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# The Lytic Cycle of Epstein-Barr Virus in the Nonproducer Raji Line Can Be Rescued by the Expression of a 135-Kilodalton Protein Encoded by the BALF2 Open Reading Frame

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**In Epstein-Barr virus (EBV)-carrying nonproducer Raji cells, the induction of the viral replicative cycle by chemical treatment is limited to only the early stage and viral DNA synthesis is totally inhibited. We previously showed the absence of two messenger RNAs that are encoded by the *Bam*HI-A fragment of the EBV genome and that correspond to open reading frames BALF2 and BARF1 in chemically induced Raji cells. Since the BALF2 gene encodes a 135-kDa DNA-binding protein which was immunoprecipitated by antibody against ICP8 protein, a key protein in herpes simplex virus replication, we asked whether the lack of productive cycle in Raji cells is due to the absence of expression of the BALF2 gene. We transfected the Raji cell line with the BALF2 gene. After chemical induction, the BALF2-transfected cells expressed not only early antigens but also late antigens. In these cultures, the viral particles were detected by electron microscopy. The expression of late antigens was completely inhibited by arabinofuranosylthymine, which is a specific inhibitor of viral DNA replication. The BALF2 gene might play an essential role in the induction of the EBV-lytic cycle.**

The identification of the essential viral genes involved in Epstein-Barr virus (EBV) DNA replication is important to the understanding of the mechanism of viral replication. In the Burkitt's lymphoma-derived Raji cell line, the early stage of the viral cycle can be induced after treatment with chemical products, but viral DNA synthesis is completely inhibited; consequently, it is impossible for this cell line to produce viral particles (3, 39). The Raji EBV genome has two major deletions: the 3.5-kb sequence covering the whole EBNA 3C gene, essential for B-cell immortalization (30), and the BZLF2 gene and the 2.9-kb sequence in the right-hand end of the *Bam*HI-A fragment (14, 25). The virus-encoded enzymes necessary for viral DNA synthesis (DNA polymerase, thymidine kinase [TK], and DNase) were, however, produced in the induced Raji cells expressing early antigens (7, 21–24). At least two early genes localized in the 2.9-kb deleted sequence of the *Bam*HI-A fragment were not transcribed during the early stage of the viral cycle induced in Raji cells (37): the BARF1 gene, encoding a 33-kDa protein capable of transforming rodent fibroblasts and human Louckes B cells (33, 34), and the BALF2 gene, encoding a 135-kDa DNA-binding protein (DBP) (p135 BALF2 protein) (37). Another open reading frame, the BALF1 sequence, is also deleted in the *Bam*HI-A fragment of the Raji EBV genome. Consequently, at least five viral genes could be suspected to be involved in this defective function. Among the proteins encoded by these genes, the p135 BALF2 protein is expected to be essential for EBV replication since this protein has about 30% amino acid sequence homology (27) with the DBP ICP8 encoded by the herpes simplex virus (HSV) genome which is essential for HSV replication (35). On the other hand, the infectious virus particles isolated from Raji cells by superinfection with P3HR1 virus possessed a normal *Bam*HI-A fragment (9) and a 135-kDa DBP which is absent in the Raji cell line was translated in these cells (1, 16, 37). We therefore examined whether the BALF2 gene is capable of restoring EBV replication in the Raji cell line.

The EBV nonproducer Raji $TK^-$ cell line (13) and EBV producer P3HR1 and B95-8 cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (20). For induction of the viral cycle, cells were grown for 3 days with 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml and 1 mM sodium butyrate (SB) (20). To inhibit the late viral cycle, arabinofuranosylthymine (ara-T) at a concentration of 50 μg/ml was added to the cultures (20, 21). A genomic BALF2 sequence and a cDNA BARF1 sequence were cloned into the pZip-Neo-SV(X) 1 vector (34, 37). Transfection was performed by the electric field-mediated DNA transfer method as described previously (31, 33). Three days after transfection, $1 \times 10^3$ to $2 \times 10^3$ cells per well were grown in six-well plates under selection in the presence of 1.2 mg of neomycin (GIBCO) per ml. Neomycin-resistant ($Neo^r$) cells were harvested from independent wells and replated as distinct cell lines. Two $Neo^r$ clones were isolated from BALF2-transfected Raji cell lines, and two other $Neo^r$ clones were also isolated from Raji cells transfected with the pZip vector alone. For obtaining the monoclonal subclones, the above-mentioned BALF2-transfected $Neo^r$ Raji cells were plated into 96-well plates with neomycin at one to two cells in 0.1 ml of growth medium per well. The cells were maintained under these conditions for 4 to 5 weeks, with medium changes once a week. $Neo^r$ cell populations were harvested from independent wells and then expanded into distinct subcell lines.

