clone after HCV inoculation with HCV from serum (●) and culture supernatant (○). The second PCR was performed for 15, 20, 25, 30 and 35 cycles. As an internal control of RT and PCR reactions, RL RNA was added to each RT–PCR reaction.

all five EBV-infected MT-2 cell clones and at 30 days post-inoculation in two of them (Figure 1). Similar results were also obtained for HepG2 and Akata cells (Figure 1).

To ensure that the observed RNA signals reflected HCV replication and not inoculated HCV, the copy number of HCV RNA was estimated semi-quantitatively by changing PCR cycles. HCV RNA from EBV-infected MT-2 cells was 850 copies on day 0 and reached a level of 2.2×10^5 copies at day 5 post-inoculation (Figure 2). These results indicated that EBV enhanced the replication of HCV.

Production of infectious HCV in EBV-infected MT-2 cells

To examine whether infectious HCV was produced in the present system, the culture supernatant was collected from EBV-infected MT-2 cells at 4 days post-inoculation and
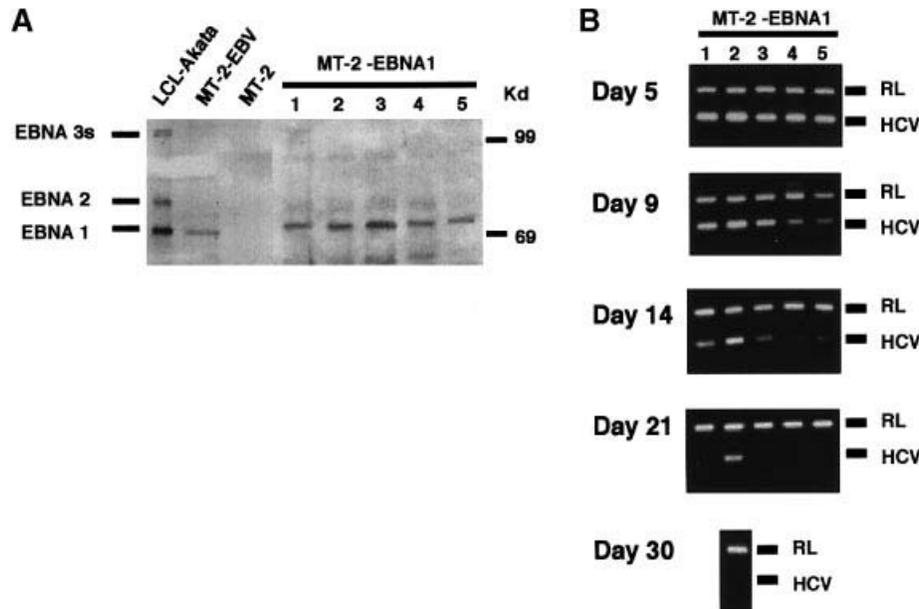
Fig. 3. HCV replication in EBNA1-positive MT-2 cell clones. (**A**) EBNA1 expression in MT-2 cell clones transfected with the EBNA1 plasmid. EBNA1 was detected by immunoblotting using a standard EBNA-positive human serum. (**B**) Detection of HCV RNA by RT–PCR in EBNA1-positive MT-2 cell clones. Cells were incubated with the HCV suspension for 2 h, washed, and resuspended in fresh medium. After the designated incubation time, a portion of the culture was harvested and assayed for HCV RNA using RT–PCR. As an internal control of RT and PCR reactions, RL RNA was added to each RT–PCR reaction.

filtered through a 0.45-μm-pore-size membrane. EBV-infected MT-2 cells (2×10^5) were inoculated with 2 ml of the filtrate containing ~10^3 HCV RNA copies/ml for 2 h at 37°C. The cells were washed with PBS and incubated in 2 ml of fresh medium. After 5 days, net HCV RNA in the MT-2 cells was calculated as 1.8×10^5 copies (Figure 2), which confirmed that production of infectious HCV had occurred in EBV-infected MT-2 cells.

