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# The role of DNA hypomethylation, histone acetylation and *in vivo* protein-DNA binding in Epstein–Barr virus-induced CD23 upregulation

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ABSTRACT

We analyzed epigenetic marks at the CD23 regulatory regions in well characterized Epstein–Barr virus (EBV)-carrying cell lines covering the major latency types. Bisulfite sequencing showed that DNA methylation is not a major regulator of EBV-induced CD23 transcription, although a wide hypomethylated DNA sequence in the regulatory regions is always present in the cell lines with high CD23 expression. Acetylated histone H3 levels at the CD23b promoter showed strong correlation with CD23b expression, while a weaker correlation could be observed at the CD23a core promoter. DMS *in vivo* footprinting at the intronic EBV-responsive enhancer and the intermediate-affinity CBF1 site at the CD23a core promoter did not reveal any significant sign of *in vivo* protein-DNA interactions, despite the presence of strong, characteristic footprints in the same DMS-treated DNA samples at the two CBF1 sites of the LMP2A-promoter. Our *in vivo* results suggest a minor role for DNA methylation, while a more important role for histone acetylation in the regulation of EBV-induced CD23 expression. Furthermore, our *in vivo* footprinting results support the complex model of CD23 induction by EBV, rather than a simple model with direct transactivation of CD23 by EBNA-2.



## 1. Introduction

Epstein–Barr virus (EBV) is an ubiquitous gammaherpesvirus, associated with several neoplasms including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, posttransplant lymphoproliferative disease and Hodgkin's disease. In type I latency only the EBV nuclear antigen 1 (EBNA-1), two protein non-coding RNAs (EBER-1 and -2) and the BamHI-A region transcripts can be detected, while in type III latency five additional nuclear antigens and three EBV-encoded latent membrane proteins (LMP) are also expressed [1].

Modulation of cellular gene expression by type III latency viral products is essential to induce and maintain B cell proliferation [2]. CD23, also known as the low-affinity IgE receptor [3] is one of the cellular genes that is rapidly induced by *in vitro* EBV infection [4]. Furthermore, immortalized lymphocytes arise only from CD23-positive EBV-infected B cells [5]. CD23 is cooperatively induced by EBNA-2 and LMP-1 [6], and both membrane-bound CD23 and the soluble sub-fragments of CD23 stimulate the growth of lymphoblastoid cell lines (LCL) [7].

Two isoforms of CD23 exist, which differ in their cytoplasmic N-terminus by six or seven amino acids and use different promoters and 5′ exons [8,9]. The regulatory region of the human CD23a gene consists of two 188 bp long, almost perfectly identical sequence elements forming an inverted repeat, which frames the core promoter (from −243 to −1, relative to the initiation site of CD23a transcription), the first exon and the 5′ end of the first intron [8,10]. Several regulatory sequences have been described within the core promoter [10,11], including an intermediate-affinity C-promoter (Cp) binding factor 1 (CBF1) site (from −172 to −166), which was suggested to be essential for the activation of CD23a transcription by EBNA-2 [12]. Other publications however showed, that EBV-induced upregulation of CD23a transcription depends on a 37 bp long enhancer sequence located in the upstream part of the first intron (intronic EBV-responsive enhancer, IEBVRE, from +247 to +283; [13–15]).

*Abbreviations:* AcH3, acetylated histone H3; BL, Burkitt's lymphoma; CBF1, C-promoter binding factor 1; ChIP, chromatin immunoprecipitation; Cp, C-promoter; DMS, dimethyl sulfate; EBNA, EBV nuclear antigen; EBV, Epstein–Barr virus; IEBVRE, intronic EBV-responsive enhancer; LCL, lymphoblastoid cell line; LMP, latent membrane protein; LMP2Ap, LMP-2A promoter; LM-PCR, ligation-mediated PCR; PCR, polymerase chain reaction; TIC, total input chromatin..

