5-Azacytidine Up Regulates the Expression of Epstein-Barr Virus Nuclear Antigen 2 (EBNA-2) through EBNA-6 and Latent Membrane Protein in the Burkitt's Lymphoma Line Rael

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Nonproductive infection of B lymphocytes by Epstein-Barr virus (EBV) is associated with a highly restricted expression of viral genes. In growth-transformed lymphoblastoid cell lines, the products of these genes include a complex of at least six EBV nuclear antigens (EBNAs) (EBNA-1 through EBNA-6) and one membrane protein (latent membrane protein [LMP]). EBV-carrying Burkitt's lymphoma (BL) biopsies and derived cell lines that have retained a representative phenotype (group I BL lines) express only EBNA-1 (M. Rowe, D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson, *EMBO J.* 6:2743-2751, 1987). We have found that EBNA-2 through EBNA-6 and LMP can be up regulated by treating the group I BL line Rael with the DNA-demethylating agent 5-azacytidine (5-AzaC). The drug acted in a time- and dose-dependent manner. EBNA-2-positive cells were detected by anti-complement immunofluorescence staining just 12 h after addition of 4 μ M 5-AzaC and reached a maximum number at 72 h, when up to 75% of the population was positive. EBNA-2, EBNA-3, EBNA-4, EBNA-4, EBNA-6, and LMP were demonstrated in immunoblots starting at 48 h. The EBV-encoded early antigens and viral capsid antigens were also induced but at a lower level. EBNA-2 and the lytic cycle-associated antigens appeared with a different time course and in largely nonoverlapping cell subpopulations, as demonstrated by double fluorescence staining. Thus, EBNA-2 expression was not restricted to lytically infected cells, nor was EBNA-2 required for entry into the lytic cycle. The coding and regulatory sequences of EBNA-2 and LMP were found to be highly methylated in Rael cells and were, as expected, demethylated after 5-AzaC treatment. These findings suggest that DNA methylation may participate in the regulation of growth transformation-associated viral genes in BL cells.

Nonproductive infection of B lymphocytes by Epstein-Barr virus (EBV) generates immortalized lymphoblastoid cell lines (LCL) that carry multiple episomal copies of the viral genome and express a restricted set of viral genes. The products of seven genes have been identified. Six EBV nuclear antigens (EBNAs), EBNA-1 through EBNA-6 (4, 6, 19, 22, 36, 43) are localized in the nucleus and form the EBNA complex, originally detected by anti-complement immunofluorescence (34). The seventh protein, latent membrane protein (LMP), is associated with the cell membrane (18, 29).

Little is known about the role of these proteins in the transformation process. EBNA-1 binds to the origin of viral DNA replication in *Bam*HI-C and is required for the maintenance and replication of plasmid DNA (52). It can also activate a viral enhancer (35). EBNA-2 participates in the initiation and maintenance of transformation, since EBV strains with deletions affecting the EBNA-2 coding region (20, 33) cannot activate resting B cells to enter the mitotic cycle (31, 32). Only EBNA-2-carrying virus strains induced the expression of B-cell activation markers in EBV-negative BL lines (3, 25). Transfection of the EBNA-2 gene into an EBV-negative BL line induced the constitutive expression of CD23 (50), an activation marker that can function in the dual capacity of a B-cell growth factor receptor (14) and, in a processed form, an autocrine B-cell growth factor (44). LMP can induce anchorage-independent growth and tumorigenicity in rodent fibroblasts (1, 51).

The regular expression of EBNA-1 through EBNA-6 and

LMP in LCLs is paralleled by a consistent cellular phenotype. All LCLs express a gamut of B-cell activation markers and grow mostly in large clumps (38, 46). In contrast, EBV-carrying Burkitt's lymphoma (BL)-derived cell lines are phenotypically heterogeneous (7, 11, 39). EBV-negative and -positive BL biopsies, established lines of EBV-negative BLs, and early in vitro passages of the EBV-positive BLs express CD10 and CD77 but no activation markers and grow in vitro as free cells (group I BLs) (37, 39). During serial propagation, the EBV-carrying BL lines often drift towards a more LCL-like phenotype, as indicated by the progressive appearance of activation markers, change of growth pattern, and finally of CD10 and CD77 (group II and group III BLs) (39). Some EBV-carrying BL lines, like the Rael cell line used in this investigation, have maintained a group I phenotype in spite of prolonged in vitro cultivation.

