*Proc. Natl. Acad. Sci. USA*
Vol. 89, pp. 905–909, February 1992
Medical Sciences

# Host-cell-phenotype-dependent control of the BCR2/BWR1 promoter complex regulates the expression of Epstein–Barr virus nuclear antigens 2–6

(Burkitt lymphoma/nasopharyngeal carcinoma/promoter usage/somatic hybrid)

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ABSTRACT **Epstein–Barr virus nuclear antigens (EBNAs) are expressed in a cell-phenotype-dependent manner. EBNA 1 is regularly expressed in all Epstein–Barr virus-carrying cells, whereas EBNAs 2–6 are only expressed in Epstein–Barr virus-carrying cells with a lymphoblastoid phenotype including group III Burkitt lymphoma (BL) lines positive for B-cell activation markers. Transcripts are initiated at the BCR2 or exceptionally at one BWR1 promoter in lymphoblastoid cell lines and group III BL lines. In group I BL lines, nasopharyngeal carcinoma, and the somatic cell hybrids, where EBNAs 2–6 are downregulated, the BCR2/BWR1 promoter complex is inactive or switched off. Upregulation of EBNAs 2–6 in group III BL cells and in 5-azacytidine-treated group I BL cells accompanies the activation of the silent BCR2/BWR1 promoters. Activation of BCR2 parallels demethylation of at least one CpG pair in the same promoter region. The activity of BCR2/BWR1 promoter complex depends on a particular B-cell phenotype. EBNA 1 transcription must be initiated at another promoter in cells that express only EBNA 1.**

Two promoters, BWR1 (in multiple copies) and BCR2, have been identified in the Epstein–Barr virus (EBV) genome that can serve as initiation sites for Epstein–Barr nuclear antigen (EBNA) mRNAs (for review, see ref. 1). mRNAs coding for EBNAs 1–6 can be generated from either one of these promoters by complex splicing from precursor RNA molecules up to 100 kilobases (kb) long (1). BWR1 and BCR2 map to a large transcription regulatory region of 7 kb (Fig. 1*a*; refs. 1 and 2). Their activity is mutually exclusive (3). After the primary infection of B lymphocytes, transcription begins at BWR1 promoters and then switches to BCR2 48–72 h after infection (4).

Lymphoblastoid cell lines (LCLs) express EBNAs 1–6 and latent membrane proteins (LMPs) 1, 2a, and 2b (5, 6). Burkitt lymphoma (BL) tumors and representative cell lines (group I BL lines) express only EBNA 1 (7, 8). EBV-carrying BL lines often drift toward a more LCL-like phenotype (group III BL lines) during culturing *in vitro* (7). This is reflected by the decrease or disappearance of BL-associated CD10 and CD77 markers and the appearance of activation markers. EBNAs 2–6 and LMPs 1, 2a, and 2b are upregulated as well. Nasopharyngeal carcinoma (NPC) represents yet a third type of EBV–host cell interaction. EBNA 1 is always expressed but EBNAs 2–6 are not. LMP 1 is detected in some 65% of NPCs (9).

We have found (10, 11) that somatic cell hybrids derived from the fusion of EBV-carrying group III BL lines or LCLs with non-B-cell lines downregulate EBNAs 2–6, LMP 1, and B-cell-specific markers. This suggested that the regulation of EBNA 1, on the one hand, and EBNAs 2–6, on the other hand, can be dissociated from each other. EBNA 1 shows a dominant constitutive expression, and EBNAs 2–6, LMPs, and B-blast-specific markers are expressed in a cell-phenotype-dependent fashion. The purpose of the present study was to examine whether the EBNA 2–6 expression depends on selective promoter activity. Our S1 nuclease analysis has shown that group I BL cells, NPC biopsies, and somatic cell hybrids that express only EBNA 1 utilize neither the BCR2 nor the BWR1 promoter. As our earlier findings (9, 12, 13) suggested that viral DNA methylation may play a role in the regulation, we investigated whether promoter activity correlated with the methylation status.