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Although epigenetic mechanisms play an important role in the regulation of several lymphoid-specific genes [16], their role in the regulation of EBV-induced CD23 expression is unexplored. Therefore we carried out a comprehensive analysis of DNA methylation patterns, acetylated histone H3 (AcH3) levels and *in vivo* protein-DNA interactions at the main regulatory regions of CD23 in a panel of well characterized EBV-positive human cell lines covering all latency types.

## 2. Materials and methods

### 2.1. Cell lines and tissue culture

All cell lines (Table 1) were maintained in RPMI 1640 medium containing 10% fetal calf serum (except MOLP-8, which was kept on 20% FCS), 2 mM glutamine, 50 units penicillin per ml and 50 μg streptomycin per ml at 5% $CO_2$ and 37 °C.

### 2.2. Real-time reverse transcription PCR

Total cellular RNA was isolated from cell cultures with TRI Reagent (Sigma) according to the manufacturer's instructions. Five μg of RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) and two oligonucleotides complementary to the human CD23 (5′-GCTCGAAGTTCCTCCAGTTC-3′) and β-actin (5′-TGTAACGCAACTAAGTCATAG-3′) mRNAs, according to the manufacturer's instructions. The relative levels of CD23a and β-actin transcripts were determined with the LC FastStart DNA Master SYBR Green I kit (Roche), while the relative level of CD23b mRNA was determined with the LightCycler Taqman Master kit (Roche) in a LightCycler 2.0 instrument (Roche) using the standard curve method, with primers listed in Table 2.

### 2.3. Immunoblotting

Total cell lysates were prepared and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting, with antibodies directed against β-actin (AC-15; Sigma–Aldrich) and EBNA-2 (PE-2; DAKO).

### 2.4. Control DNA sequencing

Genomic DNAs were amplified with PCR using the primers listed in Table 2. Both strands of the PCR products were then sequenced on a MegaBACE DNA sequencing system (GE healthcare) using dye-labeled ddNTPs, according to the manufacturer's instructions.

### 2.5. Automated genomic sequencing of sodium bisulfite-treated DNA

Bisulfite sequencing was performed essentially as described earlier [17,18], with the primers shown in Table 2. The bisulfite conversion reaction was complete, since all cytosines outside CpG-dinucleotides were converted to uracil and therefore sequenced as thymine.

### 2.6. Chromatin immunoprecipitation assay

Formaldehyde cross-linked chromatin was prepared from $2 \times 10^7$ cells and immunoprecipitated [19,20] with an antibody directed to diacetylated histone H3 (06-599; Millipore), or was mock-precipitated with non-specific antibody (SC-2027; Santa Cruz Biotechnology). Recovered DNA aliquots were quantified with the LC FastStart DNA Master SYBR Green I kit (Roche) in a LightCycler 2.0 instrument (Roche) using the standard curve method, with primers listed in Table 2.

**Table 1**
Origin and latency type of cell lines.

| Cell line | Cell type and/or stage of B cell maturation | EBV | EBV latency type, EBNA-2 expression[a] |
|---|---|---|---|
| **REH** | Acute lymphoblastic leukemia; Pro-B cell | - | |
| **RS4;11** | | | |
| **SEM** | | | |
| **LCL-721** | LCL | + | III (EBNA-2 +) |
| **IARC-171** | | | |
| **CBM1-Ral-STO** | | | |
| **KR-4** | | | |
| **DG-75** | Sporadic, EBV-negative BL; early centroblast[b] | - | |
| **BJA-B** | | | |
| **Ramos** | | | |
| **BL-41** | | | |
| **BL-41-E95B** | | + (*in vitro* infected) | III (EBNA-2 +) |
| **Rael** | Endemic, EBV-positive BL; late germinal center B cell[b] | + | I (EBNA-2 -) |
| **Mutu-BL-I-cl-216** | | | |
| **Daudi**[c] | | | III (EBNA-2 -) |
| **Mutu-BL-III-cl-99** | | | III (EBNA-2 +) |
| **Jijoye-p79** | | | |
| **Raji**[d] | | | |
| **NCI-H929** | Multiple myeloma; Plasma B cell | - | |
| **MOLP-8** | | | |
| **U-266** | | | |
| **MDA-MB-231** | Carcinoma; Epithelial cell | - | |
| **C666.1** | | + | II (EBNA-2 -) |

References for cell lines are listed in Table S1.
[a]According to Altiok et al. [32] and Salamon et al. [33].
[b]According to Bellan et al. [34] and Piccaluga et al. [35].
[c] Daudi carries a virus strain that has a deletion of the EBNA-2 gene [36]. Therefore Daudi does not express or expresses only minimal amounts of EBV latent membrane proteins [32].
[d]Raji carries a virus strain that has a deletion of the EBNA-6 gene [37].

