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The Average Number of Molecules of Epstein-Barr Nuclear Antigen 1 per Cell Does Not Correlate with the Average Number of Epstein-Barr Virus (EBV) DNA Molecules per Cell among Different Clones of EBV-Immortalized Cells

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Epstein-Barr nuclear antigen 1 (EBNA-1) is the only viral protein required to support latent replication of Epstein-Barr virus (EBV). To assess the likelihood that EBNA-1 regulates the amount of EBV DNA in a cell, we measured the average numbers of EBNA-1 molecules and EBV DNA molecules per cell in different clones of cells. The amount of EBNA-1 protein present in recently established lymphoblastoid cell lines was measured with affinity-purified anti-EBNA-1 antibodies, and viral DNA was measured by nucleic acid hybridization. The average levels of EBNA-1 protein varied little between these cell lines, whereas the average amount of viral DNA present varied substantially; consequently, these numbers were not correlated. These is no apparent relationship between amounts of EBNA-1 and viral DNA.

Infection of human peripheral B lymphocytes in vitro with Epstein-Barr virus (EBV) gives rise to immortalized B-lymphoblastoid cells (14). Following an initial apparent amplification, the viral DNA attains a copy number which varies between different clones of cells but can be quite stable over time for a given clone (but see reference 10 for an exception). The great majority of cells within a clone are said to be latently infected because they express only a limited set of viral genes that presumably are necessary for both the maintenance of the viral DNA and the expression of its immortalizing functions.

Of the 8 to 10 latently expressed genes identified to date, only one, that for Epstein-Barr nuclear antigen 1 (EBNA-1), is needed for the replication of DNAs containing the EBV latent origin of replication, *oriP* (12, 27). *oriP* also contains a transcriptional enhancer element to which EBNA-1 binds (18) and which is located in the viral genome 4 kilobase pairs from a promoter that may be used for six of the known latent proteins (4). This enhancer has been shown to increase transcription from this promoter (24) in the presence of EBNA-1. One of the genes transcribed from this promoter encodes EBNA-1, raising the possibility that EBNA-1 regulates its own synthesis.

It seems likely that a mechanism exists that links the regulation of viral DNA synthesis to that of cellular DNA synthesis because the amount of viral DNA is maintained at a constant level in proliferating cells. If EBNA-1 mediates this link, it is reasonable to propose that the amount of EBNA-1 correlates with the amount of viral DNA in a cell. On the other hand, viral DNA replication may be regulated by the cellular mechanism that controls host chromosomal replication. In this hypothesis, EBNA-1 would simply be required for the recognition of the viral origin of replication by cellular machinery. As one step towards addressing this hypothesis, we measured the average copy numbers of viral plasmids and of EBNA-1 molecules per cell in a series of recently immortalized clones of cells. The average copy number of viral DNA varied over a wide range in these cell lines, whereas the amount of EBNA-1 present varied little.

Generation of a specific antiserum against EBNA-1. The average number of EBNA-1 molecules per cell was measured in a quantitative immunoblot assay with antibodies generated to a bacterially synthesized fusion protein that contains a portion of EBNA-1. DNA encoding part of EBNA-1 was inserted into the bacterial expression plasmid pJS413 (25). This construction is a fusion with an N-terminal portion from the lambda *cro* gene and a C-terminal portion from the *lacZ* gene and includes the amino acids from residues 7 to 37 and 420 to 617 of the BKRF1 open reading frame of the B95-8 strain of EBV (1); these residues do not include internal repeat 3 (the Gly-Gly-Ala repeat region of EBNA-1). Cell extracts from JS1060 cells carrying this plasmid were prepared as described previously (3). The fusion protein was purified by binding to an aminobenzyl 1-thio-β-D-galactopyranoside–agarose (Sigma Chemical Co.) column containing buffer A (20 mM Tris hydrochloride [pH 7.5], 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 M NaCl) plus a protease mix (15). The column was washed with 15 volumes of buffer B (buffer A plus 1 M NaCl), and the fusion protein was eluted with buffer B plus 1 mg of isopropyl-β-D-thiogalactopyranoside per ml. A sodium dodecyl sulfate-polyacrylamide gel of the eluted proteins is shown in Fig. 1. Three major bands were observed: two of the bands corresponded to proteins of the size expected for β-galactosidase and for the fusion protein, and the third was of intermediate size.