BL biopsies and group I BL lines express EBNA-1 but no EBNA-2 through EBNA-6 or LMP. Drift towards a group III phenotype is associated with up regulation of these antigens (40, 41). Rescue of the virus from BL lines by cocultivation with cord blood cells leads to the full expression of all seven proteins in the resulting LCLs (40), confirming that their eclipse in BL cells depends on the host cell phenotype.

Some EBV-carrying, virus nonproducer lines can be induced to enter the lytic cycle by a variety of agents that influence the expression of differentiation markers (for a review, see reference 10). We have tested some of these agents, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), *N*-butyrate, teleocidin, 5-bromodeoxyuridine (BUdR), and 5-azacytidine (5-AzaC) for their ability to induce the expression of EBNA-2 through EBNA-6, LMP, and the lytic cycle-

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associated early antigen (EA)-viral capsid antigen (VCA) complex in the Rael line. EBNA-2 through EBNA-6 and LMP were induced by the DNA demethylating agent 5-AzaC but not by the other inducers. Our finding suggests that viral DNA methylation may participate in the host cell-dependent down regulation of EBNA-2 through EBNA-6 and LMP.

MATERIALS AND METHODS

Cell lines and culture medium. The EBV-positive BL line Rael (24) was maintained by feeding three times per week with RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 µg of streptomycin per ml, and 5% heat-inactivated fetal calf serum (complete medium). Exponentially growing cells harvested 24 h after feeding were seeded at a concentration of 3×10^5 /ml in medium containing 10 ng of TPA per ml, 300 µM *N*-butyrate (Sigma Chemical Co., St. Louis, Mo.), or a combination of the two, 10 ng of teleocidin (a gift from R. E. More, Department of Biochemistry, University of Hawaii, Honolulu) per ml, the indicated concentrations of 5-AzaC (Sigma), and 50 µg of BUdR (Sigma). At the indicated time points, the cells were harvested, live cells were counted by trypan blue dye exclusion, and dead cells were removed by Ficoll-Isopaque (Nycomed AS, Oslo, Norway) gradient centrifugation. The LCL CBC-Rael-Sto was obtained by rescue of the Rael virus into cord blood cells by cocultivation (I. Ernberg, K. Falk, J. Minarovits, T. Tursz, P. Busson, M. G. Masucci, and G. Klein, submitted for publication).

Detection of EBV antigens. (i) **Immunofluorescence.** Previously characterized sera from EBV-infected individuals were used for detection of EBNA-2 by anti-complement immunofluorescence staining (34). EBNA-2-specific reagents were prepared by absorbing the serum of an EBV-positive chronic lymphatic leukemia 17 patient with P3HR1 and Daudi cells as described (5). EA and VCA were detected by direct immunofluorescence (18), using fluorescein isothiocyanate- or tetramethyl-rhodamine isothiocyanate-conjugated sera from BL (EA) or nasopharyngeal carcinoma (VCA) patients. Two color immunofluorescence was performed in two steps: (i) EBNA-2 staining with fluorescein isothiocyanate-conjugated anti-C' antibodies and (ii) EA staining with rhodamine-conjugated BL serum.