**Table 2**
Primers and probe used in PCR.

| | |
|---|---|
| *Primers and probe for real-time RT-PCR* | |
| CD23a | 5′-CTGCTTAAACCTCTGTCTCTGAC-3′ |
| | 5′-GCTTGGATTCTCCCGATGATG-3′ |
| CD23b | 5′-GGGAGCCCAATAGAGTCAGAG-3′ |
| | 5′-CTGGCTTGGAGGATTCATTATG-3′ |
| | 5′-6-Fam-CTGCTTGTTCCAAGTTCCTGTTCTATT-BHQ-1-3′ |
| β-Actin | 5′-GGCGGCACCACCATGTACCCT-3′ |
| | 5′-AGGGGCCGGACTCGTCATACT-3′ |
| *Primers for PCR amplification of bisulfite-modified DNA* | |
| CD23a outer | 5′-CCCTCCTTCCCTCCTACTC-3′ |
| | 5′-GAATTGAGGTTTTAAGAGGGTAAG-3′ |
| CD23a inner | 5′-Univ-AAGAGAATTGGGTGAGAATTAGAG-3′ |
| | 5′-BiotinCTTCCTCTACCTCTCTCTCCTCTTC-3′ |
| | 5′-Univ-CTCTAATTCTCACCCAATTCTCTTAC-3′ |
| | 5′-Biotin-GTTGGTTATTTTTTGAGGTTTAGTTTAAG-3′ |
| | 5′-Univ-CTCTCTTCCTACTTAAACCTCTATC-3′ |
| | 5′-Biotin-ATGGTGGGGTGTTTATTGGTAAG-3′ |
| | 5′-Univ-GGTGGGGTGTTTATTGGTAAG-3′ |
| | 5′-Biotin-CTCTCTTCCTACTTAAACCTCTATC-3′ |
| CD23b outer | 5′-GGGTGTATTTGTTGTGTAGTTTAAG-3′ |
| | 5′-AACCACTTTCCCAAATATCATC-3′ |
| CD23b inner | 5′-Univ-GTTGGTAATTAGGATTTGATG-3′ |
| | 5′-Biotin-TTCCTATTCTATTTAACCTCTAACTC-3′ |
| | 5′-Univ-AACCCTCTACACTCACCCTAAC-3′ |
| | 5′-Biotin-GTGTGAGTAAGGAGGTGAGGT-3′ |
| *Primers for ChIP assay* | |
| CD23a core promoter | 5′-GTGGTATGATTCAGTGTGCAGTAAC-3′ |
| | 5′-GCCACCCGGATAACATTACAC-3′ |
| CD23b promoter | 5′-GCTGAGTGGACTGCGTTGTC-3′ |
| | 5′-CACTCCTCCCTGGCTCTGTG-3′ |
| BALF-2 coding region | 5′-GGGTCTTGTGGTAGGTGTTGAG-3′ |
| | 5′-CCAGGAACATCAAGATCAAGAAC-3′ |
| Cp | 5′-GTGCGTCGAGTGCTATCTTTGGAAC-3′ |
| | 5′-ACTTTGCGAGCCCTGCGTCTTGAG-3′ |
| *Primers for LM-PCR* | |
| CD23a core promoter | 5′-CCTAAATATGGGCTTGCCACCCGG-3′ |
| | 5′-GATAACATTACACGCATGGCCTCTCC-3′ |
| | 5′-ACACGCATGGCCTCTCCTGGTGCTC-3′ |
| CD23a IEBVRE | 5′-GCACTCACCAAGCTCATCTCAGACC-3′ |
| | 5′-CCAGATGGCCTGCTTTCACTTCCCC-3′ |
| | 5′-CAAATCAGGGACTCGAATGCCTCGAG-3′ |
| LMP2Ap | 5′-AGTTCCTGAGAGCCCAGGGGTCTC-3′ |
| | 5′-TCTCGTGCAGGTGTCCCCGGGGAAT-3′ |
| | 5′-TGTTCTCCCTGATCACCGCCCACCC-3′ |