The deletions of the BALF2 and BARF1 sequences in parental $TK^-$ Raji cells were confirmed by PCR analysis. DNA was extracted in buffer (Tris-HCl, 10 mM, pH 7.4; EDTA, 50 mM; NaCl, 150 mM; Sarkosyl, 1%; proteinase K, 20 mg/ml) as previously described (20). Two defective EBV sequences in the Raji strain—a 256-bp sequence from position 163979 to 164235 in the deleted region of BALF2 and a 679-bp sequence from position 165496 to 166192, containing a whole BARF1

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sequence (2)—were amplified as previously described, with some modifications (5). As expected, both the 256- and 679-bp amplified sequences were completely absent in the parental Raji TK$^-$ strain used in this study (14, 25), while these sequences were present in DNA from the EBV-carrying IB4 and P3HR1 DNAs (data not shown).

**Expression of viral antigens in BALF2-transfected Raji cells.** The expression of the p135 BALF2 protein in Neo$^r$ clones of Raji cells was examined by immunofluorescence with anti-BALF2 serum (Fig. 1, Not-Induced BALF2 serum). The serum revealed a nuclear immunofluorescence (Fig. 1, Raji-BALF2) similar to that already observed with BALF2-transfected rodent cells (37), while control parental Raji cells or Raji cells transfected with pZip alone gave no positive response (Fig. 1, Raji TK$^-$). The NPC serum gave a similar nuclear immunofluorescence in the BALF2 transfectant (Fig. 1, Raji BALF2, Not-Induced NPC serum). We also examined the immunofluorescence in the Raji cell line transfected by the BARF1 sequence. A membrane-cytoplasmic immunofluorescence which was similar to those previously reported for human Louckes B cells transfected with the BARF1 sequence (Fig. 2, Raji BARF1, Not-Induced NPC serum) (33) was observed with the BARF1 transfectant. Parental Raji cells gave no positive immunofluorescence with NPC serum.

When the Raji cell line and BALF2 or BARF1 of the Raji cell line were induced with TPA-SB, the level of lytic antigen expression in these cultures was around 20% of that revealed by NPC serum (Fig. 1, all cells with TPA-SB NPC serum). When these induced transfectants were tested with a monoclonal antibody against the late antigens, anti-viral capsid antigen (VCA) serum, anti-gp110, or anti-gp350, about 5 to 8% of the cell population became positive in only the BALF2 transfectant (Fig. 1, Raji-BALF2 with TPA-SB VCA$^+$, TPA-SB anti-gp110, and TPA-SB anti-gp350) while no such immunofluorescence was detected in the induced parental Raji cell line or in the induced BARF1 transfectant. These results indicate that the BARF1 sequence is unlikely to induce the late event. The distribution (in cytoplasm or whole cells) of gp110 in transfectants was similar to those already observed in the induced B95-8 cells (12). In the case of gp350, its immunofluorescence was localized on cellular membranes or in cytoplasm (Fig. 1). In the noninduced BALF2 transfectant, immunofluorescence was obtained neither with NPC serum nor with the antibodies against late antigens tested in this study (data not shown). Since both anti-VCA serum (Fig. 1, Raji-BALF2 with TPA-SB VCA$^+$) and monoclonal F3.23 antibody (anti-68-kDa VCA) (data not-shown) also gave about 5 to 8% positive responses, as was the case with earlier monoclonal antibodies against late antigens, the percentage of late-antigen-expressing cells in these induced transfectants does not exceed 5 to 8%. The induction level of late antigen after treatment with chemical inducers corresponds to the classic induction level, which is in general about half of the early-antigen-expressing cell population. The expression of late antigens induced in the BALF2 transfectants was totally inhibited by the addition of ara-T, while those of early antigens and BALF2 protein were not blocked by this compound (data not shown).

