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Different Activation of Epstein-Barr Virus Immediate-Early and Early Genes in Burkitt Lymphoma Cells and Lymphoblastoid Cell Lines

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Specific expression of the Epstein-Barr virus (EBV) immediate-early and early gene products Zta, Rta, I'ta, and MSta by a recombinant vaccinia virus system allowed us to analyze the first steps in the induction of the lytic cycle in EBV-infected Burkitt lymphoma (BL) cells and lymphoblastoid cell lines (LCLs). Significant differences in the induction of early genes were found between these cell types: whereas in BL cells the *trans* activator Zta was found to induce key steps of the early lytic cycle, only minor activities of Zta were noted in LCLs. Contrary to Zta, the *trans* activator Rta was found to be highly effective in LCLs. These observations suggest that Rta may play an important role in the activation of the early lytic cycle in LCLs, although it cannot be activated by Zta. The latter may be a reason for the lower tendency of LCLs to switch into the lytic cycle compared with BL cells or differentiated epithelial cells.

Epstein-Barr virus (EBV) infects human B lymphocytes and certain epithelial cells. Infection of B lymphocytes is predominantly latent; the virus persists lifelong in a small number of these cells which are regarded as the reservoir of the virus. In epithelial cells, on the other hand, the lytic cycle is frequently induced, leading to the production of progeny virus (1, 31). Viral latency in B lymphocytes can be disrupted in vitro by chemical agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (36) and by cross-linking of immunoglobulin (29) on the surface of latently infected cells. The switch from latency to lysis is associated with the expression of the immediate-early *trans* activators Zta (also termed Z, ZEBRA, or EB1), encoded by the open reading frame BZLF1, Rta (or R), encoded by BRLF1, and I'ta, derived from BI'LF4 (2, 22). Zta is a member of the AP-1 family of transcription activators (4, 7); Rta was shown to be an enhancer-binding factor (14). Zta and Rta induce the expression of proteins of the early group such as the MSta protein (also termed MS-EA or EB2), encoded by the open reading frame BSLF2/BMLF1, and the p138 derived from BALF2 (3, 5, 14, 34). These early events lead to a cascade of expression of about 80 viral genes, viral DNA replication, and finally the release of progeny virions and cell death (13).

Induction of the lytic cycle followed by virus production seems to be a rare event in B lymphocytes in vivo. This may be due to immunological surveillance mechanisms by which cells entering the lytic cycle are killed before progeny viruses are produced; however, disruption of latency in these cells may also be impeded by cell-specific genetic mechanisms.

Most of our knowledge about the early events during the lytic cycle stems from experiments with Burkitt lymphoma (BL) cells. However, it is doubtful whether these malignant cells are comparable to the normal resting B cells harboring the virus in healthy individuals. The initial events in the induction of the lytic cycle may differ in the latter, as a different cellular environment may well influence the activation of viral genes. Recently, differences in early activation steps between epithelial cells and BL cells were demonstrated (33).

In this investigation, we therefore compared the key steps in the induction of the lytic cycle in BL cells and EBV-positive lymphoblastoid B-cell lines (LCLs), which because of their nonmalignant state may resemble the normal EBV-infected B lymphocytes more closely. We concentrated our analysis on the very early events characterized by the expression of the Zta, Rta, and I'ta proteins. Recombinant vaccinia viruses containing the genes coding for Zta, Rta, I'ta, and MSta were used to express these proteins in different BL and lymphoblastoid cell lines and to study the influence of each of these proteins on the expression of the other immediate-early *trans* activators and the early proteins MSta and p138 encoded by BALF2. The feasibility of such an approach for gene regulation studies was recently demonstrated by Zhu et al. working with immediate-early *trans* activators of herpes simplex virus (35). Our study showed considerable differences in the early induction pathways between LCLs and BL cells.