## MATERIALS AND METHODS

**Cells.** The origin and EBV gene expression of all the cell lines is summarized in Table 1 and reviewed in refs. 10 and 11. CB-M1-Ral-STO has been established by infection with EBV rescued from Rael. The Mutu clones (cls) were kindly provided by Alan Rickinson (CRC Laboratories, Birmingham, U.K.). Mutu I clones cl 148 and cl 216 have a group I phenotype, and the group III Mutu cl 99 and cl 176 were obtained by *in vitro* culturing and selection (8). KH1 and KH2 are KR4–HeLa hybrids (11). They resemble the HeLa parent cell phenotypically and express only EBNA 1. PUTKO and DUTKO are P3HR1–K562 and Daudi–K562 hybrids, with a K562 phenotype and no B-cell markers. They express only EBNA 1. HP1 is a HL-60–P3HR1 hybrid that expresses the B-cell markers, EBNA 1, and EBNAs 3–6.

**NPC Biopsies.** C15 and CAO are nude-mouse-passaged EBV-DNA-positive African and Chinese NPCs, respectively. NPCs 1–4 are all EBV-DNA-carrying tumor biopsies, collected by local surgery in Shanghai and Guandong areas. The biopsies were stored frozen at −70°C until used for RNA isolation. C15, CAO, and NPCs 1 and 3 were LMP-positive whereas NPCs 2 and 4 were negative by immunoblot analysis with the S12 monoclonal antibody. They were all EBNA 1-positive (9).

**RNA Preparation and S1 Nuclease Assay.** Total RNA from cell lines was prepared by homogenization in guanidinium isothiocyanate and ultracentrifugation through a CsCl cushion. Total RNA from NPC material was prepared by acid-guanidinium thiocyanate/phenol/chloroform extraction.

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen; LCL, lymphoblastoid cell line; BL, Burkitt lymphoma; NPC, nasopharyngeal carcinoma; 5-azaC, 5-azacytidine; LMP, latent membrane protein; nt, nucleotide(s); cl, clone.

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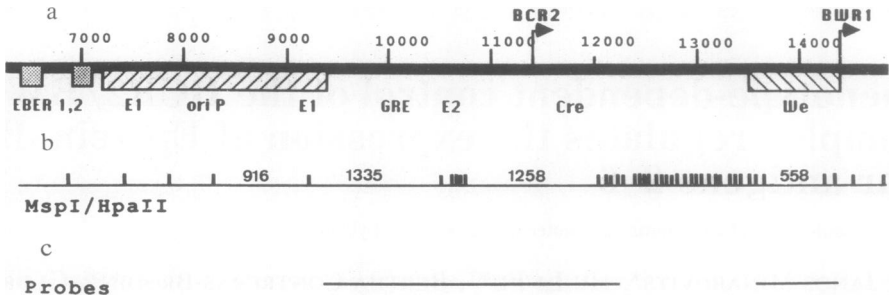


FIG. 1. (*a*) Schematic map of the EBV genome between coordinates 6000 and 15,000 with the BCR2 and BWR1 promoter regions. Arrows, promoters; hatched boxes, ori P; stippled boxes, Epstein–Barr virus-encoded RNA (EBER) open reading frames; E1, EBNA 1 binding sites; GRE, glucocorticoid receptor responsive element; E2, EBNA 2 responsive element; We, W-enhancer element; Cre, BCR2 downstream regulatory element. (*b*) Same region with the *Hpa* II/*Msp* I cleavage sites in prototype B95-8 DNA (for *Hpa* II in unmethylated DNA), and the number of base pairs of the largest fragments. (*c*) Probes for analysis of methylated CpGs. The probe covering the BCR2 region is a 2.7-kb *Sac* II fragment (positions 9516–12,291). The W-enhancer probe is an *Ava* I fragment (positions 13,827–14,460).

Synthetic oligonucleotides used for S1 nuclease protection assay were as described (3): $C_1$ 58-mer, 5′-CTGGGGGTC-TTCGGTGTCCTTGTCTCTATGCCATCTGATCTAAAA-TTTGCAGCAGAAC-3′; $W_0$ 48-mer, 5′-ATTTGTGTG-GACTCCTGGCGCTCTGATGCGACCAGAAATAGCTG-CAGG-3′. Total RNA (50–100 μg) was hybridized with labeled oligonucleotide for 16 h at 37°C in hybridization buffer containing 50% (vol/vol) formamide, treated with S1 nuclease (200 units/ml) for 30 min at 37°C, and electrophoresed in 10% denaturing polyacrylamide gels as described in detail (14). As an internal control, labeled synthetic oligonucleotide complementary to the human glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) corresponding to bases 1187–1168 was used (15). Five noncomplementary bases were added at the 3′ end: GAPDH 25-mer, 5′-TGATGGTACATGACAAGGTGATTAG-3′. The oligonucleotides were labeled at the 5′ end using T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (Amersham).

