Amplification and deregulation of *MYC* following Epstein–Barr virus infection of a human B-cell line

(B-cell neoplasia/Burkitt lymphoma/oncogene activation/viral transformation)

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ABSTRACT In Epstein–Barr virus (EBV)-positive Burkitt lymphoma (BL) the role of EBV in the translocation and deregulation of the *MYC* oncogene remains unknown. By utilizing an EBV-negative BL (BJAB) and several EBV-positive sublines derived from it by *in vitro* infection, it was possible to show that the presence of the virus was associated with altered expression and copy number of *MYC*. In the EBV-negative BJAB line, the level of MYC transcripts declined progressively as cells approached the stationary phase of growth. In contrast, in EBV-infected BJAB cells *MYC* expression remained elevated as cells entered stationary phase. This effect on *MYC* expression was reversibly linked to the presence of the virus. Furthermore, following EBV infection of BJAB cells by two different strains of EBV, amplification of *MYC* in association with the appearance of a homogeneously staining region on chromosome 8 at the mapped location of *MYC* had occurred. These studies suggest that both the deregulation of MYC transcription and the chromosomal rearrangement in the region of the *MYC* locus in this B-cell line may have occurred as a result of EBV infection.

Most Burkitt lymphomas (BL) are characterized by the presence of the Epstein–Barr virus (EBV) genome (1) and a chromosomal translocation that juxtaposes the *MYC* oncogene and an immunoglobulin gene (2–5). Both EBV and altered *MYC* function are believed to be involved in the genesis of BL, but the relationship between the presence of the virus and *MYC* dysfunction is unknown. Although deregulation of *MYC* in BL has been described (2–9) and attributed to the translocation, the large number of different breakpoints adjacent to and within the *MYC* gene in BL (2–5) has precluded the development of a consistent hypothesis concerning the mechanism by which *MYC* deregulation occurs. It is conceivable that factors other than the translocation, such as the presence of EBV, may contribute to *MYC* deregulation in BL. Indeed, it has recently been shown that EBV infection of an EBV-negative BL results in increased expression of the endogenous cellular oncogene *FGR* (10).

Studies to determine whether EBV directly affects *MYC* structure or expression are inherently difficult due to the lack of availability of clonal lines of normal nontransformed proliferating human B cells or pre-B cells. For this reason, we have used an EBV-negative BL line and several sublines derived from it by *in vitro* infection with EBV to determine whether EBV alters MYC transcription or structure. These lines should differ genetically only by the presence or absence of the EBV genome and thus provide an ideal system for examining the effects of the virus on endogenous genes. It is known that EBV infection of two EBV-negative BL lines, BJAB and Ramos, results in altered growth properties such as increased resistance to growth saturation and de-

creased serum requirements (11–15). Since *MYC* has been implicated in controlling cell growth in normal human B cells (16), it seemed plausible that the altered growth induced by EBV conversion of these lines may be related to altered *MYC* expression. Our studies show that EBV infection of the EBV-negative BL line BJAB results in deregulation and amplification of *MYC*.

MATERIALS AND METHODS

Cell Lines and Cell Culture. BJAB cells were derived from a human BL that has been previously shown to be negative for the presence of EBV as determined by nucleic acid hybridization studies and the absence of EBV nuclear antigen (EBNA) (11, 17). Karyotypic analysis of this line showed no evidence of a translocation involving chromosome 8 (17, 18). BJAB-B1 and BJAB-B958 are EBNA-positive sublines of BJAB that were derived from BJAB cells by infection with P3HR1 and B958 strains of EBV, respectively (19). BJAB-B1 was cloned from P3HR1-infected BJAB cells (20). BJAB-B1-106 and BJAB-B1-107 lines were subcloned from BJAB-B1 in this laboratory (21). The BJAB-B1-106 line is EBNA-positive, whereas the BJAB-B1-107 line is a revertant line in which all cells have lost the EBV genome as determined by the absence of EBNA (21). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum plus penicillin and streptomycin. Growth curves for each cell line were derived by harvesting cells daily and determining cell density by using an automated Coulter Counter. Viability was determined by exclusion of trypan blue.

