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Levels of Epstein-Barr Virus DNA in Lymphoblastoid Cell Lines Are Correlated with Frequencies of Spontaneous Lytic Growth but Not with Levels of Expression of EBNA-1, EBNA-2, or Latent Membrane Protein

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The process of Epstein-Barr virus (EBV)-induced transformation of human B lymphocytes results in a cell line that is a mixture of latently and lytically infected cells, with the lytic cells composing roughly 5% to less than 0.0001% of the overall population. A set of nine normal lymphoblastoid cell lines that span a 100- to 200-fold range in average EBV DNA content were studied, and the frequency with which these cells entered a lytic phase of viral growth correlated with their EBV DNA copy number (as a population average). However, neither factor correlated with the levels of expression of transcript for the viral genes EBNA-1, EBNA-2, and latent membrane protein, nor did they correlate with the levels of EBNA-2 protein and latent membrane protein. The rate at which a cell line enters into lytic growth spontaneously is therefore not dependent on the overall steady-state levels of expression of these latent-phase genes.

Epstein-Barr virus (EBV) is a lymphotropic herpesvirus that infects human B cells, enabling them to proliferate indefinitely as transformed lymphoblasts. Latent infection of lymphoblasts requires only about 10% of the viral genome, and the 172-kilobase-pair (kbp) viral genome (3) is maintained as a supercoiled plasmid (as reviewed in reference 28). Approximately 12 genes are expressed during latent infection, and these include the two EBERs (small untranslated RNAs); a set of six Epstein-Barr nuclear antigens (EBNA-1, EBNA-2, EBNA-3 [3A], EBNA-4 [3B], EBNA-5 [leader protein], and EBNA-6 [3C]); and four cytoplasmic or membrane proteins (latent membrane protein [LMP] [BNLF-1], terminal gene [LMP-2A], LMP-2B, and possibly BHRF-1) (2, 39, 50; reviewed in reference 28). Many of these genes are likely to be required for the immortalization of the B lymphoblast, the maintenance and regulation of expression from the viral genome, or the control of latency.

In cells that have entered a lytic phase of viral growth, approximately 50 to 100 viral genes may be expressed, including the early antigens (EA) and viral capsid antigens (VCA) (7, 24, 62). With a low frequency, a cell that is latently infected with EBV is spontaneously induced to enter the lytic phase of viral growth, which is reflected by its expression of EA and VCA and its amplification of the viral genome. The percentage of lytically infected cells is generally low and can range from less than 0.0001% to roughly 5% of the transformed cell population (36, 52, 64). Given the 100- to 1,000-fold amplification of the viral genome during lytic replication (17), this small subpopulation can contribute significantly to the overall EBV DNA content of a transformed cell line. Lytic growth can be induced by expression of the viral gene product BZLF-1, also known as ZEBRA (12, 16) and EB1 (10), which has the ability to activate and cooperate with other regulatory proteins (10, 19, 29, 38, 55). It is not known what triggers this cascade of expression of viral early antigen or what is the basis for the variability in its spontaneous induction.

To characterize the expression of latent-phase genes in EBV-transformed cell lines, I measured the steady-state levels of expression of three viral genes, EBNA-1, EBNA-2, and LMP. The EBNA-1 gene is encoded by viral DNA sequences lying at approximately 109 kbp on the viral genome, but its transcriptional promoters (Pc [8] and possibly also Pw [49]) lie between 64 and 98 kbp upstream. The expression of EBNA-1 is not increased in cells that have been induced to enter the lytic cycle with the drug 12-*O*-tetradecanoyl-13-phorbol acetate (63). EBNA-1 protein binds to and is necessary for the function of the putative origin of DNA replication oriP (31, 37, 41, 65). A transcriptional enhancer lying within oriP is active in the presence of EBNA-1 protein (43), and the Pc promoter is sensitive to this enhancing activity (53). The EBNA-2 gene is located at 48 kbp on the viral genome and may be transcribed from the Pw (49) and possibly Pc (8) promoters. EBNA-2 protein is necessary for the transformation of B lymphocytes (18), and it may play an important role as an activator of the expression of the cellular gene CD23 (61). The cytoplasmic levels of both the EBNA-1 and EBNA-2 transcripts are only two to three copies per cell in poly(A)$^+$ RNAs derived from IB-4 cells (22, 57) (although in this study I demonstrate that there are up to several hundred copies per cell of these transcripts in unfractionated cellular RNAs). The LMP gene is located at approximately 169 kbp on the viral genome and is transcribed from the promoter EDL1 in the direction opposite to that of EBNA-1 and EBNA-2 (15, 23). There exist differing results as to whether 12-*O*-tetradecanoyl-13-phorbol acetate induces higher levels of LMP (9, 23, 47). LMP appears to be involved in the activation of expression of CD23 and cellular adhesion molecules (60), and it both colocalizes with the protein vimentin (30) and spans the plasma membrane (4, 15, 32). LMP has the interesting and unique property among EBV-encoded genes of being able to transform rodent fibroblast cell lines to a state of anchorage independence and