Expression of Zta, Rta, I'ta, and MSta. We inserted the coding sequences of the EBV *trans* activators Zta, Rta, I'ta, and MSta in vaccinia viruses under the control of the early/late 7.5K promoter, using the protocol of Mackett and coworkers (18). Coding regions of the reading frames BZLF1, BRLF1, BI'LF4, and BSLF2/BMLF1 were cloned in the correct orientation in the plasmid construct pAvB (30) adjacent to the 7.5K promoter, flanked on either side by vaccinia virus thymidine kinase (TK) sequences. CV1 cells infected with wild-type vaccinia strain WR were transfected with the plasmid construct by the $CaPO_4$ method (10). Homogeneous recombination gave rise to TK-negative recombinants, which were selected by 5-bromodeoxyuridine in TK-negative B143 cells (19). Cells infected with these recombinant viruses were assayed for foreign gene expression by Western blotting (immunoblotting). Virus was cloned twice prior to large-scale preparations of virus stocks by serial passaging on CV1 cells. The recombinant vaccinia virus strains expressing Zta, Rta, I'ta, and MSta were called Z-Vac, R-Vac, I'-Vac, and M-Vac, respectively. Infec-

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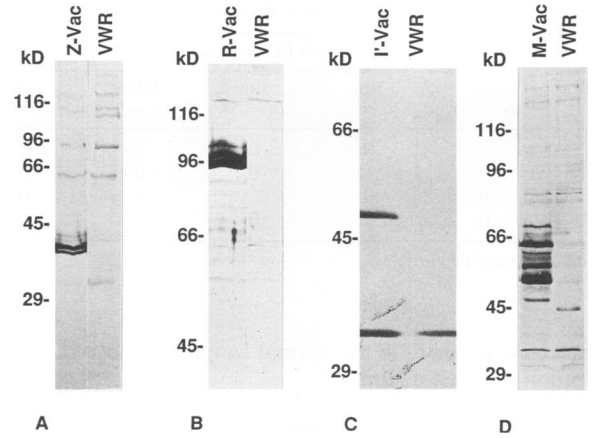

FIG. 1. Western blots demonstrating expression of the EBV *trans* activator proteins Zta, Rta, I'ta, and MSta by infection of CV1 cells with Z-Vac (A), R-Vac (B), I'-Vac (C), and M-Vac (D). Vaccinia virus wild-type strain WR-infected CV1 cells served as a negative control (VWR). Zta has a size of 35 kDa (A), Rta expression results in a band of 94 kDa (B), and I'ta is a protein of 48 kDa (C). MSta expression results in a pattern of different posttranslationally modified forms with molecular sizes of between 42 and 66 kDa (D), as described by Wong and Levine (32). Polyclonal rabbit antisera directed against Zta, Rta, I'ta, and MSta are described elsewhere (21, 22).

tion of various cell lines with the recombinant vaccinia viruses resulted in an efficient expression of the different *trans* activator proteins (Fig. 1).

LCLs obtained by immortalization of B lymphocytes with EBV strain B 95-8 and EBV-positive BL cell lines Akata (29), Eli (27), Jijoye (12), Daudi (16), P3HR-1 (12), and Raji (25) were infected with one of these recombinants at a time (2×10^6 cells at a multiplicity of infection of 5 in a 100-μl volume). Virus adsorption was allowed for 1 h at 37°C, and then 2 ml of RPMI 1640 containing 10% fetal calf serum was added. EBV gene expression was analyzed after 2 days of incubation at 37°C in 10% CO_2. The infected cells were analyzed for the expression of the immediate-early proteins Zta, Rta, and I'ta and of the early gene products p138 and MSta by Western blotting. Cells were solubilized, and an equivalent of 6×10^5 cells was loaded into one lane of a 15 or 17% sodium dodecyl sulfate-polyacrylamide gel (17). Western blotting was performed as described elsewhere (21). No significant differences in the level of expression between LCLs and BL cells were found in parallel experiments.