Primers and probe were purchased from Metabion (Martinsried, Germany). Abbreviations: 6-Fam, 6-carboxyfluorescein; BHQ-1, Black Hole Quencher 1; IEBVRE, intronic EBV-responsive enhancer; LMP2Ap, LMP-2A promoter; Univ, M13 universal primer sequence (5′-GTAAAACGACGGCCAGT-3′).

### 2.7. Dimethyl sulfate in vivo footprinting

Genomic footprinting was performed essentially as described earlier [17,18], with primers for ligation-mediated PCR (LM-PCR) listed in Table 2.

## 3. Results

### 3.1. High resolution methylation analysis of CD23 regulatory regions

First we analyzed the expression of CD23a and CD23b mRNAs, together with DNA methylation patterns of their respective regulatory regions in 12 well characterized EBV-positive human cell lines covering all latency types (Table 1). To see the potential influence of cellular differentiation state on DNA methylation patterns, the same analyses was carried out on an EBV-negative carcinoma line and ten EBV-negative B cell lines representing different stages of B lymphocyte development (Table 1).

CD23a mRNA level (Fig. 1A) was high (more than 50-fold compared to RS4;11) in all four LCLs and BL-41-E95B, and moderate (more than 10, but less than 50-fold compared to RS4;11) in two EBNA-2-positive type III BL lines Jijoye-p79 and Mutu-BL-III-cl-99. All other cell lines did not express or transcribed only a low level (less than 10-fold compared to RS4;11) of CD23a. CD23b mRNA (Fig. 1B) was highly expressed in three LCLs (CBM1-Ral-STO, LCL-721 and KR-4), and moderately in IARC-171, BL-41-E95B, Mutu-BL-III-cl-99 and Raji. All other cell lines did not express, or transcribed low levels of CD23b mRNA.

To reveal any correlation between EBNA-2 protein expression and CD23 mRNA levels, immunoblot analysis of EBNA-2 was carried out in representative cell lines (Fig. 1C). EBNA-2 protein expression was high in CBM1-Ral-STO and Jijoye-p79, slightly lower in Mutu-BL-III-cl-99, while Ramos was negative.

Since polymorphic variants may alter the results of bisulfite sequencing, we first sequenced the CD23 regulatory regions (positions −792 to +768 and −659 to +252 relative to the transcriptional initiation sites of CD23a and CD23b, respectively; [8,9]) from unmodified genomic DNAs in all cell lines. Compared to the prototype human DNA sequence (GeneBank/EMBL/DDJB ID: NC_000019.9) the results surprisingly revealed several polymorphic nucleotides at the CD23a (Table S-2A) and CD23b (Table S-2B) regulatory regions, but only one (position −44) at the CD23a regulatory region abolished a CpG, while three variants (positions −421, +209 and +220) at the CD23b regulatory region established new CpGs. None of the polymorphic variants affected previously published regulatory elements or coding sequences.

Bisulfite sequencing showed that the whole CD23a regulatory region (including the core promoter, first exon, 5′ part of the first intron and almost the entire sequence of the two 188 bp inverted repeats; from position −448 to +483; Fig. 2A) with the exception of a few CpGs (mostly in the inverted repeats and at position +377) contained only hypomethylated (<25%) or unmethylated CpGs in all LCLs, sporadic BLs, myeloma lines, two out of three pro-B lines and in the Jijoye-p79 and Raji EBNA-2-positive type III BL lines. On the other hand, in the REH pro-B line, in all of the EBNA-2-negative BL lines, and interestingly in the EBNA-2-positive Mutu-BL-III-cl-99 several highly (>50%), or moderately (25–50%) methylated CpGs could be found in the CD23a regulatory region. In the two epithelial lines the majority of CpGs in the CD23a regulatory region was highly methylated.