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TABLE 1. Expression of EBV-specific genes in lymphoblastoid cell lines that differ in EBV DNA copy number

Cell line	EBV DNA copies/cell[a]	Percent VCA or EA positive[b]		No. of transcript copies/cell[c]			Relative expression of protein[d]	
		DR	αN7	EBNA-1	EBNA-2	LMP	EBNA-2	LMP
LCL721	5	<0.1	<0.02	100 ± 13 (n = 3)	190 ± 20 (n = 2)	120 ± 20 (n = 2)	0.7, 1.0	0.24, 0.20
11/17-3	10	0.4	<0.02	60 ± 9 (n = 3)	270	50 ± 15 (n = 6)	0.45, 0.39	0.16, 0.16
11/17-5	20	0.2	<0.02	55 ± 5 (n = 3)	90	30 ± 10 (n = 6)	0.47, 0.45	0.48, 0.26
THLB-1	50	ND[e]	ND	120 ± 50 (n = 3)	250 (n = 2)	120 ± 20 (n = 2)	1.0, 1.0	1.0, 1.0
3/15-9	110	1	0.25	60	160 ± 20 (n = 2)	80	0.45, 0.27	0.22, 0.33
11/17-10	120	ND	ND	50	130 ± 50 (n = 2)	70	0.67, 0.40	0.71, 0.75
11/17-4	150	1	0.3	170 ± 100 (n = 3)	250 ± 30 (n = 2)	110 ± 60 (n = 6)	0.62, 0.49	1.2, 1.0
THLB-5	200	ND	ND	150 ± 60 (n = 3)	100 ± 30 (n = 2)	70 ± 10 (n = 2)	0.58, 0.45	0.82, 0.92
3/15-31	500–1,000	2	1.8	140	100 ± 30 (n = 2)	100 ± 40 (n = 2)	0.64, 0.47	0.51, 0.37
Ramos	≤1	≤0.1	<0.02	<1	<1	<1	Undetectable	Undetectable

[a] By dot-blot hybridization with a double-stranded [^{32}P]DNA probe spanning nt 3994 to 9516 from the EBV genome.
[b] Human antiserum DR detects VCA-positive and EA-positive cells. Mouse monoclonal αN7 detects cells expressing the 150-kDa viral capsid antigen. <, Limit of detection. By the Kendall K test, the correlation between EBV DNA copy numbers and the percentages of antigen-positive cells (for each antisera) was significant (at a level of $\alpha = 0.02$).
[c] Reported as number of copies per cell ± range, over n trials. By the Kendall K test, the correlations between the EBV DNA copy number and the levels of transcript for EBNA-1, EBNA-2, or LMP were not significant (levels of $\alpha = 0.2$, $\alpha = 0.13$, and $\alpha = 0.54$, respectively).
[d] Data presented are normalized to THLB-1 value and are presented in arbitrary units. Values to left and right of commas represent separate experiments.
[e] ND, Not done.

tumorigenicity (5, 5a, 58, 59). This feature may be predictive of a role in the transformation of human B cells by EBV.

In this study, the expression of the EBNA-1, EBNA-2, and LMP genes was characterized in nine lymphoblastoid cell clones that span a 100- to 200-fold range in their content of EBV genomes. The EBV DNA copy numbers increased along with increasing rates of spontaneous induction of lytic growth, but there was no association between higher EBV DNA copy numbers (or rates of spontaneous induction) and the levels of expression of the transcripts for EBNA-1, EBNA-2, or LMP. This finding was extended to the protein product level for EBNA-2 and LMP. Collectively, these findings suggest that cell lines arrive at their differing rates of spontaneous induction of lytic growth without any accompanying gross (i.e., detectable) modulation in their steady-state levels of expression of these latent-phase genes. In addition, the association between higher rates of spontaneous induction and higher average EBV DNA levels in the cell lines suggests that there exists some restriction on the copy number of the EBV genome during latency.

The lymphoblastoid cell lines used in this study included 11/17-3, 11/17-4, 11/17-5, 11/17-10, 3/15-9, 3/15-31, THLB-1, and THLB-5, which were cell clones transformed with EBV (B95-8 strain) and passaged in culture for less than 6 months after viral transformation (52). In addition, Ramos, an EBV-negative Burkitt's lymphoma cell line (26) was obtained from American Type Culture Collection (Rockville, Md.) and LCL721, an EBV-transformed (B95-8 strain) lymphoblastoid cell clone grown for several years in culture (25), was obtained from Robert DeMars. By dot-blot hybridization analysis of cellular DNAs, the number of copies of the viral genome in nine cell clones (LCL721, 11/17-3, 11/17-5, THLB-1, 3/15-9, 11/17-10, 11/17-4, THLB-5, and 3/15-31) was found to span a range of nearly 200-fold (Table 1). These results are in accordance with an earlier measurement of the EBV DNA copy numbers by renaturation kinetics in many of the same clones (52). In that study, it was found that the rate of release of infectious virus as measured by cocultivation analysis was not related to the overall EBV DNA copy number in a cell clone. A subsequent study by Wilson and Miller (64) highlighted the inefficiency of virus release in human lymphoblastoid cell lines, suggesting that the relationship between viral DNA copy number and rates of spontaneous induction of lytic growth should be reassessed. Preliminary studies (data not shown) with the cell clone 3/15-31 indicated that 50 μg of phosphonoacetic acid per ml could effect a fivefold drop in the number of copies of the viral genome. This would be consistent with frequent lytic induction in 3/15-31 cells (54); however, the interpretation of this experiment must be guarded because of the cytotoxic side effects of this drug.