**DNA Preparation and the Analysis of DNA Methylation.** DNA (10 μg) was digested with *Hpa* II (a CpG-methylation-sensitive enzyme) or *Msp* I (CpG-methylation-insensitive enzyme), analyzed on a Southern blot, and hybridized as described (12). A 2.7-kb *Sac* II fragment (positions 9516–12,291) and an *Ava* I fragment (positions 13,827–14,460) were used as probes for the analysis of CpG methylation in the BCR2 region and the W-enhancer region, respectively (Fig. 1*c*).

## RESULTS

**Group I BL Lines and NPC Use Neither BCR2 nor BWR1 Promoter.** No protected fragments were obtained with RNA from three group I BL lines (two clones of Mutu I, Rael, and Akata) (Fig. 2 and Table 1), indicating that neither of the promoters was used to initiate EBNA 1 transcription.

The LMP-expressing NPC tumors C15, CAO, and NPCs 1 and 3 and the LMP-negative NPC tumors NPCs 2 and 4 did not show the protected fragments with $C_1$- or $W_0$-oligonucleotide probes (Fig. 3). The limited availability of NPC RNA hampered our ability to do the test more than twice and to equalize the RNA loading. Therefore, cohybridization with a GAPDH oligonucleotide was used as an internal control of amount and quality of the NPC RNA. This showed a 20-nt protected fragment in all the samples (Fig. 3). We conclude that NPC tumors, either LMP positive or negative, use neither the BCR2 nor the BWR1 promoter.

**LCLs and Group III BL Lines Use BCR2 or BWR1 Promoter.** When hybridized with $C_1$- or $W_0$-oligonucleotide probes, LCLs and group III BL lines showed 44- or 30-nt protected fragments, respectively (Figs. 2 and 4). Only the BCR2 promoter was utilized in KR4, CB-M1-Ral-STO, IARC-167, CBC, JK-2, JY, Mutu III cl 99 and cl 176, Jijoye p79, and Jijoye M13. Only the BWR1 promoter was utilized in LCL FLEB 14-6, group III-like BL lines P3HR1, Daudi, and Namalva. In the latter the signal was weak. In the BCR2 users IARC-167 and Jijoye M13, a very weak signal was observed with the $W_0$-oligonucleotide probe. When hybridized with the $W_0$-oligonucleotide probe, both the BWR1 and BCR2 promoter users showed a protected fragment of the same size as the probe (48 nt). This may be due to hybridization of precursor EBNA mRNA that initiates at BCR2. The expression of EBNAs 1–6 in CB-M1-Ral was associated with BCR2 promoter utilization showing that the Rael-derived virus has an intact BCR2 promoter, even though it was not used in the parental Rael line. The switch of group I BL Mutu to group III was associated with the activation of the BCR2 promoter.

**BCR2 and BWR1 Promoter Activities Can Be Induced in Rael by 5-Azacytidine (5-azaC).** 5-azaC induces upregulation of EBNAs 2–6 in the Rael line (16). Both the BCR2 and BWR1 promoters were activated by 5-azaC in Rael (Fig. 2). Thus, the BWR1 promoter is intact in Rael.

**BCR2 and BWR1 Promoters Are Inactive in EBV-Carrying B-Cell/Non-B-Cell Hybrids (Fig. 5).** KR4 expressed all six EBNAs and utilized the BCR2 promoter. In the KR4–HeLa hybrids KH1 and KH2, which express only EBNA 1, neither the C nor W oligonucleotides protected any fragment specifically. None of the promoters were utilized in the Daudi–K562 (DUTKO) and the P3HR1–K562 (PUTKO) hybrids, in contrast to the parental Daudi and P3HR1 lines that utilized the W promoter. Utilization of both the BCR2 and BWR1 promoters can thus be switched off by fusing with non-B lines. EBNA 2–6 expression is downregulated in parallel. Only one of the examined hybrids, P3HR1–HL-60 (HP1), continued to express EBNAs 1–6 and B-cell markers. Interestingly, this hybrid utilized the BCR2 promoter unlike its parental line, which used the W promoter.

