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Host Cell-Dependent Regulation of Growth Transformation-Associated Epstein-Barr Virus Antigens in Somatic Cell Hybrids

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We have analyzed the expression of the three major known growth transformation-associated Epstein-Barr virus (EBV) proteins, EBNA-1, EBNA-2, and latent membrane protein (LMP), in a series of somatic cell hybrids derived from the fusion of EBV-carrying Burkitt lymphoma (BL) lines with EBV-positive or EBV-negative B-cell lines. Independently of the cell phenotype, EBNA-1 was invariably coexpressed in all EBV-carrying hybrids. In hybrids between EBV-carrying, LMP-positive and LMP-negative Burkitt lymphoma lines, LMP was expressed, indicating positive control. Two EBV-negative lymphoma lines, Ramos and BJAB, differed in their ability to express LMP after B95-8 virus-induced conversion and after hybridization with Raji cells. BJAB was permissive while Ramos was nonpermissive for LMP, although both expressed EBNA-2. The EBNA-2-deleted P3HR-1 virus gave the same pattern of LMP expression in these two cells. Our findings indicate that the expression of EBNA-1, EBNA-2, and LMP is regulated by independent mechanisms.

Epstein-Barr virus (EBV) infection of normal B lymphocytes leads preferentially to a nonlytic interaction reflected by the emergence of permanent EBV-carrying lymphoblastoid cell lines (LCLs). This transformation is apparently due to the coordinated action of a limited set of virally encoded proteins that are constitutively expressed in all LCLs. These include a family of at least six nuclear antigens (EBNAs) (6, 7, 16, 19, 33, 35, 39), and one membrane protein (LMP) (14, 41).

There is now some information on the functional properties of the three best characterized antigens, EBNA-1, EBNA-2, and LMP. EBNA-1, encoded by a transcript from the *Bam*HI K region of the viral genome (15, 40), binds to the viral Ori-P region in *Bam*HI C (34). It has been suggested that this binding is essential for the maintenance of the EBV episomes and for the control of viral replication (46). EBNA-2, encoded by the *Bam*HI WYH region of the viral genome (16), may play a role in primary B-cell activation since EBNA-2-defective viruses carried by the Burkitt lymphoma (BL) lines P3HR-1 or Daudi are unable to activate B cells into the cycle (2, 21) and cannot immortalize them (5, 18). They can still convert already established EBV-negative BL lines to a permanent EBV carrier state (22, 23). EBNA-2-carrying (but not EBNA-2-defective) viral substrains can induce the expression of B-cell activation markers in EBV-negative BL lines. In some lines, this could be also achieved by transfection with the isolated EBNA-2 gene (45). LMP has a membrane-transport-protein-like structure (10) and was found to transform some established rodent fibroblast lines (1, 44). Both EBNA-2 and LMP may serve as targets for T-cell-mediated cytotoxic reactions against EBV-transformed cells (28, 29, 42).

Prior to the dissection of the EBNA family into its individual protein components and the detection of LMP, it was taken for granted that the three known EBV-carrying virus nonproducer cell prototypes, LCL, BL, and nasopharyngeal carcinoma (NPC), express the same set of growth transformation-associated proteins. The main watershed of EBV antigen expression was seen at the borderline between the growth transformation-associated and the lytic-cycle-associated antigens. The leakiness of the nonlytic interaction differs between B-cell lines of different origins, however, suggesting a difference in host-cell-dependent control. Cord-blood-cell-derived lines are generally less permissive than LCLs of adult origin transformed by the same virus (9). Marmoset lines are more permissive than human lines. Hybridization of virus producer cells with cells of non-B lineages eclipsed the inducibility of the viral cycle (24).

Recently it was shown that the three main EBV-carrying cellular prototypes, LCL, BL, and NPC, differ in their EBV gene expression. All three express EBNA-1. EBNA-2 is regularly present in LCLs but not in BL line biopsies or in phenotypically representative BL lines (37). It is not expressed in NPC (9a). LMP is also down-regulated in BLs (40), but it is expressed in about half of the NPC biopsies (9a).

Rescue of EBV from BL (36) (I. Ernberg, K. Falk, J. Minarovits, M. G. Masucci, and G. Klein, submitted for publication) or NPC (9a) by cocultivation with normal B lymphocytes leads to full expression of EBNA-2 and LMP in the derived LCLs, indicating that their absence from the donor cells is due to cellular regulatory mechanisms. This is also consistent with the fact that EBNA-2 and LMP usually appear during the serial propagation of EBV-carrying BL lines in vitro, in parallel with the phenotypic drift towards a more LCL-like cell, as indicated by the appearance of activation markers (37).