The percentage of cells expressing lytic-phase antigens was determined for six of the cell clones by indirect immunofluorescence, using a seropositive human serum and a mouse monoclonal antibody. The human serum DR detected VCA and EA in a small percentage of the population of five of the cell lines tested (Table 1). The frequency of these VCA-positive and EA-positive cells was highest (2%) in the 3/15-31 population, carrying an average of 500 to 1,000 EBV DNA copies per cell, and undetectable (<0.1%) in the LCL721 population, carrying an average of only 5 EBV DNA copies per cell. The clones with intermediate frequencies of lytic-phase antigen expression (11/17-3, 11/17-5, 3/15-9, and 11/17-4) also had intermediate EBV DNA copy numbers. These findings were confirmed with a mouse monoclonal antibody, αN7, raised against viral nucleocapsids. The monoclonal αN7 antibody immunoprecipitates a 150-kilodalton (kDa) major capsid protein from B95-8 cells and can be used in immunofluorescence studies to detect lytically induced B95-8 cells (S. Metzenberg and B. Sugden, unpublished data). The results with this antibody were similar to that of the human antiserum (Table 1). The systematically lower percentages of positive cells detected with the monoclonal antibody may reflect the fact that it does not detect cells that are VCA negative and EA positive. These studies of lytic-phase antigen expression provided, in a single-cell assay, an indication that the frequency of spontaneous lytic induction was higher than the previously reported frequencies of infectious virus release in many of these clones.

These results were extended by a measurement of the accumulation of the transcript for the early antigen BHLF-1 in the nine EBV-transformed cell clones (Fig. 1B). BHLF-1 transcripts accumulate to high levels during the early stages of lytic induction (24), and BHLF-1 is the most frequently transcribed gene in the producer cell line B95-8 (35). If the