RNA Isolation and RNA Blot Analysis. RNA was purified from cells by pelleting cell lysates in guanidinium isothiocyanate through a cesium chloride cushion according to the method of Glisin *et al.* (22). Fifteen micrograms of total RNA was loaded per lane and separated by electrophoresis through a 6.7% formaldehyde/1% agarose gel. Molecular weight markers (*Hind*III-digested phage λ DNA) were included on every gel. RNA was transferred to nitrocellulose paper according to Southern (23) and hybridized as described by Thomas (24) in 50% formamide/5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/50 mM Na_2HPO_4/1× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/10% dextran sulfate/100 µg of denatured salmon sperm DNA per ml, with 10^6 cpm·ml^{-1} of denatured purified radiolabeled probe. After hybridization for 16 hr at 42°C, filters were washed sequentially in 2× SSC/0.1% NaDodSO$_4$ for 20 min at room temperature and 0.1× SSC/0.1% NaDodSO$_4$ for 30 min at 50°C. Filters were exposed to Kodak X-AR film. Some blots were hybridized to a second probe after washing in 5 mM Tris·HCl/0.2 mM EDTA/0.05% pyrophosphate/0.1× Denhardt's solution at 65°C for 2 hr. Relative amounts of

Abbreviations: BL, Burkitt lymphoma; EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen.

hybridization were determined by densitometry of the autoradiographs using a Joyce–Loebl automatic recording microdensitometer.

Genomic DNA Isolation and Southern Blot Analysis. Genomic DNA was isolated from cells by digestion in 0.5% NaDodSO$_4$ and 0.2 mg·ml^{-1} of proteinase K, extraction in phenol/chloroform, and ethanol precipitation. Contaminating RNA was removed by treatment with RNase A. DNA samples (10 μg) were digested to completion with the indicated restriction endonucleases and separated by electrophoresis in a 0.7% agarose gel. Molecular weight markers were included on each gel. DNA was transferred to nitrocellulose paper according to Southern (23). Hybridization was done at 68°C for 16 hr in 6× SSC/0.5% NaDodSO$_4$/0.01 M EDTA/ 200 μg·ml^{-1} of denatured salmon sperm DNA, with 10^6 cpm·ml^{-1} of purified radiolabeled probe. Blots were washed sequentially in 2× SSC for 20 min at room temperature and 0.1× SSC for 2 hr at 68°C and exposed to Kodak X-AR film. Relative amounts of hybridization were determined by densitometry of the autoradiograph.

Preparation of Probes. All human probes were purified from their plasmid vectors and radiolabeled by nick-translation using [^{32}P]dCTP to a specific activity of 10^9 cpm·μg^{-1}. Plasmids containing the human *MYC* gene and the human HLA class I (B locus) were generously provided by Philip Leder (Harvard University) and Sherman Weissman (Yale University), respectively. The purified β-globin probe was provided by Susan Baserga (Yale University). The whole EBV genomic probe was derived by nick-translation of a Charon 4A library of the B958 strain of EBV generously provided by Bill Sugden (University of Wisconsin). The EBV DNA polymerase probe was a 2.6-kilobase fragment of the putative EBV DNA polymerase gene subcloned from the Charon 4A library of EBV-B958 by Marcia Lewis in this laboratory.

EBNA Determinations. The cell lines were examined for the presence of EBNA by the anti-complement immunofluorescence method (25). The human anti-EBV serum (WCS) used for EBNA detection was shown to be reactive with the EBNA 1 protein by anti-complement immunofluorescence using a mouse fibroblast line carrying the *Bam*HI K fragment of EBV (26).

RESULTS

Although infection of the EBV-negative BL line BJAB with EBV is known to increase resistance to density-dependent growth arrest (13–15), the mechanism by which EBV alters the growth of BJAB is uncertain. Since *MYC* has been implicated in controlling cell growth in normal human B cells (16), it seemed plausible that this altered growth property may be related to enhanced *MYC* expression as cells approached growth saturation densities. To examine this hypothesis, the BJAB cell line and four sublines derived from it by infection with EBV (BJAB-B958, BJAB-B1, BJAB-B1-106, and BJAB-B1-107; see Table 1) were examined for *MYC* expression at multiple cell densities. Cultures of the five