In order to confirm the induction of p135 BALF2 protein and late antigens in TPA-SB-treated BALF2 transfectants, the cellular extracts from TPA-SB-treated B95-8 cells, used as positive control, and TPA-SB-treated BALF2 transfectants in the absence or presence of ara-T were analyzed by immunoblotting with monoclonal anti-BALF2 protein, anti-gp350/220 and two anti-VCAs, monoclonal anti-68-kDa VCA and monoclonal anti-p18 VCA (32). The results are presented in Fig. 2. A 135-kDa protein that immunoreacted with monoclonal anti-BALF2 antibody was present in positive-control B95-8 cells as well as in all BALF2 transfectants with or without ara-T. No such polypeptide was identified in untreated parental Raji cells or those treated with TPA-SB (Fig. 2A). Interestingly, the level of the 135-kDa BALF2 protein expressed in clone R30 was much higher than that in clone R25 and the treatment of these clones with TPA-SB resulted in a significant diminution of 135-kDa-protein levels. The antibodies against p18 VCA, p68 VCA, and anti-gp350/220 (Fig. 2B to D, respectively) recognized each polypeptide in the TPA-SB-treated BALF2 transfectants and in TPA-SB-treated B95-8 cells, but the corresponding polypeptides are absent in the ara-T-treated cell culture. We noticed that the intensity of gp220 in BALF2 transfectants was greater than that of gp350, contrary to that of B95-8, in which gp350 was a major polypeptide. The expression of the 135-kDa protein is likely due to the BALF2 recombinant identified in transfected cells. When DNA from BALF2 transfectants was digested by the *Bam*HI restriction enzyme and then analyzed by Southern blotting with the BALF2 sequence as probe, two bands, of 14 and 18 kb, were visualized in the BALF2 transfectant. By contrast, parent Raji cell DNA showed only an 18-kb *Bam*HI-A N het fragment. Since the 14-kb sequence, a sequence with the predicted size of the BALF2 recombinant, hybridized positively with both pZip vector alone and the BALF2 sequence, this sequence corresponds to the BALF2 pZip recombinant. However, we do not know if this recombinant plasmid is integrated in cellular DNA or stays in the episomal form (36). The presence of the BALF2 recombinant plasmid in the transfectants was also confirmed by PCR (data not shown).

As observed in the HSV growth cycle with ICP8 protein susceptible to stimulate the late gene expression (11), one might ask whether a 135-kDa protein can stimulate the expression of late antigens in noninduced BALF2 transfectants. When the immunoprecipitation experiments were carried out with cell extracts of noninduced BALF2 transfectants, two minor polypeptides (47- and 90-kDa) were immunoprecipitated with NPC serum. A 47-kDa polypeptide is also present in noninduced parent Raji cells, but its intensity is less than that in the BALF2 transfectant. Further work will be necessary to determine the natures of these polypeptides.

**EBV DNA synthesis and viral genome copy number in Raji transfectants.** The viral copy number per cell for Raji transfectants was examined after 3 days of chemical induction (Table 1). The copy number was determined by the dot-blot method, with diluted Raji TK$^-$ DNA, in which about 40 copies per cell were previously found, as the standard control (22, 37, 38). About 2.5- to 3-fold this copy number (i.e., 95 to 125 copies per cell) was found in Raji transfectants expressing about 5 to 7% gp350 antigen. We then analyzed EBV DNA synthesis in Raji transfectants with a CsCl gradient in the presence of [$^3$H]thymidine after the induction of lytic viral cycle. The distribution of tritium-labelled DNA in the CsCl gradient is presented in Fig. 3. A large peak (refraction index, 1.4002 or ~1.7010), covering the density from 1.4033 to 1.3986, was observed with the induced R25 as well as R30 BALF2 transfectants, including a viral DNA density of 1.4010 (~1.718). The BALF2 transfectant DNA gave a radioactive peak about two- to fivefold greater than that of the BARF1 or pZip transfectant. When R30 BALF2 transfectant was treated with ara-T, a great part of the high-density DNA disappeared and the residual DNA had a density of 1.3980, which represents a typical host DNA density and is similar to that of Raji DNA transfected with pZip alone or BARF1. These observations indicate that the DNA synthesis that was sensitive to ara-T