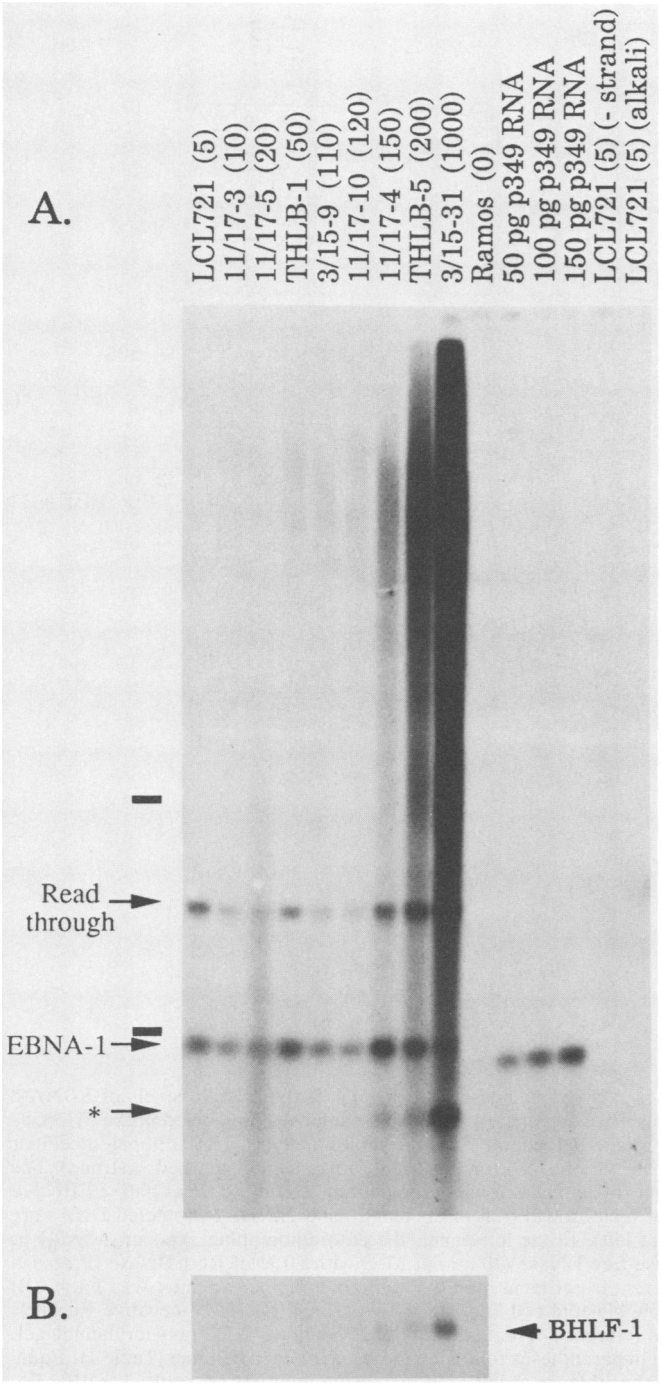


FIG. 1. Measurement of the number of copies of the EBNA-1 transcript by S1 nuclease analysis and the relative levels of BHLF-1 transcript by Northern blot hybridization. (A) EBNA-1 transcripts were detected by S1 nuclease analysis (45) (unlabeled probe M13p514 or M13p513), alkaline agarose gel electrophoresis of the protected DNAs (33), and detection by Southern blot analysis (11, 42, 51). Unfractionated cellular RNA (25 μg) was hybridized to 50 ng of M13p514 virion DNA (with one exception, discussed below). Nine EBV-transformed cell lines are analyzed in the nine lanes at left (lanes labeled with names of cell lines and EBV DNA copy numbers in parentheses, as in Table 1). The positions of readthrough (full insert length), normally cleaved EBNA-1, and putative BKRF-2 (asterisk) RNA-protected DNAs are indicated with arrows (left), as are 700- and 1,670-base markers (bars at left). The cell line Ramos (lane 10) was derived from an EBV-negative Burkitt's lymphoma. For the lanes marked p349, the indicated number of picograms of p349 RNA was mixed with 25 μg of Ramos RNA before S1 analysis (p349 RNA is transcribed in vitro [34], has the same coding sense as native EBNA-1 RNA, and spans nt 107970 to 109892 of EBV, except for an internal deletion between approximately nt 108200 and 108900). The sample marked − strand was hybridized to M13p513 in lieu of M13p514, and the sample marked alkali was pretreated with NaOH (37°C for 15 min in 0.4 M NaOH–0.02 M EDTA, followed by dilution, neutralization, and ethanol precipitation with 50 μg of *E. coli* tRNA as a carrier). The autoradiographic exposure was 66 h. (B) Samples of RNA (10 μg each) were analyzed for BHLF-1 transcript by gel electrophoresis and Northern blot analysis (1, 11) with plasmid probe p527 (17). The 10 samples shown are appropriate to the labels at the top of part panel A, and the position of the 2.5-kb BHLF-1 transcript is indicated with an arrow (at right). The autoradiographic exposure was 7 days.

BHLF-1 transcript level is taken as an overall measure of lytic growth, it is clear (Table 1 versus Fig. 1B) that higher average EBV DNA copy numbers are associated with increased spontaneous lytic growth in the cell clones.

Consideration of these results raised the question of whether a substantial variation in average EBV DNA copy number and spontaneous lytic induction could be associated with a difference in the expression of the latent-phase genes of EBV. The levels of accumulation of transcripts from three latent-phase genes (EBNA-1, EBNA-2, and LMP) were measured quantitatively in the cell lines. The steady-state levels of EBNA-1 and EBNA-2 transcript were determined by S1 nuclease analysis (Fig. 1A and 2A, respectively), and the steady-state levels of LMP transcript were determined by Northern (RNA) blot analysis (Fig. 3A). In all cases, the measurements were made upon samples of unfractionated RNAs [poly(A)$^+$ and poly(A)$^-$ not separated, and prepared by the method presented in reference 44] and were made quantitative by a side-by-side analysis of specific RNA standards prepared in vitro with SP6 polymerase (34) (see Table 1 for results of densitometric scanning of autoradiograms). To ensure that the same number of cell equivalents of RNA was tested from each cell line, an internal RNA standard was added to the cells at the time of lysis (56) and then measured quantitatively in the purified RNA samples by Northern blot analysis (data not shown).

Figure 1A shows the protected fragments detected by S1 nuclease analysis with a single-stranded probe (M13p514, a recombinant M13 bacteriophage containing nucleotide [nt] 109210 through nt 110491 from the EBV genome [3]) that overlaps the 3′ end of the EBNA-1 open reading frame. The mature transcript for EBNA-1 was detected by protection of a 790-bp fragment (Fig. 1A, EBNA-1 arrow). No transcript was detected in Ramos, a cell line derived from an EBV-negative Burkitt's lymphoma. The EBNA-1-protected fragment detected in the nine EBV-transformed cell lines is consistent with the 3′ terminus of the EBNA-1 transcript following an ATTAAA or AATAAA sequence at nt 109906 or 109937, respectively (21, 63). This transcript varied in average copy number between approximately 50 per cell in the cell line 11/17-10 and 170 per cell in the cell line 11/17-4 (Table 1). In Fig. 2A is shown a similar S1 nuclease analysis with a probe specific for the 3′ end of the EBNA-2 gene (M13p587, an M13 recombinant phage containing nt 49475 through nt 50305 from the EBV genome). Again, no signal was detected in the EBV-negative cell line Ramos, but an EBNA-2-protected fragment of 570 bases was detected in all nine EBV-positive cell lines (Fig. 2A, EBNA-2 arrow). This