(ii) **Immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) were performed as described. A total of 10^7 cells were dissolved in 1 ml of electrophoresis sample buffer. Proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (26) and blotted on to nitrocellulose paper (Schleicher & Schuell, Dassel, Federal Republic of Germany) (47). The efficiency of the protein transfer and the position of molecular weight markers were visualized by staining the filters with Ponceau-S (Sigma). Excess protein-binding sites were blocked by incubation of the blotted filters for 1 h in phosphate-buffered saline (PBS) containing 5% dried skimmed milk. The filters were subsequently incubated with the specific antibodies diluted in PBS containing 5% dried skimmed milk for 2 h at room temperature or for 16 h at 4°C. After incubation with the primary antibody, the filters were washed in PBS containing 5% dried skimmed milk and subsequently incubated with the appropriate alkaline phosphatase-conjugated second antibody (Bio-Rad Laboratories, Kemila Sollentuna, Sweden) for 2 h at room temperature. After three washes in PBS containing 0.5% Tween, the

specifically bound second antibody was detected by fast red salt precipitation in the presence of alpha naphthyl phosphate. A previously characterized serum from a healthy EBV-seropositive donor (OR: VCA, 1:320; EAD, <1:10; EAR, 1:20; EBNA-1, 1:320; EBNA-2, 1:80) was used as the source of antibodies for detection of EBNA-2. LMP was detected by the monoclonal antibody (MAb) (29) S-12 provided by D. Thorley-Lawson (Department of Pathology, Tufts University School of Medicine, Boston, Mass.). The human serum and the MAb S-12 were diluted in PBS containing 5% dried skimmed milk (dilution ratios, 1:20 and 1:1,000, respectively).

Analysis of EBV DNA methylation. The effect of drug treatment on the methylation-demethylation pattern of EBV DNA was tested by analysis of restriction fragment size variation after digestion with methylation-sensitive enzymes. DNA, prepared from frozen cell pellets by the method of Maniatis et al. (28), was digested overnight with one or a combination of two of the following enzymes: *Bam*HI, *Sma*I, *Hpa*II or *Msp*I (Amersham Sweden AB, Solna, Sweden). *Sma*I recognizes the sequence CCCGGG but does not cleave when the third base C is methylated (48). *Hpa*II and *Msp*I recognize the sequence CCGG, but *Hpa*II does not cleave when the second base C is methylated (49). The DNA fragments were separated on a 1.5% agarose gel (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and blotted onto nylon sheets (Hybond N; Amersham Sweden, AB). Cloned EBV-DNA fragments (provided by L. Rymo, Department of Medical Chemistry, Gothenburg University, Gothenburg, Sweden, and by G. Bornkamm, Institute of Microbiology and Hygiene, University of Freiburg, Freiburg, Federal Republic of Germany) were used as probes. The coordinates and the restriction maps based on the B95-8 sequence are shown in Fig. 1. The probes were labeled with [³²P]CTP (Amersham) by the oligolabeling method (12). Hybridization conditions were chosen as published before (9). Bound radioactivity was detected by fluorography with Fuji X-ray film and Fuji EAC cassettes with intensifying screens. Exposure times varied between 2 and 48 h, according to the labeling intensity.

RESULTS

Induction of EBV antigens. Rael cells were exposed to a panel of substances that were earlier reported to induce the productive cycle in various EBV-carrying lines (10). As previously reported (22, 23), untreated Rael cells did not express EBNA-2 or the lytic cycle-associated EA or VCA. EBNA-2 was detected by anti-complement immunofluorescence staining in more than 50% of the cells treated with the DNA-demethylating agent 5-AzaC (Table 1). Cells treated with TPA, *N*-butyrate, teleocidin, and BUdR remained EBNA-2 negative. A significant proportion of the 5-AzaC-treated cells expressed EA and VCA (range, 8 to 32% and 7 to 18%, respectively; Table 2), whereas EA was detected in less than 1% of the cells treated with TPA and BUdR. *N*-Butyrate and teleocidin were ineffective. Increasing concentrations of these chemicals were toxic without significant effect on EBV antigen expression (data not shown).

The EBNA-2-inducing effect of 5-AzaC was time and dose dependent and reached a maximum after 72 h in the presence of the drug (3 to 9 µM; Fig. 2). A 1-µM concentration was sufficient for maximal EA induction.