Analysis of the expression of EBV genes in virus nonproducer cells may contribute to the understanding of viral latency and EBV-associated viral transformation. Expression patterns in appropriate somatic hybrids may indicate the existence of positive as well as negative controls (12, 24, 31, 47).

In an attempt to define some of the controls that regulate EBNA-1, EBNA-2, and LMP in cells of different phenotypes, we have analyzed their expression in somatic hybrids derived from the fusion of different EBV-carrying and EBV-negative lines. Our findings show that the three antigens are independently regulated.

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TABLE 1. Expression of EBNA-1, EBNA-2, and LMP in hybrids derived from EBV-positive BL lines

Cell line (reference)	Derivation	Mol wt of indicated proteins[a]		
		EBNA-1	EBNA-2	LMP
Parent				
Raji (33)		72	98	58
Namalwa (3)		84	98	Neg[b]
P3HR-1 (17)		80	Neg	62
Daudi (23)		82	Neg	Neg
Hybrid				
RN-1 (29)	Raji × Namalwa	72, 84	98	58
RN-2 (29)	Raji × Namalwa	72, 84	98	58
RN-3 (29)	Raji × Namalwa	72, 84	98	58
Rudput (46)	Raji × P3HR-1	72, 80	98	58, 62
Rudpicat (46)	Raji × P3HR-1	72, 80	98	58, 62
8A (20)	Raji × Daudi	72, 82	98	58
Ditrud (46)	Raji × Daudi	72, 82	98	58
Namdut (38)	Daudi × Namalwa	82, 84	98	Neg
Namput (38)	P3HR-1 × Namalwa	80, 84	98	62

[a] Molecular weight in kilodaltons of EBV antigens detected by probing immunoblots of total cell extracts with EBV-antibody-positive human sera or with the anti-LMP monoclonal antibody S12.
[b] Neg, Negative.

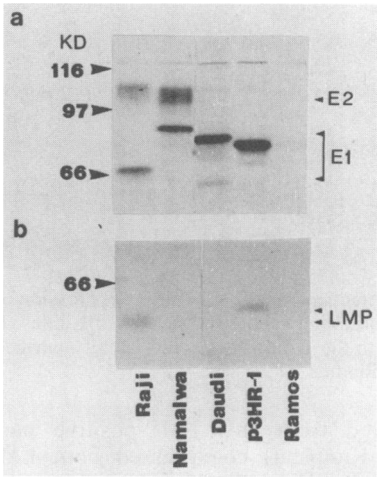

FIG. 1. Gel electrophoresis showing expression of EBNA-1, EBNA-2, and LMP in EBV-positive BL lines. Immunoblots of total cell extracts were probed with PG serum from an EBV-positive CLL patient (a) or anti-LMP monoclonal antibody S-12 (b).

MATERIALS AND METHODS

Culture medium. All cell lines were grown in 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.

Cell lines and somatic cell hybrids. Cell lines and somatic cell hybrids used in this study are listed in Table 1. All hybrids were established in our laboratory in the course of previous studies.

Sera. A sample of previously characterized serum, PG, from an EBV-positive patient with chronic lymphocytic leukemia with viral capsid antigen, diffuse component of early antigen, and EBNA titers of 1:1,280, 1:640, and >1:320, respectively, was used as the source of antibodies to detect EBNA-1 and EBNA-2. Affinity-purified rabbit anti-107 peptide antibodies (8) were also used for the specific detection of EBNA-1. LMP was detected by the S-12 monoclonal antibody (26) kindly provided by D. Thorley-Lawson.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). Cells (20×10^6 to 50×10^6) were dissolved in 1 to 2.5 ml of electrophoresis sample buffer. Proteins were separated by discontinuous gel electrophoresis according to the method of Laemmli (25) and blotted onto nitrocellulose paper (No. 401196; Schleicher & Schuell, Inc.) as previously described (43). The efficiency of the protein transfer and the positions of the molecular weight markers were visualized by staining the filters with Ponceau S (Sigma Chemical Co.). Excess protein binding sites were blocked by incubation of the blotted filters for 1 h in phosphate-buffered saline containing 5% dried skimmed milk. The filters were subsequently incubated with the specific antibodies diluted in phosphate-buffered saline containing 5% dried skimmed milk for 2 h at room temperature or 16 h at 4°C. The human serum was used at a 1:20 dilution, the affinity-purified anti-107 rabbit antibodies were diluted 1:100, and the S-12 monoclonal antibody was diluted 1:1,000. After incubation with the primary antibody, the filters were washed in phosphate-buffered saline containing 5% dried skimmed milk and subsequently incubated with the appropriate alkaline-phosphatase-conjugated second antibody (Sigma) for 2 h at room temperature. After three washes in balanced salt solution containing 0.5% Tween, the specifically bound second antibody was detected by fast red salt precipitation in the presence of α-naphthyl phosphate (Sigma).