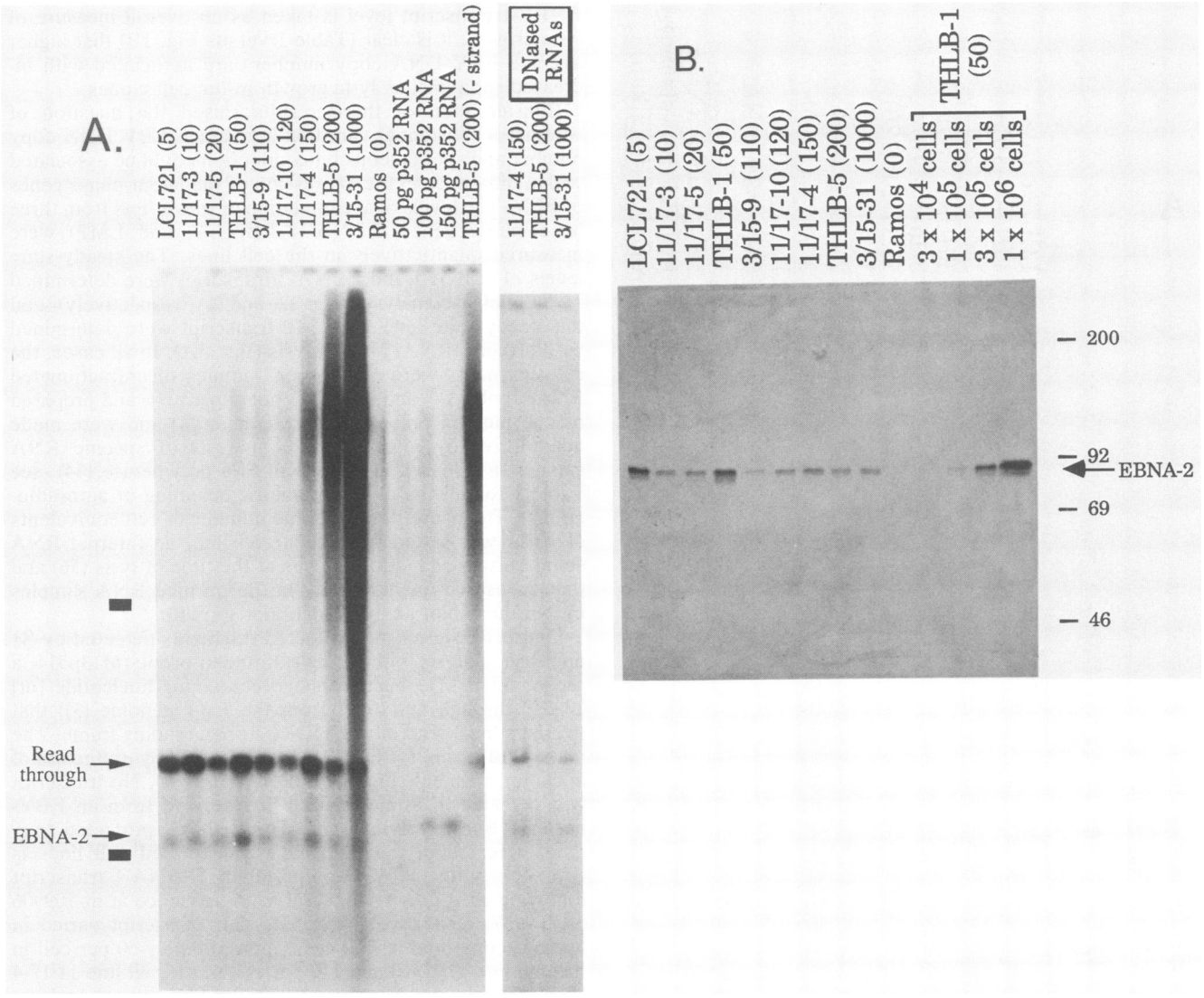
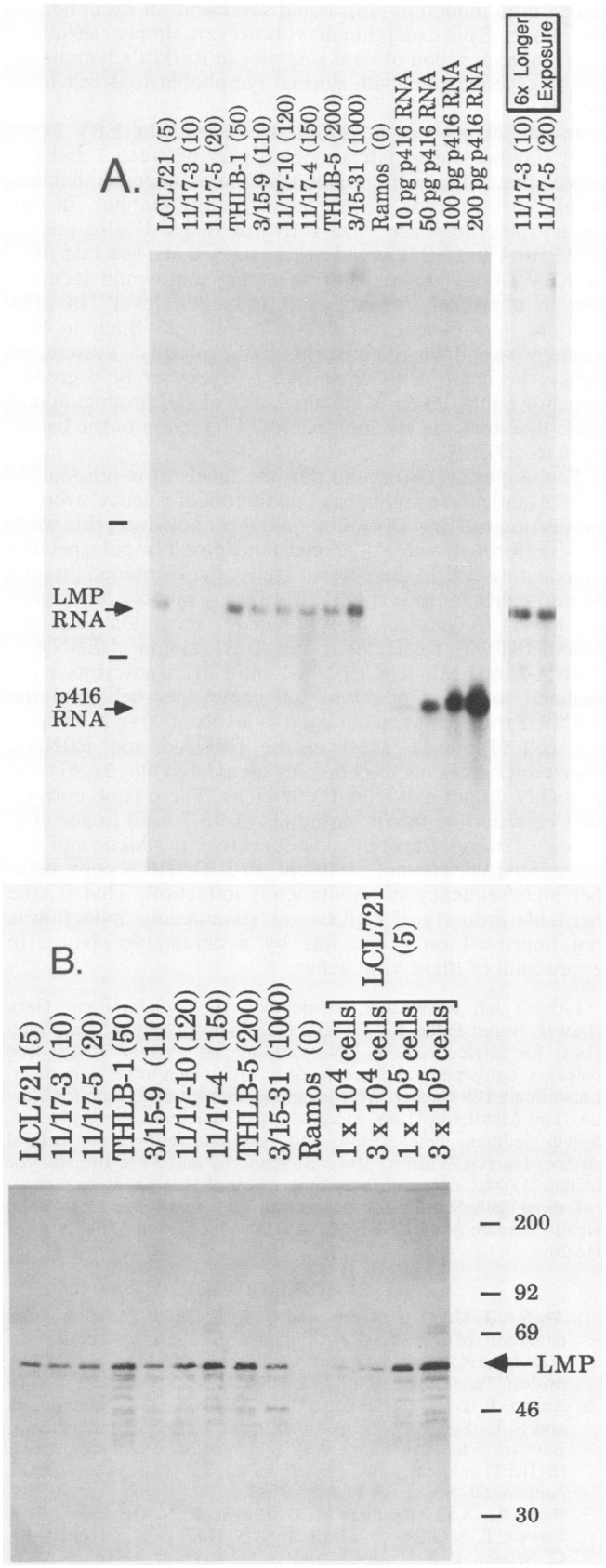