Enhancement of HCV replication by EBNA1

Among EBV-encoded genes, EBNA1, latent membrane proteins (LMP) 1 and 2A, *Bam*HI A transcripts (BARF0) and EBV-encoded small RNAs (EBERs) are expressed in EBV-infected MT-2 cells (Yoshiyama *et al.*, 1995). EBV-infected HepG2 cells express EBNA1, LMP1 and LMP2A, BARF0 and EBERs (Imai *et al.*, 1998). EBV-positive Akata cells (Komano *et al.*, 1998) express EBNA1, LMP2A, BARF0 and EBERs. We examined which EBV gene among the four EBV-encoded genes (EBNA1, LMP1, LMP2A and EBERs) was responsible for supporting HCV replication. The MT-2 cell clone was transfected with an individual EBV latent gene, and cell clones that stably expressed similar levels of EBV-infected cells were selected and analyzed. The results indicated that all MT-2 cell clones transfected with the EBNA1 gene supported HCV replication (Figure 3), whereas those transfected with other latent genes did not (data not shown).

Identification of the regions of EBNA1 necessary for supporting HCV replication

The ability of derivatives of EBNA1 to support replication of HCV was then tested in the MT-2 cell clones that stably expressed derivatives of EBNA1 (Figure 4A–C). HCV replication in d1553 and d1743 cell clones was as efficient as in wild-type EBNA1 cells. In d1745 cells, HCV RNA was detected only at 5 days post-inoculation

at a level of 10^3 RNA copies/10^5 cells. Other derivatives of EBNA1 could not support HCV replication. These results indicate that amino acids 40–90 and 328–641 of EBNA1 are necessary to support HCV replication.
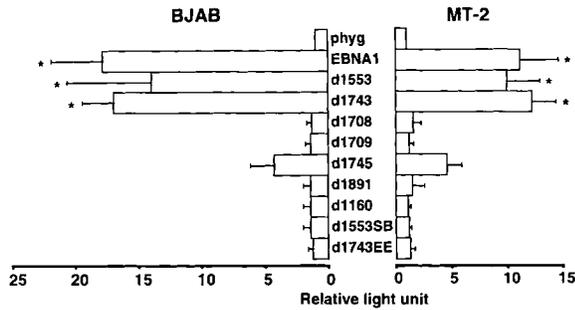
As shown in Figure 4A, the regions of EBNA1 required to support HCV replication were mostly consistent with those reported previously for transactivation (Yates and Camilo, 1988; D.Mackey and B.Sugden, unpublished data). To confirm the correlation between the activity of transactivation and that supporting HCV proliferation, the activation of transcription from the *ori*P-*Bam*HI C-Luc reporter by EBNA1 derivatives was measured in MT-2 and BJAB cells. Significantly higher numbers of relative light units compared with the control mock-transfected cells were recognized in the MT-2 and BJAB cells transfected with plasmids d1553 and d1743 (Figure 5). The relative numbers of light units in the MT-2 and BJAB cells transfected with plasmid d1745 were 4.6 ± 1.3 and 4.3 ± 1.9, respectively. Thus, the ability of each plasmid to transactivate the reporter gene correlated well with the ability to enhance HCV replication.

Discussion

The present study indicated that HCV replication was promoted by EBV and that EBNA1 was responsible for supporting HCV replication. Furthermore, deletion analysis of the EBNA1 gene showed good correlation between the transactivational activity of the EBNA1 gene and its activity supporting HCV replication.

EBNA1 is a DNA-binding protein that binds to clusters of specific sequences within the origin of latent viral DNA replication (*ori*P; Rawlings *et al.*, 1985; Reisman and Sugden, 1985). The protein is required for maintenance and replication of EBV plasmids in EBV-infected cells (Yates *et al.*, 1984, 1985). EBNA1 has also been shown