cell lines were examined over 4 successive days for MYC mRNA expression by RNA blot analysis. Cells were initially cultured on day 0 at densities such that each cell line would be in the same phase of growth when harvested on subsequent days. Thus, each cell line was in early exponential phase on day 1, midexponential phase on day 2, late exponential phase on day 3, and early stationary phase on day 4 (Fig. 1A). At low cell densities (early exponential phase) there was no difference in *MYC* expression in the EBV-positive and -negative BJAB lines (Fig. 1 *B* and *C*; Fig. 2, lanes 1). However, as cell density increased, MYC mRNA levels decreased progressively in the EBV-negative BJAB parent line and revertant subclone of BJAB-B1 (BJAB-B1-107) (Fig. 1 *B* and *C*; Fig. 2 *B* and *D*). In contrast, MYC mRNA levels were maintained as cell density increased in the EBV-positive sublines of BJAB (Fig. 1 *B* and *C*; Fig. 2 *A*, *C*, and *E*). Thus, when the cell lines were compared at the late exponential phase of the growth curve (day 3), *MYC* expression in the EBV-positive sublines was 5- to 20-fold greater than in the two EBV-negative lines (the parental and the revertant line). This experiment extended our previously reported observation made only at low cell densities (18). To show that the progressive decrease in *MYC* expression in the EBV-negative cell lines did not simply reflect an overall decrease in mRNA content as the cells approached stationary phase of growth, the blots were washed and probed with an HLA class I gene probe (Fig. 2). The levels of MYC mRNA were normalized to the amount of HLA class I mRNA (Fig. 1D). In all five cell lines, including the two EBV-negative lines (BJAB and BJAB-B1-107), HLA class I expression remained stable as cell density increased. These experiments confirmed that the presence of EBV was associated with the specific maintenance of *MYC* expression as the cells approached the stationary phase of the growth curve, whereas in the EBV-negative parental line, *MYC* expression decreased progressively as cell density increased. Furthermore, this alteration in *MYC* expression in the EBV-positive sublines was reversibly linked to the presence of the EBV genome, since loss of the virus (subclone BJAB-B1-107) was associated with reversion of the pattern of *MYC* expression to that of the original EBV-negative parental line.

To determine whether the observed change in *MYC* expression after EBV conversion of BJAB was associated with rearrangement or amplification of the *MYC* gene, Southern blot analysis using the MYC probe was done on each cell line (Fig. 3). Endonuclease digestion with *Eco*RI or *Bam*HI indicated that there was no rearrangement of MYC in any of the five lines. However, in all sublines that were carrying the EBV genome (strain P3HR1 or B958), as well as the EBV-negative revertant subclone of BJAB-B1 (BJAB-B1-107), the *MYC* gene was amplified 5- to 10-fold relative to the parental BJAB line and normal lymphocytes. To determine accurately the amount of amplification, the same blots were washed and probed with other gene probes (*FOS* and β-globin), and the amount of hybridization of *MYC* relative to other genes was determined by densitometry. These studies confirmed that *MYC* was amplified 4- to 5-fold in BJAB-B958

Table 1. Origin and EBV status of cell lines

Cell line	Parental cell line	Superinfecting strain of EBV	EBNA	EBV DNA	Ref.
BJAB	—	—	Absent	Absent	11, 17
BJAB-B958	BJAB	B958	Present	Present	19
BJAB-B1	BJAB	P3HR1	Present	Present	19, 20
BJAB-B1-106	BJAB-B1	P3HR1	Present	Present	21
BJAB-B1-107	BJAB-B1	P3HR1	Absent	Absent	21