**Correlation Between Promoter Utilization and Demethylation.** We have found (12, 13) that the EBV genome is hypomethylated in LCLs but highly methylated in BL biopsies and BL lines. To examine whether the activation of the BCR2 promoter correlates to changes in the methylation of this region, we compared group I and group III Mutu clones. We found that the activation of the BCR2 promoter in the group III clones was accompanied by the demethylation of one CpG site in the promoter region (Fig. 6*a*). There was no corresponding change in the methylation pattern of the W-enhancer–BWR1 promoter region (Fig. 6*b*).

Table 1. Origin, EBV-gene expression, and promoter utilization of cell lines

| Cell line | EBV | EBNA-1 | EBNA-2 | EBNA-3, -4, and LMP | BWR1 | BCR2 |
|---|---|---|---|---|---|---|
| Group I BL | | | | | | |
| Rael | + | + | − | − | − | − |
| Akata | + | + | − | − | − | − |
| Mutu I (cl 216) | + | + | − | − | − | − |
| Mutu I (cl 148) | + | + | − | − | − | − |
| Group III BL | | | | | | |
| Mutu III (cl 176) | + | + | + | + | − | + |
| Mutu III (cl 99) | + | + | + | + | − | + |
| P3HR1[a] | + | + | −[b] | + | + | − |
| PUT[a] | + | + | −[b] | + | + | − |
| Jijoye p.79 | + | + | + | + | − | + |
| Jijoye M.13 | + | + | + | + | − | + |
| Namalva | + | + | + | + | + | − |
| Daudi[a] | + | + | −[b] | +[c] | + | − |
| DUT[a] | + | + | −[b] | +[c] | + | − |
| Rael plus 5-AzaC | + | + | + | + | + | + |
| LCLs | | | | | | |
| CB-M1-Ral-STO | +[d] | + | + | + | − | + |
| CBC JK-2 | +[e] | + | + | + | − | + |
| KR4 | + | + | + | + | − | + |
| IARC-167 | +[f] | + | + | + | − | + |
| JY | + | + | + | + | − | + |
| FLEB 14-6 | +[g] | + | + | + | + | − |
| NPC | | | | | | |
| C15 | + | + | − | Only LMP | − | − |
| CAO | + | + | − | Only LMP | − | − |
| NPC 1 | + | + | − | Only LMP | − | − |
| NPC 2 | + | + | − | − | − | − |
| NPC 3 | + | + | − | Only LMP | − | − |
| NPC 4 | + | + | − | − | − | − |
| Non-B-cell | | | | | | |
| K562 | − | − | − | − | − | − |
| HL-60 | − | − | − | − | − | − |
| HeLa | − | − | − | − | − | − |
| Bjab | − | − | − | − | − | − |
| Hybrids | | | | | | |
| PUTKO (PUT–K562) | + | + | − | − | − | − |
| DUTKO (DUT–K562) | + | + | − | − | − | − |
| HP1 (PUT–HL-60) | + | + | −[b] | + | − | + |
| KH1 (KR4–HeLa) | + | + | − | Only LMP | − | − |
| KH2 (KR4–HeLa) | + | + | − | Only LMP | − | − |

[a]Universal fusers derived from P3HR1 (PUT) or Daudi (DUT) by selection in oubain and 6-thioguanine.
[b]P3HR1, PUT, and HP1 carry the same virus strain that has a deletion of the EBNA 2 gene and, therefore, does not express EBNA 2. Daudi and DUT carry another virus strain that has also deleted the EBNA 2 gene.
[c]Daudi and DUT do not express LMP.
[d]Immortalized by virus from the group I BL line Rael.
[e]Immortalized by virus from the mouthwash of a healthy child.
[f]Established by transformation with B95-8 EBV.
[g]Established by the transformation of human fetal liver cells with B95-8 EBV.