EBNA-2, EBNA-3, EBNA-4, EBNA-6, and LMP could be identified on immunoblots of 5-AzaC-treated Rael cells probed with EBV-antibody-positive human sera and with the

FIG. 1. Probes used to study EBV DNA methylation by Southern blot hybridization. Horizontal black lines between numbers in boxes indicate the probes that were used in Fig. 5 and 6. Small vertical bars indicate the restriction sites detected by the methylation-sensitive enzymes *Sma*I (a through d) or *Hpa*II (e). Major fragments resulting from complete digestion with these enzymes are indicated above the probe line in base pairs. Two identified enhancers are indicated by open boxes within the probes. The major latent promoters are indicated by black flags. The early promoter for the LMP is indicated with an open flag. Ori-P and the exons for EBNA-2 and LMP are indicated by stippled boxes beneath the probes. The maps are based on the B95-8 sequence. The sizes of the major fragments detected by these probes were similar in Rael and B95-8 cells. (a) *Bam*HI C probe from the *Eco*RI site 7032 to the *Bam*HI site 13215. The *Sma*I sites are indicated by vertical bars. (b) *Bam*HI W probe covering the whole *Bam*HI W fragment. The coordinates of the first *Bam*HI W repeat are shown. *Sma*I sites are indicated by vertical bars. (c) *Bam*HI Y probe covering the whole *Bam*HI Y fragment. The *Sma*I sites are indicated by vertical bars. (d) pM *Bam*H2 probe covering the 3' part of the EBNA-2 exon. The probe is smaller than the smallest fragment (1,689 bp) detected after complete *Sma*I cleavage. (e) LMP probe obtained by cleavage of the *Eco*RI Dhet EBV fragment with *Mlu*I. The *Hpa*II-*Msp*I sites are indicated by vertical bars.

LMP-specific MAb S-12 (Fig. 3). Several polypeptides were detected in the 35- to 55-kilodalton size range with EBV-antibody-positive (Fig. 3 and 4) but not with EBV-negative sera (data not shown). Some of these are probably lytic-cycle-associated antigens. The regularly interspaced bands may also correspond to the EBNA-5 ladder detected by the JF186 MAb in B958 virus-infected cells (13). This possibility could not be confirmed, since the Rael virus-encoded EBNA-5 does not react with this MAb due to strain variation of the target epitope (13).

TABLE 1. EBNA-2, EA, and VCA induction in Rael cells

Treatment	% Cells ^a positive for:			
	EBNA	EBNA-2	EA	VCA
Untreated	100	0	0	0
TPA	100	0	<1	0
<i>N</i> -Butyrate	100	0	0	0
TPA + <i>N</i> -butyrate	100	0	<1	0
Teleocidin	100	0	0	0
5-AzaC	100	55	25	10
BUDR	100	0	<1	ND ^b

^a Rael cells were seeded at a concentration of 3×10^5 in complete medium or medium containing 10 ng of TPA per ml, 300 μ M *N*-butyrate, or a combination of the two, 10 ng of teleocidin per ml, 4 μ M 5-AzaC, and 50 μ g of BUDR per ml. After 72 h, the cells were counted and dead cells were removed by Ficoll-Isopaque centrifugation. Staining was performed as described in Materials and Methods. One representative experiment of three is indicated.

^b ND, Not determined.

Time course of EBV antigen induction. The results of one representative experiment are shown in Table 2 and Fig. 4. EBNA-2-positive cells were detected by anti-complement immunofluorescence staining just 12 h after the addition of 5-AzaC. The number of positive cells increased to 65% in 72 h and, in spite of massive cell death, remained high during the whole observation period (Table 2). EA-positive cells were detected only after 24 h, and their number did not increase with time.

Immunoblots of 5-AzaC-treated cells showed a similar time course for the induction of EBNA-2 through EBNA-6 and LMP (Fig. 4). After 48 h, the pattern of expression was similar to that observed in the LCL CBC-Rael generated by the same virus. The late appearance of EBNA-3, EBNA-4, and EBNA-6 suggested by Fig. 4 was not confirmed in repeated experiments and probably reflects variations in the efficiency of protein transfer or differences in antibody titers against the various EBNA components.