Presence or absence of intracellular EBNA was determined by anti-complement immunofluorescence. Presence or absence of intracellular EBV DNA was determined by Southern blot analysis using whole EBV genome and the EBV DNA polymerase gene as probes.
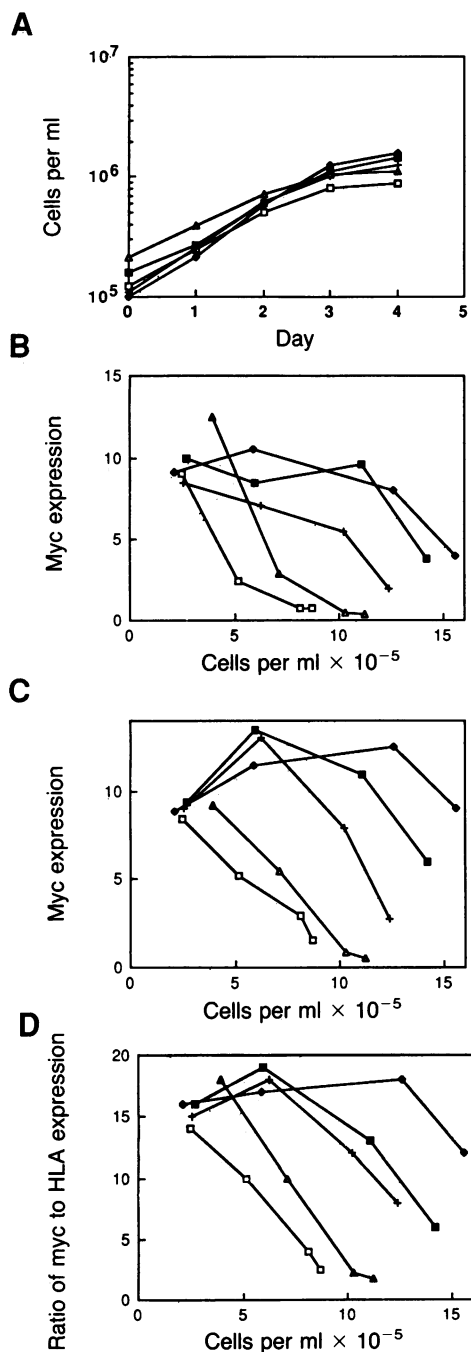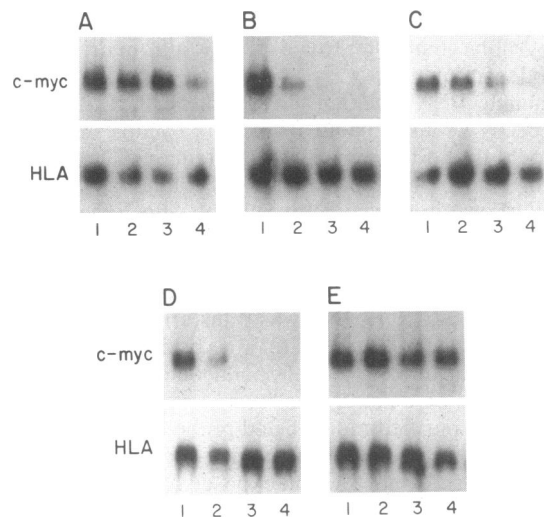
FIG. 2. RNA blot analyses of MYC and HLA class I expression in two EBV-negative and three EBV-positive BJAB lines over 4 successive days of culture. (*A*) BJAB-B1-106; (*B*) BJAB-B1-107; (*C*) BJAB-B1; (*D*) BJAB; (*E*) BJAB-B958. Lane numbers indicate the day of culture on which cells were harvested for RNA preparation.

FIG. 1. Cell density and *MYC* expression in two EBV-negative and three EBV-positive BJAB lines examined over 4 successive days of culture. □, EBV-negative parental line BJAB; △, EBV-negative revertant line BJAB-B1-107; ◆, EBV-positive line BJAB-B958; ■, EBV-positive line BJAB-B1; +, EBV-positive line BJAB-B1-106. (*A*) Growth curves. Cell viabilities as determined by exclusion of trypan blue were >90% for all five cell lines on days 1–3. On day 4, viabilities were as follows: BJAB-B1, 96%; BJAB-B958, 98%; BJAB-B1-106, 97%; BJAB-B1-107, 75%; BJAB, 80%. (*B* and *C*) Relative amounts of MYC transcripts on 4 successive days of culture from duplicate experiments. Relative amounts of MYC mRNA from RNA blot analysis were quantified by densitometry of autoradiographs using an automatic recording microdensitometer and expressed in arbitrary units, which are proportional to mRNA amounts on the ordinate axis. Data in *B* were derived from RNA blot shown in Fig. 2. (*D*) Ratio of MYC to HLA class I mRNA on 4 successive days of culture. RNA blots were hybridized to the MYC probe, washed, and rehybridized to the HLA class I probe. Relative amounts of MYC (*C*) and HLA class I mRNA were determined by densitometry.

and 8- to 10-fold in BJAB-B1 and its subclones (BJAB-B1-107 and BJAB-B1-106) relative to BJAB and normal lymphocytes. Furthermore, karyotypic analysis of the five lines provided cytologic corroboration of an amplification event involving the *MYC* gene after EBV infection (Fig. 4). In all four sublines, including the revertant line subcloned from BJAB-B1 (BJAB-B1-107), there was a large homogeneously staining region involving the terminal portion of 8q in the region of the mapped location of the *MYC* gene (27–29). In contrast, chromosome 8 appeared normal in the parental BJAB line. There was no evidence of a translocation involving chromosome 8 in any of the lines. Furthermore, no other chromosomal abnormalities that were associated with the presence of EBV were observed.