## DISCUSSION

The dominant constitutive EBNA 1 expression contrasting to the cell-phenotype-dependent regulation of EBNAs 2–6 suggested to us that EBNAs 2–6 were separately regulated at the promoter level. We have now found that either BCR2 or BWR1 were activated when EBNA 2–6 expression was upregulated, whereas both were silent in all cells that only expressed EBNA 1: the BL group I Rael, Akata, Mutu clones, the NPCs, and the KH1, KH2, PUTKO, and DUTKO B-cell–non-B-cell hybrids (Table 1). Thus, EBNA 1 transcription must be initiated at another promoter in cells that express only EBNA 1. It is likely that this corresponds to the promoter identified by Sample *et al.* (17) in the Mutu line, localized just upstream of *Bam*HI F/Q boundary.

All B-cell markers are downregulated after fusion of B cells with a variety of non-B-cell phenotypes (10, 11, 18). When cells expressing tissue-specific genes are fused with cells of a different kind, a frequently observed result is a selective block of transcription. In our material, B-cell markers were extinguished when EBV-carrying B-cell lines KR4, P3HR1, or Daudi were hybridized with non-B-cell lines. In contrast to the B-cell parent that expressed all proteins of the EBNA 2–6 complex, the hybrids expressed EBNA 1 only. In this respect EBNAs 2–6 were regulated like B-cell-specific genes. We have found that the BCR2/BWR1 complex is switched off in parallel. The HP1 hybrid was not subject to downregulation of the tissue-specific genes and EBNAs 1, 3, 4, and 6. The activity of the BCR2 promoter in this hybrid further confirms the link between cell phenotype and promoter regulation.

The switch from the group I to the LCL (group III) phenotype of the Mutu cells and 5-azaC treatment of Rael or transfer of EBV from the same BL line to a LCL background (CB-M1-Ral) resulted in upregulation of all the EBNA proteins and

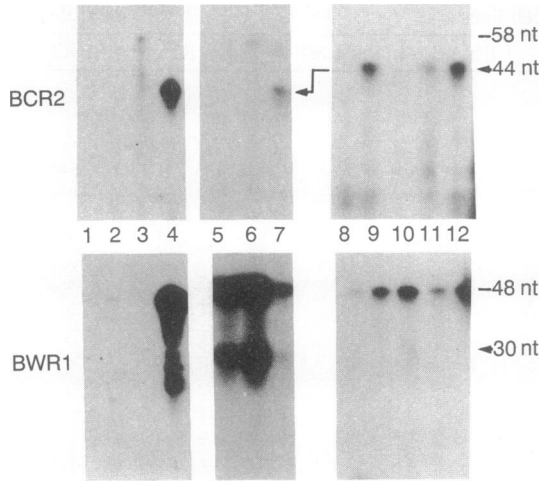


FIG. 2. S1 nuclease protection analysis of BCR2 (*Upper*) and BWR1 (*Lower*) activity. Lanes: 1, Rael; 2, Akata; 3, Mutu I (cl 216); 4, Rael plus 5-azaC; 5, P3HR1; 6, Daudi; 7, Jijoye M13; 8, Mutu I (cl 148); 9, Jijoye p79; 10, Namalva; 11, Mutu III (cl 99); 12, Mutu III (cl 176). Positions of the free oligonucleotide probes and specific protected fragments are indicated by bars and arrowheads, respectively. nt, Nucleotides.

activation of the BCR2/BWR1 complex. As NPCs are of epithelial origin, the concomitant silence of BCR2 and BWR1 promoters and EBNAs 2–6 in those further supports the dependence of these promoters on a particular B-cell phenotype.

Like Woisetschlaeger *et al.* (3, 4), we found that the BCR2 and BWR1 promoters were used in a mutually exclusive way, although a very weak signal was detected in a few BCR2 promoter users (IARC-167 and Jijoye M13) with the $W_0$ oligonucleotide probe. This may originate from a small subpopulation of cells.

One CpG pair in the BCR2 region became demethylated in parallel with the BCR2 activation. This is either the site at position 12,056 (B95-8 coordinate) or the site upstream of the