Gene expression in BL cells. As expected, in BL cell lines, the Zta protein showed a dominating influence on the expression of other transactivators and of p138. In all BL lines studied, infection with the recombinant vaccinia virus Z-Vac led to high expression of the *trans* activators Rta (Fig. 2A and B) and MSta (Fig. 3A and B) and significantly increased the expression of p138 (Fig. 4A and B) in four cell lines from low basal expression. No clear effect on p138 was observed in the cell line P3HR-1, which constitutively expresses this protein at a high level, and in the Raji line, which has a deletion of the BALF2 gene coding for p138 (data not shown). In none of the BL lines could induction of the I'ta protein by Zta be seen. Infection of BL cells with R-Vac, M-Vac, or I'-Vac showed only minor or no influence on the expression of other EBV proteins. Low amounts of MSta protein were expressed in P3HR-1 cells infected with R-Vac (not shown); no other effects of Rta, MSta, and I'ta on the different *trans* activators or p138 were observed in the six BL lines studied (Fig. 2A and B, 3A and B, and 4A and B).

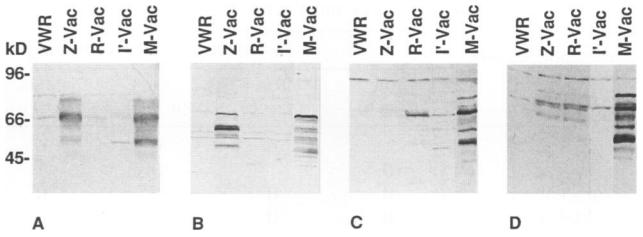

FIG. 3. Expression of MSta in BL lines and LCLs. BL lines Jijoye (A) and Eli (B) and LCLs derived from two donors (C and D) were infected with the recombinant vaccinia virus constructs Z-Vac, R-Vac, I'-Vac, and M-Vac and with vaccinia virus wild-type strain WR (VWR). Blots were stained with an MSta-specific polyclonal rabbit antiserum (21).

Gene expression in LCLs. A clearly distinct induction pattern was seen when EBV-transformed LCLs were infected with the recombinant vaccinia virus constructs. In these cells, the influence of Zta was considerably lower. Only the MSta protein was weakly expressed in two of five LCLs infected with Z-Vac (Fig. 3D), whereas none of the other transactivators nor p138 could be detected in these cells. On the other hand, Rta, which had only a marginal effect on the expression of EBV proteins in BL cells (see above), led to the expression of MSta in four of five LCLs and of p138 in three of five LCLs (Fig. 2C and D, 3C and D, and 4C and D). No clear effect on p138 expression was demonstrable in two lines, as these cells already expressed this protein at a high level (data not shown). The transactivators I'ta and MSta behaved similarly as in BL cells:

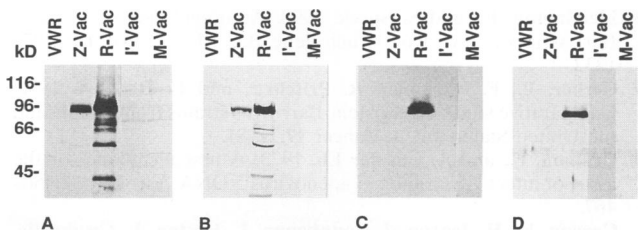

FIG. 2. Expression of Rta in BL lines and LCLs. BL lines Jijoye (A) and Eli (B) and LCLs derived from two donors (C and D) were infected with the recombinant vaccinia virus constructs Z-Vac, R-Vac, I'-Vac, and M-Vac and with vaccinia virus wild-type strain WR (VWR). Blots were stained with an Rta-specific polyclonal rabbit antiserum (21).

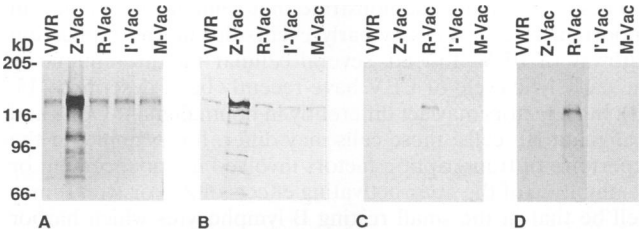

FIG. 4. Expression of p138 in BL lines and LCLs. BL lines Jijoye (A) and Eli (B) and LCLs derived from two donors (C and D) were infected with the recombinant vaccinia virus constructs Z-Vac, R-Vac, I'-Vac, and M-Vac and with vaccinia virus wild-type strain WR (VWR). Blots were stained with a monoclonal antibody reactive against p138 (24).