To corroborate the previously reported presence or absence of the EBV genome in these lines, all five isogenic lines were examined for the presence of EBNA by anti-complement immunofluorescence and EBV DNA by Southern blot analysis using the whole EBV genome and the EBV DNA polymerase gene as probes. The BJAB-B958, BJAB-B1, and BJAB-B1-106 lines were positive for both EBNA and EBV DNA (Fig. 5). In contrast, the BJAB parental line and the revertant line (BJAB-B1-107) were negative for EBNA and
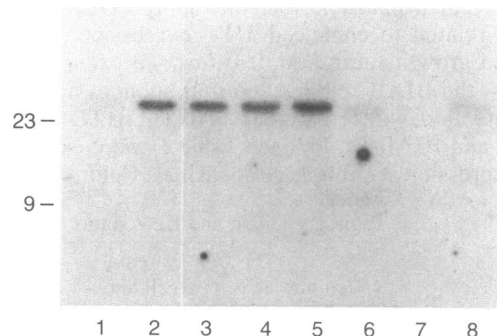

FIG. 3. Southern blot analysis of BL lines and normal lymphocytes using the human MYC probe. DNA samples (10 μg) were digested to completion with *Bam*HI. DNA sizes (in kilobase pairs) are indicated on the left of the autoradiograph. Lanes: 1, BJAB; 2, BJAB-B958; 3, BJAB-B1; 4, BJAB-B1-106; 5, BJAB-B1-107; 6, EBV-positive BL line, Raji; 7 and 8, human peripheral blood lymphocytes.

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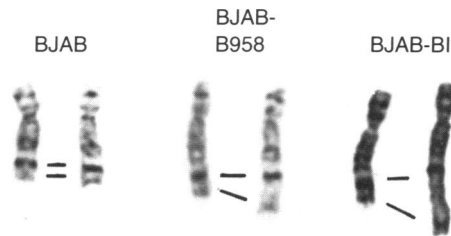*Proc. Natl. Acad. Sci. USA 84 (1987)* 5841

BJAB

BJAB-
B958

BJAB-BI

FIG. 4. Selected pairs of chromosome 8 from the BJAB parent line and two EBV-converted sublines, BJAB-B958 and BJAB-B1. Trypsin-Giemsa banding of chromosomes arrested in metaphase was done according to standard methods. A chromosome 8 pair from each cell line is shown. Both chromosomes 8 from the parent BJAB line and one chromosome 8 from the EBV-converted sublines (shown on the left in those pairs) are normal in appearance. The second chromosome 8 from the EBV-converted sublines (shown on the right in those pairs) contains an expanded homogeneously staining region that is indicated by the lines that enclose the normal band 8q24 on the normal (*Left*) chromosome 8 and the expanded homogeneously staining region on the abnormal (*Right*) chromosome 8. Although not shown, the appearance of chromosomes 8 from the two subclones of BJAB-B1 (BJAB-B1-106 and BJAB-B1-107) was identical to the chromosomes in BJAB-B1 shown here.

showed no detectable EBV DNA by the Southern analysis with either probe at a level of sensitivity of 0.5 copy per cell.