Fig. 4. HCV replication in MT-2 cell clones expressing derivatives of EBNA1. (**A**) Schematic diagram of wild-type EBNA1 and derivatives of EBNA1. The Gly-Gly-Ala repeats (90–328) and nuclear localization signal (379–391) are shown in the upper column. The black bar in each EBNA1 derivative indicates a deleted region. The hatched bars in the lower columns are location limits for known functions of EBNA1 (Yates and Camilo, 1988; Snudden *et al.*, 1994). (**B**) EBNA1 expression in MT-2 cell clones transfected with EBNA1 derivatives. EBNA1 was detected by immunoblotting using an mAb for EBNA1. (**C**) Detection of HCV RNA by RT–PCR in MT-2 cell clones expressing EBNA1 derivatives. Cells were incubated with the HCV suspension for 2 h, washed, and resuspended in fresh medium. After the designated incubation time, a portion of the culture was harvested and assayed for HCV RNA by RT–PCR. As an internal control of RT and PCR reactions, RL RNA was added to each RT–PCR reaction.

to promote transcriptional transactivation via binding to the enhancer sequence within *ori*P (Yates and Camilo, 1988). It is possible that EBNA1 promotes the transcription of foreign genes when the genes have the cognate sequence for EBNA1 binding, although no promoter of a cellular gene that has cognate sequences for EBNA1 binding and is transactivated by EBNA1 has been found.

The dependence of virus replication on helper viruses is well known in adeno-associated virus (AAV). It requires co-infection with an unrelated helper virus, either an adenovirus (Ad) or a herpesvirus, for productive infection in cell culture (Berns, 1996). Genetic analysis of helper functions has been most extensive for Ad, and indicates that all of the Ad helper functions affect gene expression, i.e. Ad early region 1A protein is required for transactiv-

ation of AAV gene expression. Therefore, it is conceivable that EBNA1 promotes HCV replication via its transactivating function.

Recent research has revealed that EBNA1 can bind to RNA, at least *in vitro*, and might therefore be capable of controlling gene expression at the post-transcriptional level (Snudden *et al.*, 1994). The domain of EBNA1 responsible for binding RNA is called the RGG motif (Figure 4A). However, the RGG motif domains of EBNA1 seem to be dispensable for promoting HCV replication because d1553SB and d1743EE plasmids with intact RGG motifs did not support HCV replication. HCV is presumed to replicate in the cytoplasm and not in nuclei (Rice, 1996). In contrast, EBNA1 is localized in nuclei. Therefore, we assume that EBNA1 influences HCV replication not

Fig. 5. Transactivation ability of EBNA1 derivatives. The *ori*P-*Bam*HI C-Luc reporter plasmid and each plasmid of EBNA1 derivatives were co-transfected into BJAB and MT-2 cells. After 2 days of transfection, cells were harvested and the relative light units of luciferase activity were measured. The results are expressed as fold transactivation relative to that of mock (phyg)-transfected cells. Data presented represent averages of three experiments. *$p < 0.0009$ compared with the level of mock-transfected cells.

directly but indirectly by inducing transcription of the cellular gene.

Throughout the world, HCV is the major etiologic agent of post-transfusional non-A, non-B hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989). Persistent HCV infection is frequently associated with liver cirrhosis and hepatocellular carcinoma (HCC) (Ohkoshi *et al.*, 1990; Saito *et al.*, 1990). However, carcinogenesis is a multistep process, and although these viruses may be essential contributing factors, alone they are insufficient for hepatocarcinogenesis. In a previous study, we have demonstrated that EBV is detected in 37% of HCC tissues and especially frequently in cases with HCV, and that hepatocytes become a target of EBV infection (Sugawara *et al.*, 1999). The present results point to the possibility that EBV may be involved in the pathogenesis of HCV-related HCC by enhancing HCV replication. Moreover, the growth-promoting properties of EBV are well known. EBV-infected cells are targets of EBV-specific cytotoxic T cells (Rickinson and Kieff, 1996). Thus, EBV infection could either contribute directly to tumorigenic potential or could contribute to HCC by exacerbating the inflammatory process in liver tissue, although we have not yet obtained direct evidence of this.

Materials and methods

Cell lines and cell culture
The cell lines used for the assay included MT-2 (Miyoshi *et al.*, 1981), HepG2 (Aden *et al.*, 1979) and Akata cells (Takada, 1984; Takada and Ono, 1989). The Akata cells were subcloned and 100% EBV-positive clones and completely EBV-negative clones were isolated (Shimizu *et al.*, 1994).