Correlation between EBNA-2 and EA-VCA induction. The results of eight separate experiments are presented in Table 3. EBNA-2 was detected in 39 to 72% of the cells (mean, 56 ± 4). EA was detected in 6 to 23% (mean, 19 ± 3), and VCA was detected in 8 to 18% (mean, 11 ± 2). The percentage of EA- and VCA-positive cells was lower than the frequency of EBNA-2-positive cells in each experiment. Moreover, EBNA-2 and EA appeared in different cells, as a rule. Double fluorescence staining demonstrated that between 70 and 100% of the EBNA-2-positive cells were EA negative. Conversely, between 50 and 100% of the EA-positive cells did not express EBNA-2.

EBV DNA methylation in untreated and 5-AzaC-treated cells. *Sma*I (Fig. 5)- and *Msp*I-*Hpa*II (Fig. 6)-digested DNA was hybridized to EBV DNA probes covering the control and coding regions of EBNA-2, *Bam*HI C, W, and Y, and the 5' end of *Bam*HI H, respectively (Fig. 5), or the coding region of LMP, *Eco*RI Dhet (Fig. 6). The coordinates of the probes, the relevant cleavage sites, and the positions of the relevant promoters based on the B95-8 sequence are shown in Fig. 1, a through e. These regions of EBV DNA were highly methylated in untreated Rael cells. As expected, 5-AzaC treatment lead to demethylation of CpG sites, reflected by the appearance of low-molecular-weight bands in the DNA from treated cells. All the bands corresponding to the major fragments predicted on the basis of the B95-8 sequence were detected in blots of DNA from 5-AzaC-treated Rael cells.

TABLE 2. Time course of EBNA-2 and EA induction

Antigen	% Cells ^a positive at:										
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	Day 4	Day 5	Day 6	Day 9
EBNA-2	0	0.5	0.5	20	40	60	65	70	60	75	90
EA	0	0	5	5	10	10	10	7	7	ND	ND
% Recovery	0	100	115	128	130	135	141	157	75	40	10

^a Rael cells were seeded at a concentration of 3×10^5 /ml in medium containing $4 \mu\text{M}$ 5-AzaC and were incubated at 37°C in a 5% CO_2 atmosphere. After 72 h and 6 days, the cells were reseeded at the original concentration in 5-AzaC-free medium. At the indicated time points, the cells were harvested, viable cells were separated by Ficoll-Isopaque centrifugation, and portions were used for fluorescence staining or for sodium dodecyl sulfate-gel electrophoresis (Fig. 4). One representative experiment out of three is indicated.

DISCUSSION

Six of the seven EBV-encoded antigens that are regularly detected in EBV-transformed LCLs of nonmalignant origin (EBNA 2 through EBNA-6 and LMP) are down regulated in EBV-carrying BL cells with a representative (group 1) phenotype (40, 41). The mechanism of this phenotype-dependent down regulation is not known. Our finding that EBNA-2 through EBNA-6 and LMP can be up regulated by treating a representative BL line (Rael) with the DNA demethylating agent 5-AzaC but not by a variety of other inducers is the first clue about the mechanism that may be involved.

The relationship between the antigen-inducing effect of the drug and its ability to induce DNA demethylation was confirmed by the finding that the 5' flanking region and the exons for EBNA-2 and LMP were highly methylated in Rael and became hypomethylated after 5-AzaC treatment. Elsewhere, we have shown that the same regions of EBV DNA were hypomethylated in LCLs, including the CBC-Rael-Sto line obtained by rescue of the Rael virus into cord blood cells (Ernberg et al., submitted).

The fact that 5-AzaC was the only inducer among several potential inducers that could trigger the appearance of EBNA-2 through EBNA-6 and LMP is noteworthy. The drug acts by direct inhibition of cytosine methylation caused by incorporation of the base analog modified at the 5' position (21). This results in genomewide demethylation of both cellular and viral DNA. It was found previously that

FIG. 2. Dose response to 5-AzaC. Cell viability (■) and percentage of EBNA-2 (▨)- and EA-positive (□) cells were monitored in Rael cells cultured for 3 days in medium containing different concentrations of 5-AzaC. Untreated cultures were more than 99% viable with no EA- or EBNA-2-positive cells. One representative experiment of three is indicated.