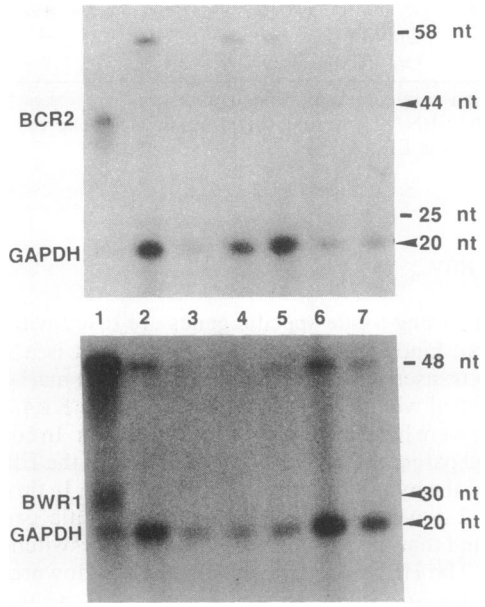


FIG. 3. S1 nuclease analysis of BCR2 (*Upper*) and BWR1 (*Lower*) activity in NPC. Lanes: 1, control; 2, C15; 3, CAO; 4, NPC 1; 5, NPC 2; 6, NPC 3; 7, NPC 4. CB-M1-Ral-STO and Daudi were used as positive controls for BCR2 and BWR1 analysis, respectively. Total RNA (20–100 $\mu$g) from NPC tumors was used. As internal control, a GAPDH probe was added to the hybridization mixture.

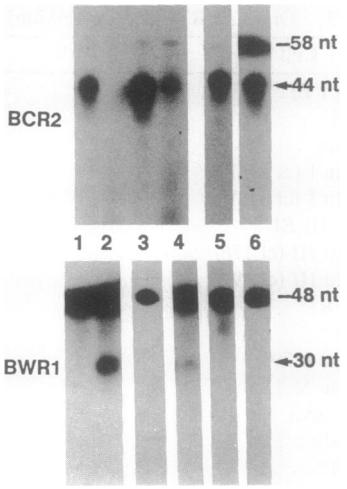


FIG. 4. S1 nuclease analysis of BCR2 and BWR1 promoter activity in LCLs. Lanes: 1, CBC JK2; 2, FLEB 14-6; 3, CB-M1-Ral; 4, IARC-167; 5, JY; 6, KR4.

promoter at position 10,798 (see Fig. 1*b*). This confirms our previous observations (9, 12) that CpG methylation of control regions may correlate to regulatory events in latent gene expression.

LCLs and group III BL lines normally use BCR2 for EBNAs 2–6 transcription, after an initial 48- to 72-h period when BWR1 is used during primary infection of virgin B lymphocytes (4). We found exceptional BWR1 activity in four cell lines, three group III-like BL lines (Daudi, P3HR1, and Namalva) and FLEB 14-6. These are particular cell types that may provide clues to the mechanisms governing BCR2/BWR1 promoter switch. FLEB 14-6 is a LCL of pro-B origin with no immunoglobulin gene rearrangement (19) and with an intact BCR2 from the B95-8 virus. Transcriptional control in this line has prevented the switch from BWR1 to BCR2. Namalva is exceptional in that it carries integrated virus genomes. Daudi and P3HR1 have large deletions affecting the whole EBNA 2 exon and the 3′ end of EBNA 5. The parental BL line (Jijoye p79 and M13), from which P3HR1 once was cloned, has an intact region from EBNA 5 to EBNA 2 and uses the BCR2 promoter. EBNAs 2 and/or 5 may thus be directly or indirectly involved in the switch control. EBNA 2 was recently shown to interact with a DNA sequence upstream of the BCR2 promoter (20). HP1, the

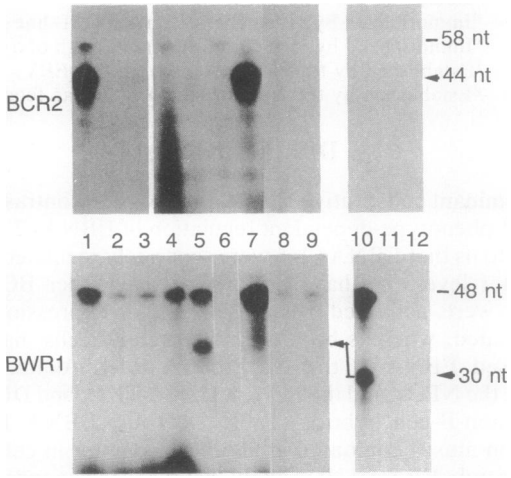


FIG. 5. S1 nuclease analysis of BCR2 and BWR1 promoter usage in somatic cell hybrids and their parental cell lines. Lanes: 1, KR4; 2, KH1; 3, KH2; 4, HeLa; 5, PUT; 6, PUTKO; 7, HP1; 8, HL-60; 9, K562; 10, DUT; 11, DUTKO; 12, Bjab.