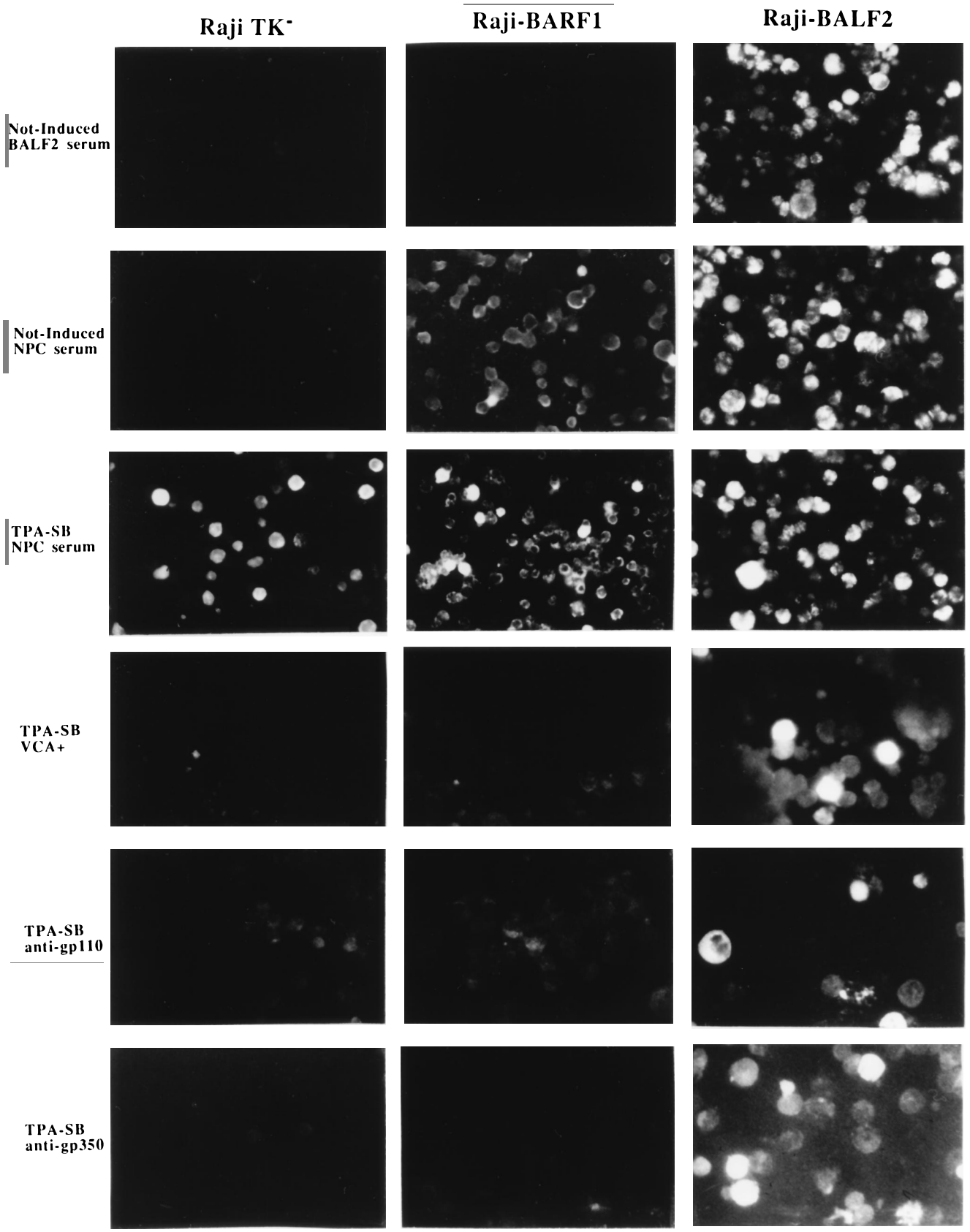


FIG. 1. Immunofluorescence analysis of EBV early and late antigens. Parental Raji cells or BALF2 or BARF1 transfectants were treated or not with TPA-SB for 72 h. Samples containing $1 \times 10^6$ Raji $TK^-$ cells or transfectant clones were air dried onto glass slides, acetone fixed, and incubated respectively with a 1:100, 1:100, 1:100, 1:100, or 1:2 dilution of anti-BALF2, NPC sera (anti-early antigen and VCA), anti-VCA serum, monoclonal anti-gp110 (from E. Kieff, Harvard Medical School), or monoclonal anti-gp350/220 antibody (from E. Kieff), respectively followed by fluorescein isothiocyanate-conjugated anti-human immunoglobulin or anti-mouse immunoglobulin (15).