DISCUSSION

We have shown that EBV conversion of the EBV-negative BL, BJAB, is associated with altered MYC transcriptional expression that can only be appreciated by examining the cells at multiple points along their growth curves. As the cells approached the stationary phase of their growth curves, *MYC* expression diverged strikingly between EBV-positive and -negative BJAB lines. This change in *MYC* expression was reversibly linked to both the presence of the viral genome and altered growth properties, since loss of virus was associated with reversion of the pattern of *MYC* expression and the growth properties to that of the parental line. This was further substantiated by examining a second revertant line (BJAB-B1-101) that had been subcloned from BJAB-B1 (21). This line displayed a pattern of *MYC* expression relative to its growth curve that was similar to that of the parental EBV-negative line BJAB (data not shown). It is possible that enhanced *MYC* expression as cells entered the stationary phase of growth may be the mechanism by which EBV alters the growth characteristics of the BJAB line. Alternatively, the observed change in the pattern of *MYC* expression may simply be a consequence rather than a cause of altered
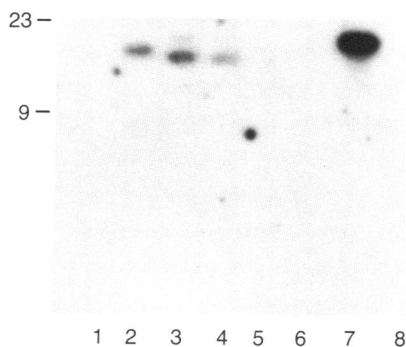

FIG. 5. Southern blot analysis of BL lines and normal lymphocytes using the EBV DNA polymerase probe. DNA samples (10 μg) were digested to completion with *Eco*RI. DNA sizes (in kilobase pairs) are indicated on the left of the autoradiograph. Lanes: 1, BJAB; 2, BJAB-B958; 3, BJAB-B1; 4, BJAB-B1-106; 5, BJAB-B1-107; 6, human lymphocytes; 7, EBV-positive BL line Raji; 8, human lymphocytes.

growth in the EBV-positive lines. These two hypotheses cannot be distinguished from these experiments. Furthermore, it is not known if the sustained levels of MYC transcripts in EBV-positive lines were associated with sustained expression of the MYC protein as cells approached stationary phase.

Unexpectedly, infection of this EBV-negative BL by EBV (strain B958 or P3HR1) was associated with amplification of the *MYC* gene. This appears to be the first report of *MYC* amplification in association with the presence of EBV. Although this observation supports the hypothesis that EBV caused or promoted the chromosomal rearrangement in the region of the *MYC* locus in this cell line, other explanations must be considered. It is possible that the amplification of *MYC* occurred in some cells prior to infection with EBV, and successful conversion by EBV was then favored in those cells with amplified *MYC*. Alternatively, the amplification event may have occurred after EBV conversion as a consequence of cloning or prolonged maintenance in tissue culture. However, the finding that the amplification event occurred in two independent cell lines that were each infected separately by different viral strains (P3HR1 and B958) lends support to a causal relationship. Furthermore, we have examined an additional cloned cell line, BJAB-A5, which was derived by infection of BJAB with the P3HR1 strain of EBV (20) and found an 8-fold amplification of *MYC* in this line as well. Moreover, the amplification event in these cell lines is unlikely to be related to the duration of tissue culture passage, since four of the lines with amplified *MYC* (BJAB-A5, BJAB-B1-106, BJAB-B1-107, and BJAB-B1-101) have a much shorter history of tissue culture passage (kept in frozen storage since 1978) than the EBV-negative parental line (BJAB) and the other two converted lines (BJAB-B958 and BJAB-B1). Amplification or translocation of MYC does not appear to occur after *in vitro* immortalization of human umbilical cord lymphocytes by EBV (unpublished observations). It is conceivable that the host B cell (or pre-B cell) must be in a precise state of differentiation or proliferation for a structural change in the region of the *MYC* gene to occur.

Amplification of *MYC* is not a sufficient explanation for the altered *MYC* expression observed in the EBV-converted BJAB lines, since loss of the virus was associated with reversion of the pattern of *MYC* expression but not reversion of the amplification event. This result suggests that the presence of EBV may be necessary to deregulate *MYC* expression in the BJAB line even after the amplification of *MYC*.

The finding of altered *MYC* expression in association with EBV conversion of an EBV-negative BL cell line raises the possibility that EBV may be more directly involved in the deregulation of *MYC* in EBV-positive BL than has been proposed previously. Deregulation of *MYC* may result from a direct effect of EBV on MYC transcription, or, alternatively, may be an indirect consequence of altered growth caused by the virus. Furthermore, the observation that the *MYC* gene becomes amplified in association with EBV infection of this line lends support to the hypothesis that a chromosomal rearrangement in the region of the *MYC* locus may occur as a result of EBV infection. This hypothesis may be relevant to the pathogenetic relationship of EBV and the translocation of MYC in EBV-positive BL.

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