RESULTS

Expression of EBNA-1, EBNA-2, and LMP in the EBV-carrying parental lines. Figure 1a shows EBNA-1 and EBNA-2 expression of the four EBV-positive parental BL lines. EBNA-1 of the expected size was detected in all four lines with the EBV-positive human serum sample, PG, and corroborated with other characterized EBV-positive human sera (OR and KF) (data not shown). EBNA-2 was also detected in Raji and Namalwa as a band of approximately 98 kilodaltons but not in Daudi and P3HR-1, which are known to carry EBNA-2-deleted viral substrains. LMP was detected as an approximately 58-kilodalton band in Raji and a 62-kilodalton band in P3HR-1 (Fig. 1b). Daudi and Namalwa were LMP negative.

The drug-resistant parental sublines of Raji, P3HR-1, and Daudi showed the same pattern of EBNA-1, EBNA-2, and LMP expression as in the original lines (data not shown).

Expression of EBNA-1, EBNA-2, and LMP in cell hybrids. (i) Hybrids between EBV-positive BL lines. The results obtained with nine cell hybrids derived from the fusion of the Raji, Namalwa, P3HR-1, and Daudi lines are summarized in Table 1. EBNA-1 of both parents was coexpressed in the hybrids (Fig. 2), confirming the earlier report by Gergely et al. (11). Hybrids between the EBNA-2-defective P3HR-1 or Daudi and the EBNA-2-positive Raji or Namalwa expressed EBNA-2 (Fig. 3a). This is an example of genetic complementation. The level of expression of EBNA-2 varies among the Namalwa hybrids (Namput and Namdut) (Fig. 3a). Our panel does not contain hybrids derived from the fusion of cells with an intact but down-regulated EBNA-2 gene and an EBNA-2-positive line. The dominant relationships of the host-cell-dependent down-regulation of EBNA-2 remain to be examined on hybrids specially constructed for this purpose. More informative data are available on LMP. The

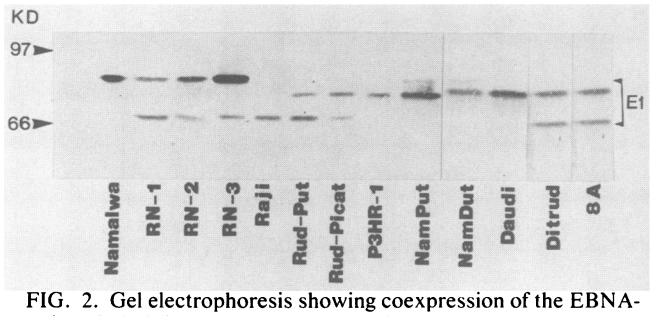

FIG. 2. Gel electrophoresis showing coexpression of the EBNA-1 antigen in hybrids between EBV-positive BL lines. Immunoblots were probed with the EBNA-1-specific, affinity-purified rabbit anti-107 peptide antibodies.

hybrids derived from two LMP-positive parental lines (Rudput and Rudpicat) coexpressed both LMP proteins almost identical in size (Fig. 3b). The LMP expression of Raji is dominant over the nonexpression of Daudi (8A and Ditrud). Although Daudi does not express LMP, the protein can be induced by sodium butyrate (B. Contreras-Salazar, B. Ehlin-Henriksson, M. G. Masucci, and G. Klein, manuscript in preparation). The Namalwa line is known to carry an intact LMP gene (13). Therefore, we cannot exclude the possibility that the failure to detect LMP in the Namalwa-derived hybrids is caused by antigenic variation. The combined findings on the Daudi-Raji and Namalwa-P3HR-1 hybrids suggest that LMP expression is under positive control.