Methylation mapping of the CD23b regulatory region (from position −399 to +97; Fig. 2B) showed a wide hypo-, or unmethylated region 5' from the initiation point of transcription in all LCLs and MOLP-8, while all other lines contained highly methylated CpGs in this region. Position −109 was hypo-, or unmethylated in all lines except C666.1, where this cytosine was highly methylated.

### 3.2. Analysis of acetylated histone H3 levels at the CD23 promoters

Next we analyzed if transactivation of CD23 by EBV is accompanied by the modulation of AcH3 levels at the CD23 promoters. Therefore the levels of AcH3 were measured with chromatin immunoprecipitation (ChIP) assay at the CD23a core promoter, CD23b promoter, BALF-2 coding region (a lytic-cycle gene that is inactive during latent infection and contains low levels of AcH3 [20]) and Cp of EBV, in five well characterized, EBV-positive lines with different latency types (Table 1), CD23 mRNA levels (Fig. 1A and B) and promoter methylation patterns (Fig. 2A and B). We detected low levels of AcH3 at all regions examined in type I/II lines. The coding region of BALF-2 contained low level, while the CD23a core promoter and Cp contained moderate levels of AcH3 in type III cells. CD23b promoter was only moderately enriched in AcH3 in Mutu-BL-III-cl-99, while it was highly enriched in CBM1-Ral-STO (Fig. 3).