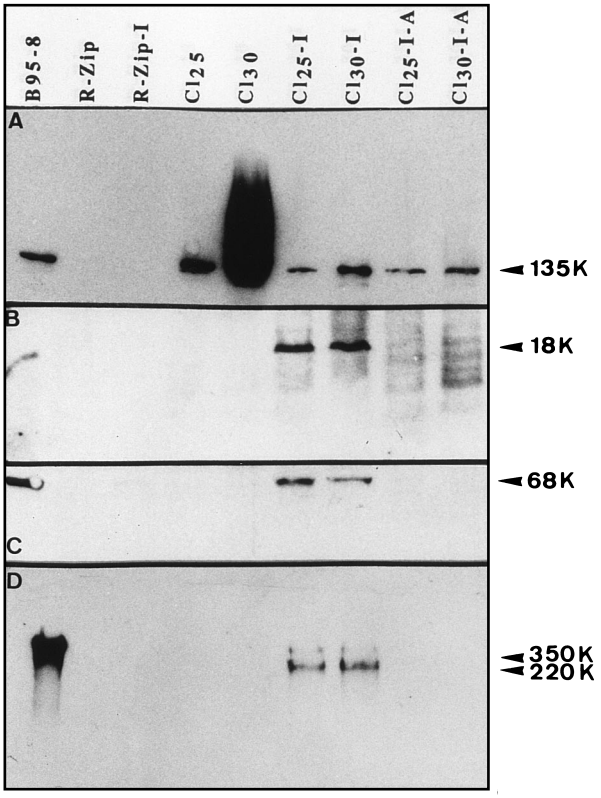


FIG. 2. Immunoblots of induced or noninduced Raji-TK$^-$, BALF2 transfectants, and B95-8 cells. Cell extracts (50 μg each) at the concentrations indicated below were separated on polyacrylamide gels (10 by 6 by 0.01 cm) and blotted onto reinforced nitrocellulose. Nonspecific protein-binding sites on blotted filters were blocked by incubation overnight in phosphate-buffered saline with 0.1% Tween 20 (PBS-Tween) containing dried 5% skim milk. The filters were subsequently incubated overnight at 4°C with antibody diluted in PBS–1% skim milk. The antibodies used were monoclonal OT13B (kindly provided by J. Middeldorp, Organon Teknica, Boxtel, The Netherlands) for anti-BALF2 (dilution, 1:1,000) (A), polyclonal OT15I (32) (J. Middeldorp) for anti-p18 VCA (dilution, 1:500) (B), monoclonal F3.23 (Biosoft, Paris, France) for anti-p68 VCA (dilution, 1:100) (C) and monoclonal OT6 (J. Middeldorp) for anti-gp350/220 (dilution, 1:500) (D). After being washed, the filters were incubated with peroxidase-labelled anti-mouse antibodies for 2 h at room temperature and were washed again in PBS-Tween. The antigen-antibody complexes were then visualized with an enhanced chemiluminescence system (ECL; Amersham) as instructed by the manufacturer. Lane 1, induced B95-8; lane 2, noninduced Raji cells transfected by pZip vector alone; lane 3, induced pZip-Raji transfectant; lane 4, BALF2 transfectant R25; lane 5, BALF2 transfectant R30; lane 6, induced BALF2 transfectant R25; lane 7, induced BALF2 transfectant R30; lane 8, induced BALF2 transfectant R25 with ara-T; lane 9, induced BALF2 transfectant R25 with ara-T. Anti-gp350/220 recognized both of the two major envelope glycoprotein molecules, gp350 and gp220. The polyacrylamide concentrations of the gels were 7.5% for gp350/220, BALF2 protein, and p68 VCA and 15% for p18 VCA.

treatment occurred in BALF2 transfectants and is likely to be of viral origin.