FIG. 2. Measurement of the number of copies of the EBNA-2 transcript by S1 nuclease analysis and of the relative levels of EBNA-2 protein product per cell by Western blot analysis. (A) EBNA-2 transcripts were detected by S1 nuclease analysis (unlabeled probe M13p587 or M13p586), alkaline agarose gel electrophoresis of the protected DNAs, and detection by Southern blot analysis. Unfractionated cellular RNA (25 μg, or 10 μg, right three lanes) was hybridized to 50 ng of M13p587 virion DNA (or M13p586, for the sample marked − strand). The description of the samples is identical to that in the legend to Fig. 1A. In vitro-synthesized p352 has the same coding sense as native EBNA-2 RNA and spans nt 48865 to 49909 of the EBV genome. The positions of readthrough (full insert length) and EBNA-2-protected DNAs are indicated with arrows (left), as are 500- and 1,800-base markers (bars at left). In the left panel, the autoradiographic exposure was 44 h. Samples in the three lanes in the right panel were pretreated with RNase-free DNase (10 min at 37°C with 20 U of RQ1 DNase [Promega Biotec, Madison, Wis.] per ml). The right panel was generated in a different experiment, and the autoradiographic exposure was 160 h. (B) EBNA-2 protein was detected in lysates of nine EBV-transformed lymphoblastoid cell lines (lanes 1 to 9) and one EBV-negative Burkitt's lymphoma cell line, Ramos (lane 10) by Western blot analysis (method in reference 4) of a sodium dodecyl sulfate–7.5% polyacrylamide gel. The approximate number of copies of the EBV genome per cell is shown in parentheses following the name of each cell line (Table 1). Equal quantities (3×10^5 cell equivalents) of cell lysates were loaded on each the 10 lanes at left, and increasing numbers (3×10^4, 1×10^5, 3×10^5, 1×10^6) of cell equivalents of THLB-1 lysate were loaded as marked at right. After electrophoresis, the polyacrylamide gel was transferred to nitrocellulose and stained with a rabbit polyclonal anti-EBNA-2 antiserum kindly provided by Georg Bornkamm. Molecular mass markers (at right) were Rainbow markers (Amersham Corp., Arlington Heights, Ill.).

protected fragment size is consistent with a 3′ terminus of the EBNA-2 transcript following a AATAAA site at nt 50003 (49). The levels of expression of EBNA-2 transcript varied between 90 per cell in the cell line 11/17-5 and 270 per cell in the cell line 11/17-3 (Table 1). These levels of EBNA-1 and EBNA-2 transcript in unfractionated RNA samples are roughly 100-fold higher than previously reported in poly(A)$^+$ RNA from the cell line IB4 (22, 57). From a comparison of EBNA-1 and EBNA-2 transcript accumulation with the cellular properties (both presented in Table 1), it is evident that there is no correlation between the variation in expression of EBNA-1 or EBNA-2 transcript and the average EBV DNA copy number in a cell line, nor is there a correlation with the frequency of spontaneous induction of the viral lytic cycle. As will be presented later, this principle was found to be true for the LMP transcript as well.