TABLE 1. EBV early lytic cycle activation steps observed following infection with recombinant vaccinia viruses

Recombinant vaccinia virus used for infection	No. of BL cell lines[a] expressing:					No. of LCLs expressing:				
	Zta (n = 6)	Rta (n = 6)	I'ta (n = 6)	MSta (n = 6)	p138 (n = 4)	Zta (n = 5)	Rta (n = 5)	I'ta (n = 5)	MSta (n = 5)	p138 (n = 3)
Z-Vac		6	0	6	4		0	0	2	0
R-Vac	0		0	1	0	0		0	4	3
I'-Vac	0	0		0	0	0	0		1	0
M-Vac	0	0	0		0	0	0	0		0

[a] Number of cell lines in which the respective protein was induced by the indicated recombinant vaccinia virus.

no activation of any other EBV protein was observed in LCLs infected with M-Vac, whereas in one of five LCLs infected with I'-Vac, induction of MSta protein was found (Fig. 3C). Table 1 summarizes the results obtained in BL lines and LCLs.

The immediate-early gene products Zta, Rta, and I'ta are known to induce the expression of early gene products such as MSta and p138 (3, 5, 14, 22, 34). Zta can be transcribed from two promoters (20, 28) which induce two different transcripts: they contain either the reading frame BZLF1 alone or the two reading frames BZLF1 and BRLF1 as a bicistronic message, the latter giving rise to the expression of Zta and Rta. Zta has been shown to stimulate transcription from either of the two promoters (8, 28), thus inducing the expression of Rta as well as enhancing its own expression. The activation of the productive cycle by transfection of Zta (26) demonstrated the key role of this protein for the replication of EBV. So far, Zta has been found to be the only *trans* activator able to induce the lytic cycle when acting alone (6, 11, 26). However, this mechanism, demonstrated so far mostly in BL cells, does not seem to work in LCLs, as shown clearly by our results. This observation agrees well with earlier observations that LCLs seem to be less permissive for lytic infection than BL cells (9, 23). In LCLs, Rta may play an important role in lytic activation, as documented by the activation of the early genes BSLF2/BMLF1 coding for MSta and BALF2. Rta seems to act alone in this case. Zta alone is obviously not able to induce the full lytic cycle, possibly because of its failure to activate Rta expression. The induction of Zta by Rta is theoretically possible by activation of the transcription unit for the 2.8-kb RNA which contains the message for Rta and Zta (28). This, however, does not seem to play a role under these conditions, as in no case could the expression of Zta be detected after infection with the vaccinia virus construct leading to Rta expression. On the other hand, in BL cells, the induction of Zta always led to Rta expression; probably the presence of Zta and Rta together mediates the induction of MSta and p138 in BL cells. However, the expression of Rta alone had no effect on the induction of the later proteins.

These experiments demonstrate that cellular factors play an important role in the very early events during the replication pathway of EBV. Indeed, several cellular factors influencing the early lytic cycle of EBV have recently been described (15, 33). Such factors may act differently in nonmalignant LCLs and malignant BL cells; these cells may differ, for example, in the repertoire of transcription factors involved in enhancement or in inhibition of the *trans*-activating effects of Zta or Rta. It may well be that in the small resting B lymphocytes which harbor EBV in the healthy host, factors similar to those in nonmalignant LCLs are active (or missing). This would explain why productive infection in these cells, which could lead to viremia and potential endogenous reinfection, seems to be a rare event. The low inducibility of the lytic cycle in these cells may be due to the long coevolution of virus and host, as the result of a selective pressure in the normal lymphocyte to avoid viral replication and cell death.

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