**The presence of virus particles.** Two methods were used to ascertain whether the transfectants in this study produced EBV particles. In the first method, the presence of EBV in live parental Raji cells was examined with a monoclonal gp350/220 antibody after 1 h of contact at 4°C with the virus isolated from 1 liter of TPA-SB-treated Raji transfectants (29). The only cells for which membrane immunofluorescence was observed were virus-treated Raji cells with the monoclonal antibody to gp350/220 (data not shown). No such immunofluorescence was obtained with Raji cells treated with normal medium or with 200-fold-concentrated culture media from noninduced BALF2 transfectants and induced parent Raji cells. In the second method, chemically induced transfectant was examined by electron microscopy (17). As shown in Fig. 4, the EBV-like particles (those with empty centers or dense cores, indicated with arrowheads in the figure) were visible only in the nuclei of the BALF2 transfectants (clone R19). Never were such viral particles observed in parental Raji cells, nor were such particles observed in BARF1-transfected cells after chemical induction. From these observations we hypothesize that the lack of 135-kDa protein encoded by the BALF2 open reading frame in

TABLE 1. EBV genome copy numbers[a]

| Raji cell type | gp350-expressing cells (%) | Copy no./cell |
|---|---|---|
| Parental | 0 | 40 |
| pZip 2 | 0 | 39 |
| pZip 4 | 0 | 37 |
| DBP 1 | 5 | 105 |
| DBP 19 | 5 | 98 |
| DBP 25 | 7 | 125 |

[a] The cell lines were induced with TPA-SB for 3 days, and the EBV genome copy number per cell was determined for the extracted DNAs as previously reported (5, 38). DNA (5 μg) from each specimen was linearized by digestion with *Bam*HI in order to facilitate fixation onto nitrocellulose filters. A serial dilution of DNA from the Raji TK$^-$ cell line, whose viral genome copy number was found by Ooka et al. (24) to be around 40 per cell, was treated in the same way to serve as a standard for viral DNA quantitation. The relative EBV genome copy number was determined by dot-blot analysis as described by Zhang et al. (38) and Bouzid et al. (5), with Raji TK$^-$ DNA as the standard control (22).

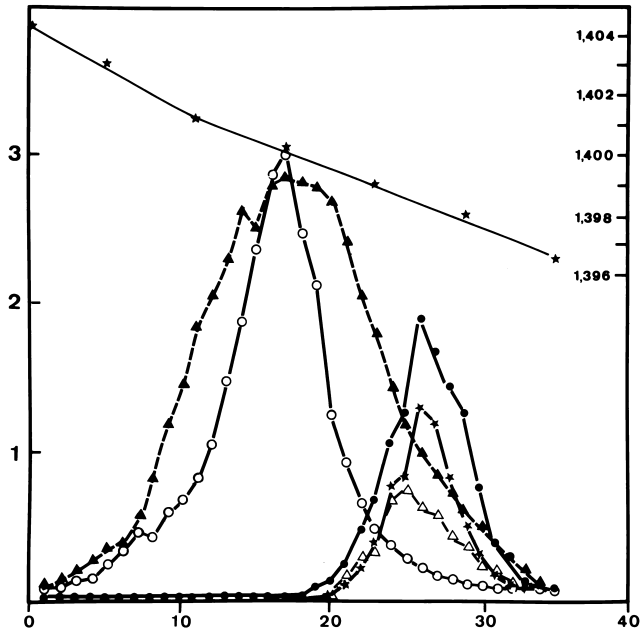


FIG. 3. Cells were cultured in the presence of [$^3$H]thymidine (2 μCi per ml of culture medium) at the time of chemical induction. The labelled DNAs from BALF2, BARF1, and pZip vector transfectants, extracted by the Sarkosyl-proteinase K method (20), were analyzed by neutral CsCl gradient centrifugation. Centrifugation was performed in a Beckman ultracentrifuge at 20°C in a 50 Ti rotor for 72 h at 33,000 rpm. Fractions were collected from each gradient, precipitated by cold trichloroacetic acid, and counted. Abscissa, fraction number; ordinate, left, [$^3$H]thymidine incorporation ($10^3$ cpm); right, refraction index (refraction idex for EBV DNA 1.4010, corresponding to ~1.718). Symbols: ○, DNA from induced clone R25 BALF2 transfectant; ▲, DNA from induced clone 30 BALF2 transfectant; △, DNA from induced clone R30 BALF2 transfectant treated with ara-T; ★, DNA from induced BARF1 transfectant; ●, DNA from induced pZip vector transfectant.