The purified fusion protein was used to immunize rabbits, and the resulting antisera were purified by negative and positive selection on immunoaffinity columns (3). The selected antibodies reacted only with the largest of the three forms of the fusion protein used to immunize the rabbits (Fig. 1). This result indicates that passage of the antisera over a β-galactosidase affinity column removed antibodies made to epitopes found on the lower-M_r forms of the fusion

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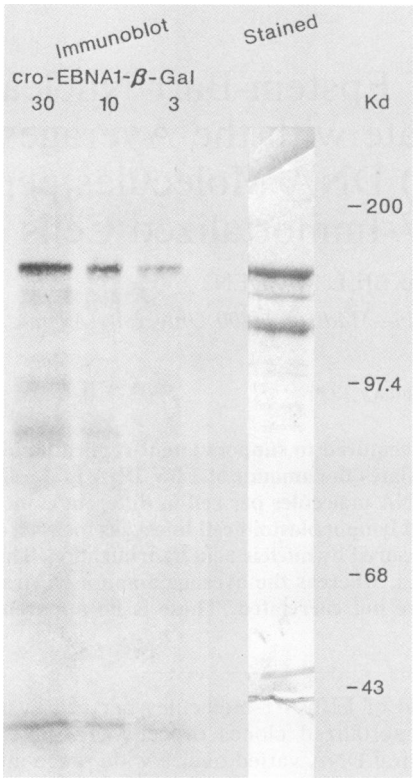

FIG. 1. Purification of the *cro*–EBNA-1–β-galactosidase protein. Protein was purified from bacterial extracts as described in the text and fractionated on a 7.5% sodium dodecyl sulfate-acrylamide gel. Gels were either stained with Coomassie blue (Stained) or electrophoretically transferred to nitrocellulose and detected with antibodies raised to and affinity purified with the fusion protein (Immunoblot). The numbers at the top of the leftmost three lanes refer to nanograms of protein loaded. Kd, Kilodaltons.

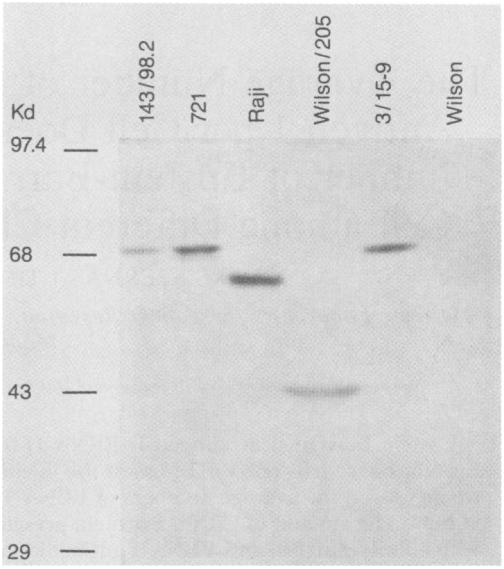

FIG. 2. Specificity of anti-EBNA-1 antibodies. Lysates of 2×10^6 (Wilson/205) or 3×10^5 (all others) cells were fractionated on 7.5% sodium dodecyl sulfate-acrylamide gels, electrophoretically transferred to nitrocellulose, and probed with anti-EBNA-1 antibodies purified as described in the text. The molecular masses of size standards are indicated at the left in kilodaltons (Kd).