(ii) Hybrids between EBV-positive BL lines and EBV-negative B-lymphoma lines. EBNA-1 and EBNA-2 were expressed in the hybrids derived from the fusion of the EBV-positive BL lines Raji and P3HR-1 with the EBV-negative BL line Ramos and with the B-lymphoma line BJAB (Table 2; Fig. 4a). In contrast, LMP was expressed in the Raji-BJAB hybrid (designated 83) but not in the Ramos-Raji and Ramos-P3HR-1 hybrids (Ratramud and Ramput) (Fig. 4b). Thus, BJAB and Ramos introduce a permissive and a nonpermissive environment, respectively, for LMP independently of EBNA-1 and EBNA-2 expression.

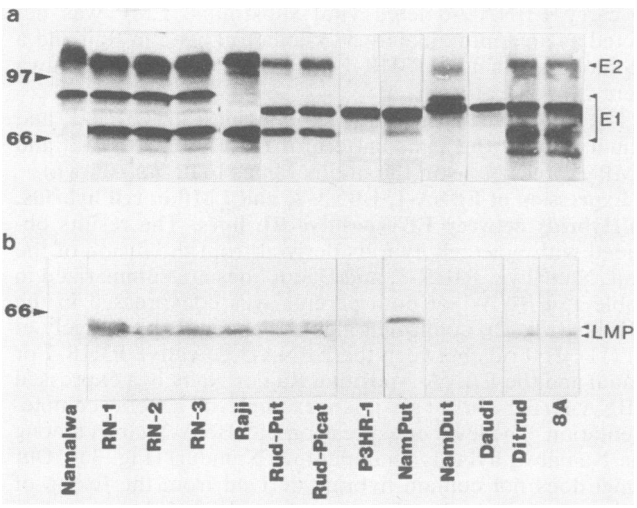

FIG. 3. Gel electrophoresis showing dominant expression of EBNA-2 and LMP in hybrids between EBV-positive BL lines. Immunoblots were probed with the EBV-positive human serum PG with high titers of anti-EBNA-2 (a) or the anti-LMP monoclonal antibody S-12 (b).

TABLE 2. Expression of EBNA-1, EBNA-2, and LMP in hybrids derived by fusion of EBV-positive BL lines and EBV-negative B-lymphoma lines

Cell line (reference)	Derivation	Mol wt[a] of indicated proteins		
		EBNA-1	EBNA-2	LMP
Parent				
Raji (33)		72	98	58
P3HR-1 (17)		80	Neg	62
Ramos (27)		Neg	Neg	Neg
BJAB (32)		Neg	Neg	Neg
Hybrid				
83 (20)	Raji × BJAB	72	98	58
Ratramud (37)	Raji × Ramos	72	98	Neg
Ramput (38)	P3HR-1 × Ramos	80	Neg	Neg
Converted sublines				
Ramos/E95-M (22)[b]	B95-8 converted	82	100	Neg
BJAB/B95-8 (4)	B95-8 converted	82	100	62
Ramos/HR1-K (22)[c]	P3HR-1 converted	80	Neg	Neg
BJAB/HR1-K (4)	P3HR-1 converted	80	Neg	62

[a] Molecular weight in kilodaltons. Neg, Negative.
[b] Five different B95-8-virus-converted Ramos sublines were tested.
[c] Five different P3HR-1-virus-converted Ramos sublines were tested.

An analogous difference was noticed in the corresponding EBV-converted sublines. Both the B95-8 and the P3HR-1 virus-converted BJAB sublines expressed LMP, whereas the corresponding Ramos convertants were LMP negative (Fig. 4).

DISCUSSION

It was recently shown that EBV-carrying tumor cell lines (BL and NPC) only express part of the EBV-encoded antigens present in LCLs of normal origin (9a, 35). When the