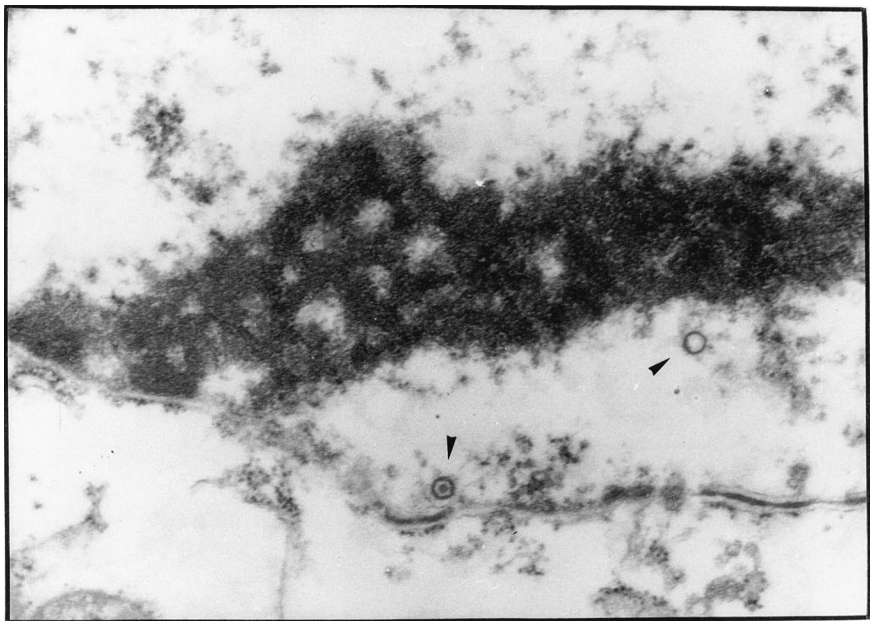

FIG. 4. Electron micrograph of BALF2-transfected Raji cells after 72 h of TPA-SB treatment. Capsids have empty centers or dense cores and are indicated by arrowheads. Magnification, ×40,000.

Raji cells is likely to be linked to the incapacity of this cell line to induce the lytic cycle. As previously observed with EBV producer cell lines (20, 21), the addition of ara-T to induced BALF2 transfectants completely inhibited the induction of late antigens. Since viral DNA synthesis is a necessary prerequisite for late-antigen expression, the production of late antigens in Raji cells should be a consequence of the viral DNA replication which was restored by BALF2 protein.

Previous works have shown that ICP8 protein, homologous to the 135-kDa BALF2 protein, has several functions during viral DNA replication: its binding to monocatener DNA promotes the progression of the replication complex by eliminating the secondary structure of DNA (6, 10, 19, 26, 28). Recent studies have demonstrated that the physical interaction between ICP8 and the C-terminal region of the herpes simplex virus type 1 origin-binding protein could play an important role in origin recognition and unwinding during herpes simplex virus type 1 DNA replication (4). By analogy with ICP8 protein, the 135-kDa BALF2 protein, in association with a viral or cellular origin-binding protein equivalent to the HSV origin-binding protein (not yet identified in the EBV system) and DNA polymerase in lytic EBV DNA synthesis (8), could intervene in the organization of the replication site. The Raji virus strain possesses the genes encoding the DNA replication proteins, except for BALF2 protein: DNA polymerase, DNA polymerase processivity factor, and primase-helicase complex (8, 24). Viewed in these contexts, it seems likely that the defectiveness of viral lytic DNA replication in Raji cells could be explained by the disfunction of ori-Lyt due to a lack of BALF2 protein. It is therefore interesting to know whether the 135-kDa protein is eventually complexed with viral or cellular origin-binding protein to recognize the lytic replication origin.

From the results presented here, it seems unlikely that the other defective genes in the Raji cell line could intervene directly in the switch-on of late stage, and the products of these genes would be necessary for other functions associated or not with viral DNA replication. With regard to this point, it should be mentioned that the EBV copy number found in the BALF2 transfectant was somehow lower than that previously observed for EBV producer P3HR1, in which about the same percentage of VCA was expressed (24). However, there are no explanations for these phenomenons at this time. Further study will be necessary to know if this is because of the difference of cell lines or the lack of some other defective gene whose expression would be necessary for viral DNA replication.

The Raji transfectants will be a good tool for studying not only the mechanism of lytic induction by p135 BALF2 protein but also the roles of other defective genes in the viral life cycle.

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