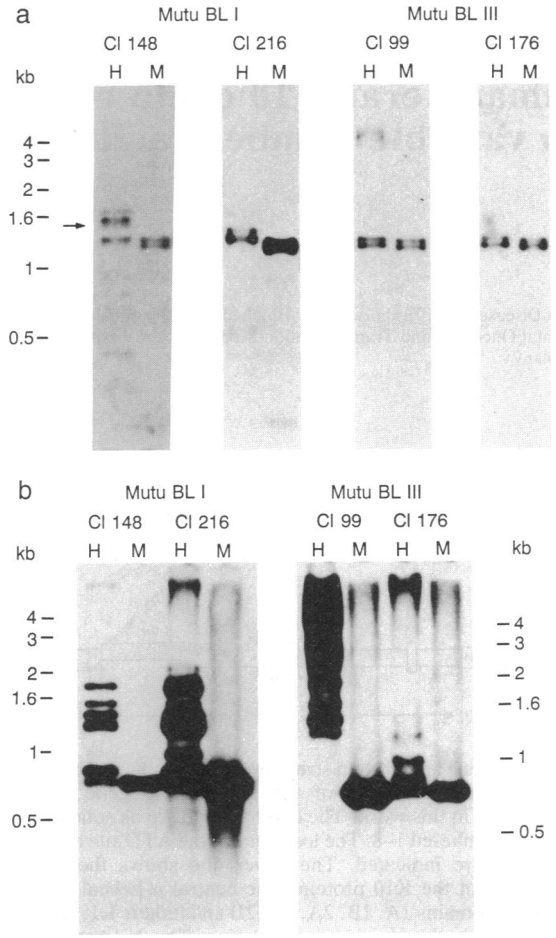


FIG. 6. Southern blots showing methylation pattern in the BCR2 and BWR1 promoter regions in group I and group III Mutu clones by comparison of *Msp* I (M) and *Hpa* II (H) cleavage sites. (*a*) A 2.7-kb probe covering the BCR2 region was used (see Fig. 1*c*). The BCR2 (coordinate 11,305) is localized in a 1258-base-pair *Msp* I fragment (coordinates 10,798–12,056 of prototype B95-8 DNA; see Fig. 1*b*). A fragment of similar size was detected in the *Msp* I-cleaved DNAs from all four Mutu clones (solid triangles). This fragment was not detected in the *Hpa* II-digested DNAs of group I clones. It was detected in the DNA of the group III clones (open triangles). Two *Hpa* II fragments were detected in the group I BL lines: 1.5 kb (in Cl 148) and 1.45 kb (in Cl 216). This indicates the presence of methylated CCGG sequences 5′ and/or 3′ of the BCR2. The upstream 1335 bp *Msp* I fragment (coordinates 9105–10,440 in B95-8) was unmethylated in all four clones as indicated by *Hpa* II–*Msp* I fragments of identical size. (*b*) A 0.5-kb probe covering the BWR1 region was used. The BWR1 promoter (coordinate 14,352) is localized in a 558-base-pair *Msp* I fragment (coordinates 13,954–14,512) of B95-8 DNA. This region is partially methylated in Mutu BL group I Cl 148 and Cl 216 and in Mutu BL group III Cl 176 as indicated by the presence of *Hpa* II fragments with both identical and larger size than that of the *Msp* I control. Mutu BL III Cl 99 is highly methylated in the region since no fragments with similar size was detected.

P3HR1–HL-60 hybrid, switched to BCR2 promoter activity after fusion. Thus the P3HR1 genome must have an intact C promoter and the putative dependence on EBNA 2 and/or 5 for switching can be overcome by cellular factors.

We are grateful to Drs. A. B. Rickinson (Birmingham, England), S. Speck (Boston), and M. Uno (Tokyo) for the Mutu clones, JY cell lines, and FLEB 14-6, respectively, and to Dr. D. A. Thorley Lawson for monoclonal antibody S12. This investigation was supported by Public Health Service Grants 2R01 CA 30264 and 1R01 CA 52225 awarded by the National Cancer Institute, Department of Health and Human Services, the Swedish Cancer Society, and the Swedish Medical Association.

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