proteins. The specificity of the immunoaffinity-purified serum was established when it was used in immunoblots to probe extracts of EBNA-1-positive cell lines (Fig. 2). The serum reacted with a band of the expected molecular mass in cells expressing the form of EBNA-1 derived from the B95-8 strain of EBV (cell lines 143/98.2 [27] and 721 [9]) and with the appropriate lower-M_r bands of forms with fewer or no copies of the Gly-Gly-Ala repeat (cell lines Raji [17] and Wilson/205 [27]). No signal was detected in extracts of the EBNA-1-negative cell line Wilson (data not shown). This antiserum was, therefore, specific for the EBNA-1 protein, and its reactivity was not dependent on the presence of the Gly-Gly-Ala repeat region, which was lacking in the EBNA-1 protein expressed in Wilson/205 cells. This latter point is important because different isolates of EBV encode EBNA-1 proteins with different numbers of Gly-Gly-Ala repeats. Antibodies that bind this repeat unit would bind differentially the EBNA-1 proteins encoded by these isolates.

Measurement of EBNA-1 and EBV DNA copy numbers. The average amount of EBNA-1 protein present per cell was measured in several cell lines propagated for long times. Ramos (11) and Wilson (provided by Ian MacGrath) are EBV negative, and Raji is an EBV-positive Burkitt lymphoma; Wilson/205 and 143/98.2 are cell lines transfected with vectors that express EBNA-1. In addition, the level of EBNA-1 was measured in recently immortalized cell clones. These clones were generated when adult B lymphocytes were infected with the B95-8 strain of EBV, cloned in soft agarose, and subsequently maintained in culture for less than 50 population doublings (22, 23). Extracts of cell lines were prepared, and their proteins were separated electrophoretically and assayed on immunoblots as described previously (3) (Fig. 3). The amount of EBNA-1 in each cell extract was measured by a comparison of the signal derived from it with signals derived from known amounts of the *cro*–EBNA-1–β-galactosidase fusion protein diluted into an EBV-negative cell extract. The comparisons of the signals were made with a laser densitometer. The affinity-purified anti-EBNA-1

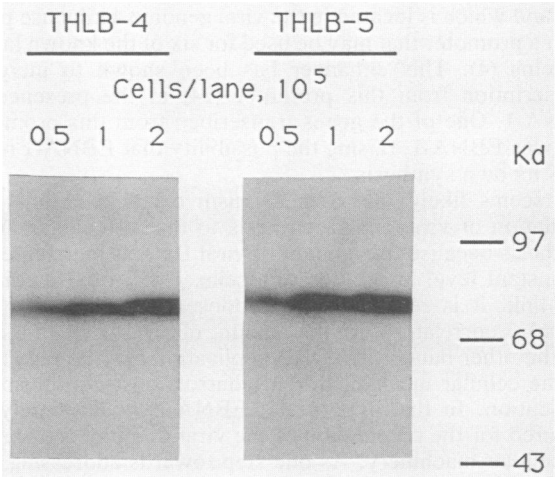

FIG. 3. Measurement of the number of EBNA-1 molecules per cell in lymphoblastoid cell lines. Immunoblots of cell lysates were prepared as described in the legend to Fig. 1. Densitometric scans of these blots were compared with a standard curve prepared from the EBNA-1 fusion protein to generate the values in the text and Table 1. Kd, Kilodaltons.

TABLE 1. Comparison of average amounts of EBNA-1 protein and EBV DNA copies per cell[a]

Cell line	No. of:	
	EBNA-1 molecules/cell	EBV DNA molecules/cell
11/17-3	31,000	10
11/17-5	43,000	15
721	35,000	20
THLB-1	25,000	30
Raji	37,000	45
GG68	31,000	50
3/15-9	44,000	60
THLB-4	25,000	100
THLB-5	29,000	100
3/15-31	41,000	400
Ramos	0	0
Wilson	0	0

[a] Amounts of EBNA-1 protein and EBV DNA were determined as described in the legend to Fig. 1 and the text, respectively. Values are the averages of two to five determinations.

antibody did not react detectably with 100 ng of β-galactosidase itself (data not shown). The results of probing these blots with the immunoaffinity-purified antibodies are expressed as the average numbers of molecules per cell (Table 1). The numbers of EBNA-1 molecules per cell for cell lines 721, Raji, Wilson/205, and 3/15-9 were (mean ± standard deviation) 35,000 ± 27,000 (seven determinations), 37,000 ± 21,000 (nine determinations), 8,000 ± 3,000 (six determinations), and 44,000 ± 16,000 (four determinations), respectively. The variation in number between the different EBV-positive lymphoblastoid cell lines tested was less than a factor of two, ranging from 25,000 to 44,000 molecules per cell. These numbers are of the same order of magnitude as that found for the average number of molecules of the transcription factor SP1 per cell (8).