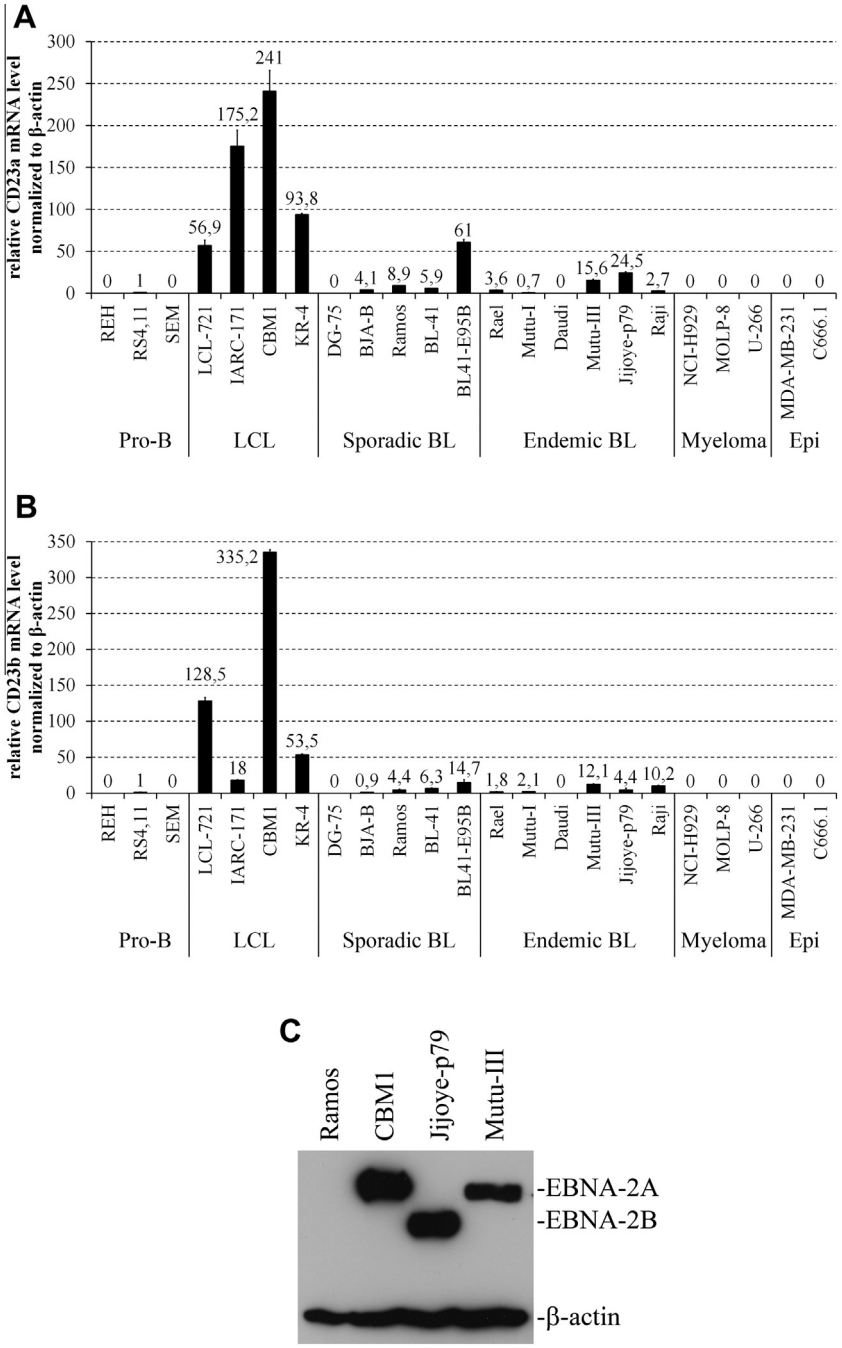


**Fig. 1.** Relative levels of CD23 mRNA and EBNA-2 protein expression. Relative CD23a (A) and CD23b (B) mRNA levels normalized to β-actin, quantified by real-time RT-PCR. Results are average of three biological replicates. (C) Immunoblot analyses of total cell lysates with antibodies directed against EBNA-2 and β-actin. Abbreviations: BL, Burkitt's lymphoma lines; CBM1, CB-M1-Ral-STO; Epi, epithelial cell lines; LCL, lymphoblastoid cell lines; Mutu-I, Mutu-BL-I-cl-216; Mutu-III, Mutu-BL-III-cl-99; Myeloma, multiple myeloma lines; Pro-B, pro-B cell lines.

### *3.3. Analysis of in vivo protein-DNA interactions at the EBV-responsive elements of the CD23a regulatory region*

Published *in vitro* experiments have charted an intermediate-affinity CBF1 binding site at the CD23a core promoter [12] and an intronic enhancer sequence [13–15] as crucial elements in the upregulation of CD23a by EBV. To elucidate *in vivo* protein-DNA interactions at these two EBV-responsive elements of the CD23a regulatory region, dimethyl sulfate (DMS) *in vivo* footprinting combined with LM-PCR was carried out in the same set of EBV-positive cell lines used in the analysis of AcH3 levels. We chose the method of footprinting, since it localizes *in vivo* protein binding at single nucleotide resolution, while the single nucleosomal resolution of ChIP is insufficient for the precise mapping of EBNA-2-CBF1-DNA interactions. Surprisingly, footprinting at the intermediate-affinity CBF1 binding site of the CD23a core promoter and at the intronic EBV-responsive enhancer did not reveal any significant sign of *in vivo* protein-DNA interaction in all five cell lines (Fig. 4A and B), while using the same DMS-treated DNA samples we could detect strong, characteristic footprints at the two CBF1 sites of the LMP-2A promoter (LMP2Ap) of EBV (Fig. 4C; positive control).