MT-2 and Akata cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (40 U/ml) and streptomycin (280 μg/ml) at 37°C in a 5% humidified CO_2 atmosphere. HepG2 cells were maintained in Dulbecco's modified Eagle's medium–high glucose under the same conditions as MT-2 and Akata cells.

EBV infection of MT-2 cells and HepG2 cells
An Akata cell clone infected with drug-resistant EBV was treated with anti-immunoglobulin antibody for 48 h. Cell-free EBV was obtained by centrifuging the induced cell cultures and filtering the supernatants through a 0.45 μm filter. For EBV infection, the supernatants were used at a dilution of 1:10 in growth medium. MT-2 cells and HepG2 cells (10^6) were suspended in 1 ml of diluted EBV supernatant for 90 min, pelleted, resuspended in fresh medium and incubated for 2 days. Cells were then plated into 96-well, flat-bottom plates at 10^4 cells/well with

complete medium containing 500 μg/ml G418 (Gibco). Cells were fed every 5 days until colonies emerged (2–3 weeks).

RT–PCR for HCV detection
To detect positive-stranded HCV RNA, RNA was extracted from 10^5 cells at various days post-inoculation. One-tenth of the RNA was used for complementary DNA synthesis with 10 pmol of antisense primer 319R (5′-TGCTCATGGTGCACGGTCTA-3′, corresponding to nucleotides 329–348 of HCV-J; Kato *et al.*, 1990). PCR amplification was performed for 35 cycles using primers 319R and 196 (5′-CCATGGC-GTTAGTATGAGTG-3′, corresponding to nucleotides 83–102 of HCV-J). An internal primer pair, 104 (5′-AGAGCCATAGTGG-TCTGCGG-3′, corresponding to nucleotides 122–141 of HCV-J) and 197R (5′-CTTTCGCGACCCAACACTAC-3′, corresponding to nucleotides 246–265 of HCV-J), was used for the second round of PCR (35 cycles).

As an internal control of RT and PCR reactions, the RNA template was generated from the Renilla Luciferase (RL) plasmid (Promega, Madison, WI) and added to each RT–PCR reaction. RL-RNA (5 pg, corresponding to 10^7 copies) and 2 pmol of antisense primer Rluc1R (5′-ATGGCAACATGGTTTCCACG-3′) were added prior to each RT reaction. The primer pair for the first round of PCR amplication was Rluc1 (5′-TGTGCCACATATTGAGCCAG-3′) and Rluc1R (2 pmol each). Rluc2 (5′-TTGGTATGGGCAAATCAGGC-3′) and Rluc2R (5′-CTTCTTCAGATTTGATCAACGC-3′) were used for the second round of PCR (2 pmol each).

One-fifth of the amplified product was electrophoresed in 2% agarose gel and stained with ethidium bromide. The sizes of the amplified DNA products from HCV RNA and RL RNA were 144 and 276 bp, respectively.

For quantitative estimation, a second PCR was performed by changing PCR cycles as described previously (Mizutani *et al.*, 1996). Briefly, the 5′ untranslated repeat (UTR) of positive-strand HCV RNA was synthesized by *in vitro* transcription from cDNA clones containing sequences from nucleotides 19 to 488 of the 5′ terminus of the HCV-J genome. RNA was serially diluted 10 times and amplified by PCR for between 35 and 10 cycles. The amount of HCV RNA was estimated by determining the highest cycle number of the PCR at which the amplified product was below the saturation level: 30 cycles, 10 copies; 25 cycles, 10^2; 20 cycles, 5×10^2; 15 cycles, 10^3; 10 cycles, 10^4. Each PCR cycle consisted of annealing at 55°C for 45 s, primer extension at 72°C for 1 min and denaturation at 94°C for 1 min.