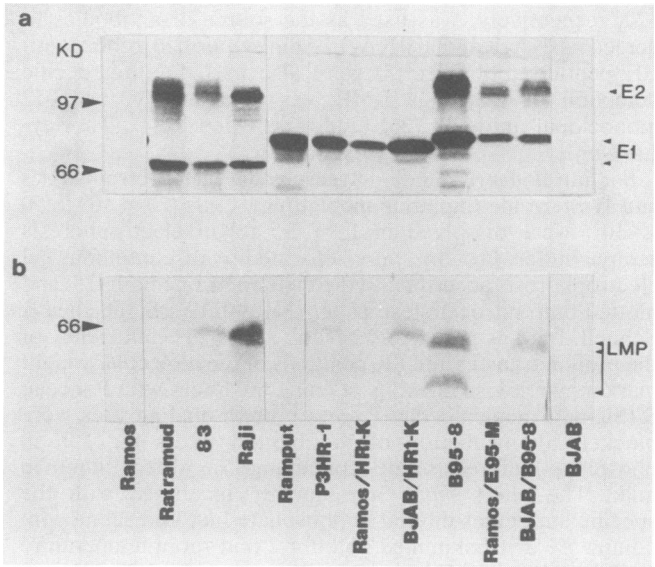

FIG. 4. Gel electrophoresis showing expression of EBNA-1, EBNA-2, and LMP in hybrids between EBV-positive BL and EBV-negative B-cell lines, and in vitro B95-8- and P3HR-1-converted lines. Four additional B95-8-converted sublines of Ramos showed the same pattern of EBV antigen expression as that of Ramos/E95-M. For more information, see Fig. 3 legend.

tumor-cell-associated virus is rescued into B cells, the derived LCLs express the complete antigen pattern characteristic of other LCLs (9a, 36, 37; Ernberg et al., submitted). This indicates that the expression of certain viral genes depends on the cellular phenotype. Our somatic hybrid studies represent a first step towards the analysis of this regulation.

EBNA-1 is invariably expressed in all EBV-carrying cells, indicating that it is insensitive to host-cell-type-dependent controls. This was reaffirmed by the somatic hybrid studies. In B and B-cell hybrids, EBNA-1 was codominantly expressed as expected (11). In most cases, the EBNA-1 proteins were expressed at the same level in the hybrids as in each corresponding parental line. The two hybrids derived by the fusion of Namalwa with P3HR-1 and Daudi were exceptional in this respect, however. In Namput and Namdut, the Namalwa EBNA-1 was underrepresented. There was also a lower expression of EBNA-2, particularly in the Namdut hybrid. Namalwa contains only two copies of the EBV genome integrated into chromosome 1 (13). The apparent down-regulation of the Namalwa-derived EBV products may therefore reflect a gene dosage effect. Hybrids between EBNA-2-defective and EBNA-2-carrying BL lines showed simple genetic complementation. The question of whether the phenotypic down-regulation of EBNA-2 in a type I BL line would be released after fusion with an EBNA-2-permissive cell or imposed on the partner could not be studied, because of the lack of suitable hybrid combinations.

The most interesting information was obtained from the study of LMP expression. We could not confirm the recent suggestion that LMP synthesis was dependent on EBNA-2 expression (30). The independent control of the two antigens was reaffirmed in several ways. The EBV-negative Ramos line was permissive for EBNA-2 after infection with B95-8 virus, but it did not express LMP. This was consistent with the fact that the Raji × Ramos hybrid expressed the Raji-derived EBNA-2 but not LMP. Both antigens are expressed in the Raji parent. In contrast to Ramos, the EBV-negative BJAB lymphoma line was permissive for EBNA-2 and LMP, both after EBV conversion and after hybridization with Raji. It is particularly noteworthy that the P3HR-1-virus-converted BJAB was defective for EBNA-2 but nevertheless expressed LMP, providing additional evidence for the independent control of the two antigens.

The negative influence of Ramos on LMP expression is in line with our previous findings on other EBV-negative BL lines. LMP was expressed at either a very low level or not at all in B95-8-virus-converted sublines of BL28 and BL41 (27). Only one of four EBV-converted BL41 sublines, BL41/95, expressed LMP at a similar level as that of the LCLs. This line had also undergone the most pronounced LCL-like phenotypic shift (42a).

While these findings indicate that EBV-negative BLs can exert a negative control on LMP expression, the hybrids between the LMP-expressing P3HR-1 and Raji cells and the EBV-carrying but LMP-negative BL line Daudi indicate that positive controls exist as well. LMP was expressed in all four hybrid combinations.

It is conceivable that Ramos and other EBV-negative BL lines have maintained a negative regulatory factor that characterizes typical BL lines, whereas their EBV-carrying counterparts may have relaxed this negative regulation, but differ with regard to the activity of a positive regulatory factor.

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