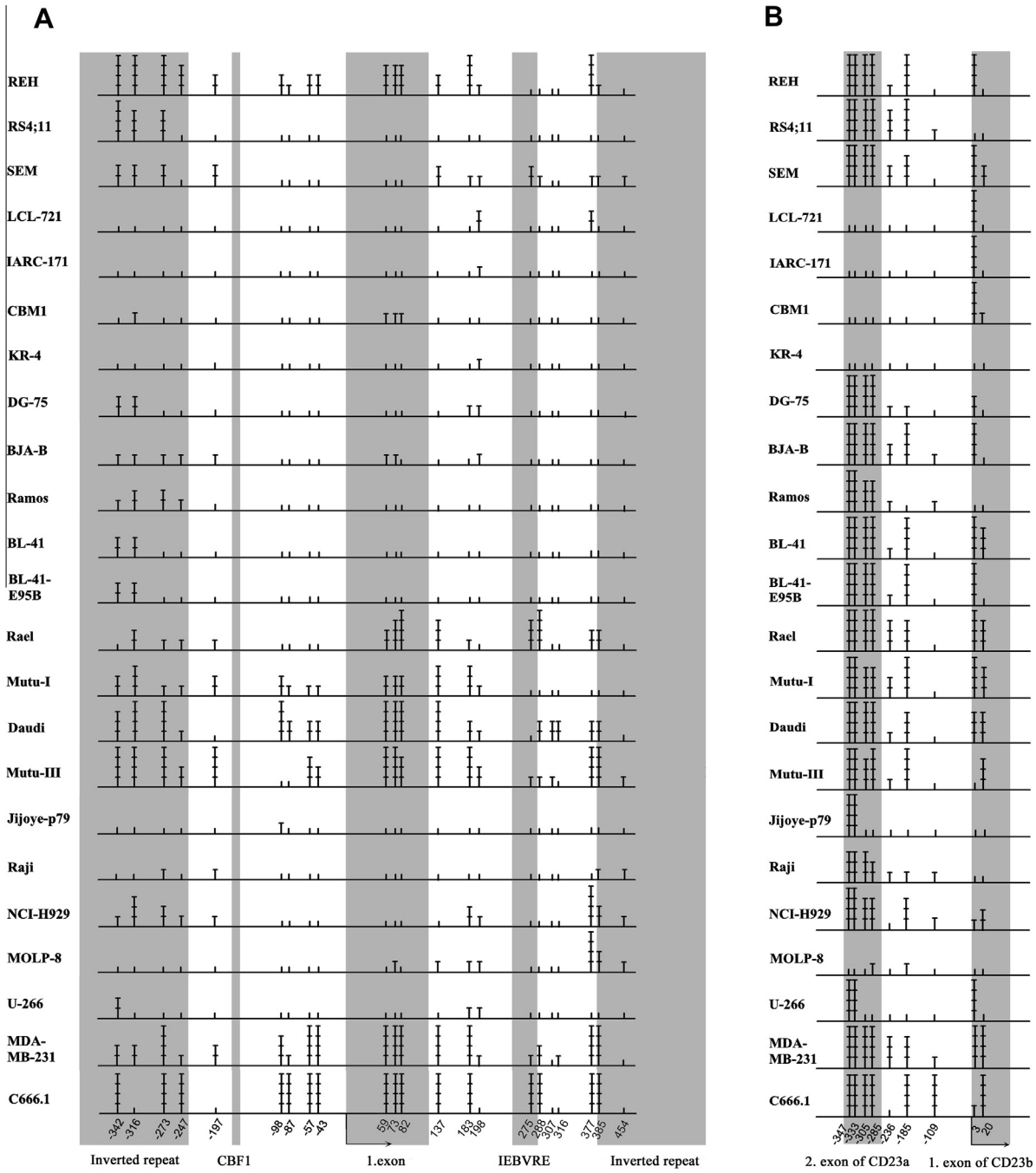


**Fig. 2.** Methylation patterns of the CD23 regulatory regions. Summary of methylation patterns in the CD23a (A) and CD23b (B) regulatory regions. Numbers and sticks indicate positions of cytosines within CpG dinucleotides, relative to the transcriptional start site of CD23a or CD23b. The degree of methylation of cytosines is indicated by the height of the sticks as follows: stick only, 0%; one horizontal line, 0–25%; two lines, 25–50%; three lines, 50–75%; four lines, 75–100%. Shadings represent regulatory elements. Abbreviations: CBF1, CBF1 binding site; CBM1, CB-M1-Ral-STO; IEBVRE, intronic EBV-responsive enhancer; Mutu-I, Mutu-BL-I-cl-216; Mutu-III, Mutu-BL-III-cl-99.