Immunoblot analysis
For protein preparation, cells were lysed in SDS–PAGE loading buffer. B-lymphoblastoid cells immortalized by Akata EBV (LCL-Akata), EBV-positive Akata cells and BJAB cells (EBV-negative) were used as controls. After being boiled for 5 min, equal amounts of protein (10 μg) were separated in 8 or 10% polyacrylamide gels and transferred to nitrocellulose membranes. Expression of EBNA1 was examined with reference human serum containing antibodies to EBNAs or a monoclonal antibody (mAb) to EBNA1 (1H4; Grässer *et al.*, 1994). The blots were developed using the enhanced chemiluminescence (ECL) method (Amersham) according to the manufacturer's protocol.

Generation of MT-2 cell clones expressing EBV-encoded gene products
The plasmids used for EBV latent genes were as follows. EBO-pSKneo carries the SV40 promoter-driven neomycin-resistant gene, EBNA1 gene and *ori*P sequence of B95-8 origin (Margolskee *et al.*, 1988). For LMP2A, total RNA was extracted from a B-lymphoblastoid cell line immortalized by B95-8 EBV and the complementary DNA was synthesized using 10 pmol of a 3′-primer specific for LMP2A: TTA-GCGCTGCTGCAGCTATGGGGTCCCTAG, which was then amplified with the 3′-primer and 5′-primer: GGATATCGGAATTCCAGAATG-AGGTGG. The amplified products were cloned into pTarget vector (Promega) using the TA cloning technique. The plasmid for *Bam*HI A transcripts was generated as described previously (Sadler and Raab-Traub, 1995) and cloned into pTarget vector. For EBERs, the *Eco*RI K fragment of EBV (Akata strain) was cloned into pcDNA3 (Invitrogen®). pcDNA3 was also used to generate MT-2 cell clones resistant to G418. Twenty micrograms of DNA were electrophoresed into 5×10^5 MT-2 cells using an Electro Cell Manipulator 600 (BTX) at room temperature in cuvettes having a 4 mm electrode gap as described previously (Yoshiyama *et al.*, 1995). Cells were then plated into 96-well, flat-bottom plates at 10^4 cells/well with complete medium containing 500 μg/

ml of G418. Cells were fed every 5 days until colonies emerged (2–3 weeks).

Generation of MT-2 cell clones expressing derivatives of EBNA1

The vector plasmid for derivatives of EBNA1 (Figure 4A) included the hygromycin B phosphotransferase gene, the cytomegalovirus (CMV) immediate-early promoter and the UTR of exon 1 of the CMV immediate-early gene (Middleton and Sugden, 1994; Kirchmaier and Sugden, 1997). All plasmids were gifts from Drs B.Sugden and D.Mackey (McArdle Laboratory for Cancer Research) and have been described in detail previously (Mackey and Sugden, 1999), except for d1553SB and d1743EE, which we made ourselves. After electrophoresis, cells were cultured as described above in complete medium containing 150 µg/ml of hygromycin B (Gibco). Protein expression of the MT-2 cells was ascertained by immunoblot analysis (Figure 4B).

Luciferase assay

For reporter plasmid construction, the *Bam*HI C fragment of EBV DNA (B95-8 origin) was digested with *Eco*RI and *Sfi*I, and an ~3.0 kb fragment was obtained. It was cloned into the *Sfi*I site of PGV-B2 (Picagene®; Toyo Ink MFG. Co. Ltd), which contained the luciferase gene. For transient transcription assays in which activation of transcription from the *ori*P-*Bam*HI C-Luc reporter was measured in MT-2 cells and BJAB cells, 5 µg of *ori*P-*Bam*HI C-Luc were cotransfected with 10 µg of each plasmid of EBNA1 or EBNA1 derivatives into the MT-2 and BJAB cells, using a lipofectamine reagent (Gibco) according to the manufacturer's instructions. After transfection, cells (10^5) were suspended in RPMI-1640 medium containing 10% fetal calf serum, harvested after 72 h incubation and lysed. The assay was performed as described previously (Kirchmaier and Sugden, 1997). The luciferase light unit from each clone expressing EBNA1 or one of the EBNA1 derivatives was compared with that of mock-transfected cells and analyzed by Student's *t*-test with Bonferroni's correction.

Acknowledgements

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