Several features of the S1 nuclease assays presented in Fig. 1A and 2A are worth noting. A small protected fragment of approximately 600 to 640 bases, likely to represent the lytic-phase transcript for the BKRF-2 gene (63), was detected in cell lines containing the highest average EBV DNA copy numbers and rates of lytic growth (in Fig. 1A, marked with an asterisk and arrow). The dark smear of hybridizing material in the 3/15-31 and adjacent lanes of Fig. 1A and 2A was due to the small quantities of DNA (roughly 0.1%) that contaminated the RNA preparations, as pretreatment of the sample with RNase-free DNase before hybridization with the recombinant M13 probe abolished the smear (for example, in the three rightmost lanes of Fig. 2A, where 10 μg of DNase-treated RNAs from 11/17-4, THLB-5, and 3/15-31 were tested). In the S1 nuclease analyses of both EBNA-1 and EBNA-2 transcripts, a protected fragment equal in size to the DNA insert of the M13 clones was detected (readthrough arrow, Fig. 1A and 2A). RNA protection of the cloned DNA was responsible for at least some of the signal, as the readthrough fragments were detected when samples were pretreated with RNase-free DNase (Fig. 2A); most of the signal could be abolished by pretreatment of the sample with alkali (Fig. 1A); and the readthrough fragments were less readily detected with M13 clones containing the EBV DNA inserts in the opposite orientations (− strand probes M13p513 [same coding sense as the EBNA-1 transcript; Fig. 1A] and M13p586 [same coding sense as the EBNA-2 transcript; Fig. 2A]). These findings indicated that transcription did not always terminate after the AATAAA sites at the 3′ end of the EBNA-1 and EBNA-2 gene, and the readthrough transcripts were stable enough to accumulate to high levels (roughly 50 copies per cell of the EBNA-1 readthrough transcript and 250 copies per cell of the EBNA-2 readthrough transcript).

The levels of expression of LMP transcript were measured by Northern blot analysis (1) of unfractionated RNA samples (Fig. 3A). No signal could be detected in RNA from the EBV-negative cell line Ramos, but the 2.8-kb LMP transcript was detected in each of the nine EBV-positive cell lines tested (note longer exposure of 11/17-3 and 11/17-5 lanes presented at right in Fig. 3A). The transcript for the

FIG. 3. Measurement of the number of copies of the LMP transcript per cell by Northern blot analysis and of the relative levels of LMP product per cell by Western blot analysis. (A) Equal amounts (5 μg) of unfractionated RNA from 10 lymphoblastoid cell lines were analyzed by gel electrophoresis, transfer to ZetaProbe membrane, and hybridization to a ^{32}P-radiolabeled probe (p335.2, which spans nt 169423 to 167487 from the EBV genome [except for deleted bases between nt 169397 and 168478]). The description of samples is the same as in the legend to Fig. 1A. In vitro-synthesized p416 RNA spans the same EBV nucleotides as p335.2 and has the same coding sense as native LMP RNA. The autoradiographic exposure in the left panel was 23 h. The two lanes at right show a 207-h exposure (six times more ^{32}P disintegrations than that shown on the left) of the 11/17-3 and 11/17-5 lanes. (B) LMP was detected by Western analysis (method in reference 4) of a sodium dodecyl sulfate–9% polyacrylamide gel, as described in the legend to Fig. 2B. In the 10 lanes at left, 3×10^4 cell equivalents of lysate were loaded on the polyacrylamide gel. The description of samples is the same as in the legend to Fig. 2B. The four lanes at right show the analysis of increasing numbers (3×10^3, 1×10^4, 3×10^4, and 1×10^5) of cell equivalents of LCL721, as marked. The ladder of minor bands below the LMP band is a reproducible finding with this antiserum (4). A rabbit polyclonal anti-BNLF-1 antiserum was kindly provided by Vijay Baichwal. Molecular mass markers (right) were the same as described in the legend to Fig. 2B.

truncated form of LMP comigrates with the full-length transcript by Northern analysis (23), and so the single band detected in Fig. 3A may contain two RNA species. However, as shown below, the truncated LMP product is only detectable in 3/15-31 cells (and then only as a minor species), so it is likely that the band detected in Fig. 3A is predominantly the full-length transcript. Quantitative analysis (Table 1) of LMP transcript indicated that the levels of accumulation varied between 50 and 120 copies per cell, in close agreement with the previously reported value for the levels of LMP transcripts in poly(A)$^+$ RNA of IB4 cells (15). The variation in accumulation of LMP transcript did not correlate with the average EBV DNA copy number or with the rate of spontaneous lytic growth.