In contrast to this finding, the number of EBV genomes was found to vary over a much wider range in these same cell lines. Total cellular DNA was spotted onto a nitrocellulose filter and probed with a *Bam*HI-*Sfi*I fragment spanning base pairs 3994 to 11405 of the DNA of the B95-8 strain of EBV, and samples were quantified by densitometric scanning of autoradiograms and comparison with samples containing known amounts of EBV DNA. The average number of EBV DNA molecules per cell varied 40-fold, from 10 to 400 copies per cell (Table 1). Furthermore, the average amount of EBNA-1 did not correlate with the average number of genome copies present per cell. This same observation has been made with viral RNA: the amount of RNA homologous to the EBNA-1 gene per cell is rather constant in a set of cell lines similar to those studied here (13).

Because the average copy number of the EBV genome covers at least a 40-fold range, the number of genes encoding the EBNA-1 protein is equally variable per cell in these clones. By analogy with genes residing in regions of amplified chromosomal DNA (20), it might be expected that an increase in the copy number of a gene would yield a corresponding increase in the amount of the protein encoded. It is equally possible, given that EBNA-1 is the sole viral protein required to support replication from *oriP*, that the copy number of EBV genomes would be determined by the amount of EBNA-1 synthesized. Both of these interpretations presume a correlation between levels of EBV DNA and EBNA-1 protein; however, there is no such relationship in the clones of cells examined in this study.

One explanation for the 40-fold range in average DNA copy number per cell is a variable number of lytic cells in a population composed primarily of latently infected cells. The recently immortalized cell lines used in this study were examined previously for the proportion of lytic cells (22, 23). All of them had less than 1 cell per 2×10^4 cells that released infectious virus, approximately 1,000-fold fewer than the B95-8 line used to derive the virus with which they were infected. Four of seven of these lines did release detectable virus, indicating that they were capable of undergoing a productive lytic cycle. In these four lines, there was no correlation between virus released and the average number of EBV DNA molecules per cell (Table 1) (22, 23). However, it is possible that the clonal populations studied may contain different numbers of cells that support the lytic phase of the life cycle but that do not release infectious virus (26). This possible heterogeneity could mask a meaningful correlation between EBNA-1 and EBV DNA copy numbers in the latently infected subpopulations.

Previous attempts to measure the EBNA-1 content of EBV-immortalized cells have used human antisera from EBV-positive donors (5, 6, 21) in assays that did not allow the detection of EBNA-1 specifically. These antisera recognize a number of latently and lytically expressed viral proteins in addition to EBNA-1. Because patterns of viral protein expression vary between different cell isolates and because titers to different antigens vary between different antisera, these studies do not provide a specific measure of the levels of EBNA-1. In addition, major epitopes of EBNA-1 recognized by these antisera are within the Gly-Gly-Ala repeat region (19), a region that varies in size. The antibodies used here were specific for EBNA-1 and should have been unaffected by various lengths of the Gly-Gly-Ala region.

These experiments do not rule out a role for EBNA-1 in controlling DNA copy number, because only the total amount of EBNA-1 in a cell population was measured. It is possible that EBNA-1 either requires a posttranslational modification to function or can form part of a complex in which it is inactivated, as has been shown for other transcriptional activating factors (2, 16). EBNA-1 has been shown to be phosphorylated (7), but it is not known what effect this modification has on the function of the protein. If EBNA-1 regulates the copy number of EBV replicons, then this function of EBNA-1 might be detected by expressing it from an inducible promoter and monitoring the extent to which its induction affects the copy number of these replicons.

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