5-AzaC can induce the lytic cycle in a variety of EBV-carrying cell lines (2). Other agents that are known to induce the lytic cycle, such as TPA, *N*-butyrate, teleocidin, and BUdR (8, 42, 45) were also found to induce cellular and viral DNA demethylation. With TPA and *N*-butyrate, Szyf et al. (45) showed that, in spite of their capacity to induce EA, these agents were unable to induce hypomethylation in certain cell lines, like Raji. When induced, hypomethylation occurred late and was associated with a period of arrest in DNA replication. They suggested that, in contrast to 5-AzaC, these inducers trigger a cellular mechanism for DNA hypomethylation and their effect may be, therefore, determined by the state of cell differentiation.

FIG. 3. Induction of EBNA-2 through EBNA-6 and LMP in Rael cells. Total cell extracts were electrophoresed through a 7.5% sodium dodecyl sulfate-polyacrylamide gel with a 3.5% top gel. After transfer to nitrocellulose filters, the blots were probed with a polyvalent human serum obtained from a healthy EBV seropositive donor or with the anti-LMP monoclonal antibody S-12. The molecular size standards in kilodaltons were myosin (200), β -galactosidase (116.3), phosphorylase B (92.4), bovine serum albumin (66.2), and ovalbumin (42.7). The identification of the different EBV antigens was based on comparison with previously characterized cell lines and published data (22). The EBNA-3, EBNA-4, and EBNA-6 bands that are faint in the photograph were clearly detected in the blot.

FIG. 4. Time kinetics of EBNA-2 through EBNA-6 and LMP induction. Rael cells were cultured in medium containing 4 μ M 5-AzaC. After 72 h, the cells were transferred to 5-AzaC-free medium and the concentration was readjusted to 3×10^5 cells per ml. At the indicated time points, 5×10^6 cells were collected and processed as described. Immunoblots were probed with the OR serum or with the anti-LMP monoclonal antibody S-12. The LCL CBC-Rael-Sto, obtained by transforming cord blood lymphocytes with the Rael virus, was included for comparison. The equal intensity of the 72-kilodalton double band detected by the EBV-positive human serum in all samples confirms that the same protein concentration was loaded in each slot. The different mobility of this signal compared with the blot shown in Fig. 3 is caused by the different length of the run and slight difference in the cross-linking of the gel. The percentages of EA- and EBNA-2-positive cells determined at each time point are shown in Table 2.

We have found that TPA and BUdR could induce only low levels of EA in Rael cells. This is in line with the previously reported resistance of Rael cells to BUdR treatment and P3RH1 superinfection (23, 24). In contrast, 5-AzaC-treated cells expressed not only EBNA-2 through EBNA-6 and LMP but also EA and VCA. The EBNA-2- and EA-positive populations were quite distinct, however. Thus, induction of EBNA-2 through EBNA-6 and LMP was not, or not necessarily, followed by entry into the lytic cycle. Conversely, EBNA-2 synthesis was not required for EA induction. This implies that the 5-AzaC-treated cells may choose between two distinct pathways of EBV antigen expression. The expression of EBNA-2 appears to be linked to B cell activation, including the induction of B-cell growth factor receptors and B-cell growth factor production (14, 44, 50). This may facilitate the expansion of virus-carrying B blasts. It may not be required, however, to initiate the cascade of nonstructural and structural antigens that leads to virus production; it may even counteract it. It is likely that EBV, similar to other viruses that have adapted to dual modes of life, latency, and lytic cycle, has distinct sets of genes specialized for one or the other. The expression of these genes is probably regulated by host cell-specific interactions. Ricksten et al. (A. Ricksten, A. Olsson, T. Andersson, and