The relative levels of expression of EBNA-2 protein and LMP were determined for the set of nine lymphoblastoid cell clones by Western blot (immunoblot) analysis. Rabbit anti-EBNA-2 and anti-LMP (BNLF-1) antisera detected 88- and 62-kDa proteins, respectively, in each of the nine EBV-positive cell clones but not in the EBV-negative Burkitt's lymphoma cell line Ramos (Fig. 2B and 3B). In Fig. 3B, trace amounts of the lytic-phase 55-kDa truncated LMP (47) can be detected as a minor species in 3/15-31 cells (migrating slightly slower than the 46-kDa marker). The right four lanes of Fig. 2B and 3B show the levels of EBNA-2 and LMP detected when threefold-increasing amounts of THLB-1 or LCL721 cell lysate were tested. These control lanes demonstrated that the signals detected in the nine EBV-positive lymphoblastoid cell clones were within the linear range of detection of the assay.

The steady-state levels of these two proteins per cell were determined quantitatively by densitometric scanning of the developed blots, and the results of these analyses are shown in Table 1. The results of two experiments are presented side-by-side, to indicate that there exists some fluctuation in the assay. The variation in EBNA-2 protein and LMP expression was approximately fourfold and sevenfold, respectively among the nine EBV-positive cell clones (Table 1). After comparison of these data with the properties of the cells (also presented in Table 1), it is evident that there is no correlation between the variation in steady-state levels of EBNA-2 protein or LMP with the overall EBV DNA copy number or with the frequency of spontaneous induction of lytic growth. These results therefore confirm and extend the findings made at the level of transcript accumulation. In a finding similar to this, the accumulation of EBNA-1 protein does not depend on the average EBV DNA copy number (T. Middleton and L. Sternås, manuscript in preparation).

From this study (and others), it is evident that the frequency with which cells are spontaneously induced varies among different cell lines that are of clonal origin. However, it is not known what genetic or epigenetic switches establish the frequency. This study demonstrates that high average EBV DNA copy numbers are associated in in vitro-transformed cell lines with high frequencies of spontaneous induction. There are at least two possible models for this association. In one model, the copy number of the EBV genome during latency might be restricted to only one to five copies per cell, and the large variation in average genome copy number (as in Table 1) might be due to the 100 to 1,000-fold amplification of the viral genome in the small percentage of lytically infected cells (17). Consequently, if a cell line had a higher rate of spontaneous induction, it would necessarily have a higher average EBV DNA copy number as well, and 99% of the cells in a population might contain only 1% of the overall EBV DNA. The cell line Raji (40), with 50 copies of the viral genome per cell and no spontaneous lytic induction (13), could represent an exception to this model. This exception may, however, simply reflect the aberrant expression of viral genomes in Burkitt's lymphoma cell lines compared with normal lymphoblastoid cell lines (46, 48).

In the second model of this association, the EBV DNA copy number during latency could vary between 1 and 100 copies per cell, and the frequency of spontaneous induction would be a function of this varying copy number. In this model the lytic-phase *trans*-activator BZLF-1 (also known as ZEBRA and EB1) would be transcribed at a low rate from all EBV DNA templates during latency and would accumulate to a critical "spontaneous induction level" in cells carrying a sufficient number of templates. Such a viral strategy would be reminiscent of the phage λ system, in which the decision to enter into lysogeny or lytic growth depends on the levels of accumulation of *c*III product and in part, therefore, on the multiplicity of infection of the bacterium (6, 27).

Ernberg et al. (14) found that the levels of expression of EBNA antigen(s), by indirect immunofluorescence, were in proportion to EBV DNA copy number; however, this work was performed with polyclonal (and possibly polyspecific) human antisera. In the present study, the combined properties of higher (or lower) EBV DNA copy number and higher (or lower) frequencies of spontaneous induction were not associated with particular levels of expression of EBNA-1, EBNA-2, or LMP. The EBNA-1 and LMP transcripts accumulated to levels of 50 to 150 copies per cell, and the EBNA-2 transcript accumulated to levels of 90 to 270 copies per cell (Table 1). Most of the EBNA-1 and EBNA-2 transcript copies may not be polyadenylated (20, 22, 57) and probably do not exit from the nucleus. There is no correlation between the minor variations (3- to 7-fold) in accumulation of these transcripts and protein products and the substantial variations (>100-fold) in EBV DNA copy number and frequency of spontaneous induction. That is, the heritable property of high or low spontaneous induction is not imprinted on a cell line by a detectable change in expression of these viral genes.

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