TABLE 3. Correlation between EBNA-2 and lytic cycle induction^a

Experiment	% Cells positive for:					
	EBNA-2	EA	VCA	EBNA-2 ⁺ /EA ⁺ ^b	EBNA-2 ⁺ /EA ⁻ ^c	EA ⁺ /EBNA-2 ⁻ ^c
1	72	10	10	0	100	100
2	55	10	10	5	91	50
3	51	24	18	ND ^d	ND	ND
4	52	32	ND	16	70	50
5	57	21	7	ND	ND	ND
6	39	29	ND	7	83	73
7	65	6	ND	0	100	100
8	55	18	8	ND	ND	ND

Mean $\pm$ SE 56 $\pm$ 4 19 $\pm$ 3 11 $\pm$ 2

^a Rael cells were cultured for 3 days in the presence of 4 μ M 5-azaC. Untreated Rael cells were EBNA-2- and EA-negative.

^b Double staining was performed in two steps: (i) EBNA-2 staining with fluorescein isocyanate-conjugated anti-C' antibodies and (ii) EA staining with rhodamine-conjugated BL serum.

^c The percentages of EBNA-2⁺-EA⁻ and EA⁺-EBNA-2⁻ cells were calculated from the percentages of EBNA-2⁺, EA⁺, and double-fluorescent cells by the formula: 1 - (percent double fluorescent cells/percent single fluorescent cells) $\times$ 100.

^d ND. Not determined.

L. Rymo, Nucleic Acids Res., in press) have recently shown that the EBNA-2 gene is under the control of a tissue-specific enhancer, located in *Bam*HI W. Nuclear extracts from the BL line DG75 and from HeLa cells showed a different binding pattern to the enhancer region. Thus, the

FIG. 5. Methylation pattern of the control and coding regions for EBNA-2. DNA from untreated and 5-AzaC-treated (4 μ M for 3 days) Rael cells was digested with 5 U of *Bam*HI per μ g of DNA and then with 10 U of *Sma*I per μ g of DNA. Cleaved DNA was electrophoresed in a 1.5% agarose gel and transferred to nylon filters as described. The blots were hybridized with ³²P-labeled EBV DNA probes covering the *Bam*HI C, W, and Y regions and part of the EBNA-2 exon as described in Fig. 1, a through d. The bars indicate the position and size (in kilobases) of each major fragment based on comparison with molecular weight markers (*Hind*III cleaved lambda DNA and a 1-kb ladder, Bethesda Research Laboratories). Maps are given in Fig. 1a through d.

FIG. 6. Methylation pattern of the LMP coding region. DNA from untreated and 5-AzaC-treated Rael cells were digested with excess *HpaII* or *MspI*. Cleaved DNA was electrophoresed in a 1.5% agarose gel, transferred to nylon filters, and hybridized to the LMP probe described in Fig. 1e. Bars and numbers indicate the positions and sizes of two of the major fragments.

expression of EBNA-2 may be regulated by cellular factors present only in B cells.

Our findings indicate that methylation of EBV DNA may provide a further mechanism for the control of transformation-associated genes in B cells at different stages of activation or differentiation. BL cells resemble phenotypically the CALLA-positive, BLA-positive, and activation marker-negative subpopulations of the germinal centers (16). They differ from activated immunoblasts in numerous aspects. In addition to down regulation of EBNA-2 through EBNA-6 and LMP, they also show selective down regulation of major histocompatibility complex class 1 antigens and of cell adhesion molecules (15, 30). We have previously proposed that the BL cell may be phenotypically representative of its normal precursor (30). If so, the down regulation of EBNA-2 through EBNA-6 and LMP in BL cells may reflect a specific aspect of the viral strategy in the corresponding normal cell. Blood and tonsil B lymphocytes from healthy EBV-seropositive donors have been shown to harbor the virus. The low-density B cell fraction contains cells that can release transforming virus *in vitro*, while the high-density fraction contains small resting B lymphocytes that are capable of direct outgrowth into EBV-carrying LCLs (27). It is tempting to speculate that the EBV gene expression of these cells may be similar to that of BL cells. Down regulation of all viral antigens except EBNA-1 might provide a distinct advantage for the virus, since it could minimize the risk for immune rejection and could favor prolonged viral latency.

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