## 4. Discussion

CD23 plays an essential role in EBV-induced B cell transformation. Our results now confirm the role of type III EBV proteins in the upregulation of CD23 transcription, as high or moderate levels of CD23 mRNAs could be observed only in EBV infected, EBNA-2-positive, latency type III lines. The huge differences in CD23 expression between type III lines (Fig. 1A and B), despite the nearly equally strong EBNA-2 protein levels (Fig. 1C), however show that type III EBV proteins are necessary, but not sufficient for high CD23 expression, and suggest a role for epigenetics in the regulation of EBV-induced CD23 expression.

Our high-resolution methylation mapping shows, that high level CD23 expression in type III EBV latency always associates with an extended hypo-, or unmethylated area in the corresponding regulatory region. On the other hand, CD23a in Raji and CD23b in Jijoye-p79 are expressed at low levels, despite the presence of extended hypomethylated regulatory regions and type III EBV proteins, suggesting that additional factors besides DNA hypomethylation may also be essential for high level CD23 expression in EBV infected B cells. Furthermore, the moderate level of CD23 expression, despite the relatively high average level of DNA methylation at the CD23 regulatory regions in Mutu-BL-III-cl-99, together with the relatively low average level of methylation at the CD23a

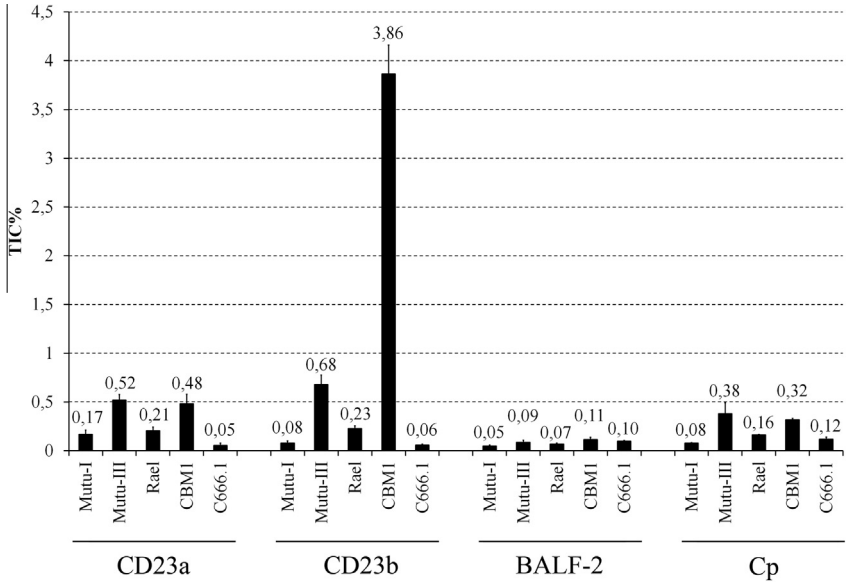


**Fig. 3.** Levels of acetylated histone H3 at the CD23 promoters. ChIP analysis of AcH3 levels at the CD23a core promoter, CD23b promoter, BALF-2 coding region and Cp. Results are average of three biological replicates expressed as the percentage of input DNA (TIC, total input chromatin). In all lines the amount of mock-precipitated DNAs were less than 0.028 percent of TIC at all four regions examined (data not shown). Abbreviations: BALF-2, BALF-2 coding region; CBM1, CB-M1-Ral-STO; CD23a, CD23a core promoter; CD23b, CD23b promoter; Mutu-I, Mutu-BL-I-cl-216; Mutu-III, Mutu-BL-III-cl-99.

regulatory regions in type I BL lines suggests that DNA methylation is not a major regulator of EBV-induced CD23 transcription in B cells, but rather only slightly influences the strength of transactivation by EBV. This is in contrast with the strong impact of DNA methylation on the regulation of several latent EBV promoters in the same cell lines [17,18,20,21]. Furthermore, the analysis of EBV-negative lines suggests that the observed cell type specific methylation differences may simply reflect the different B cell maturation stages of the cell lines.

Besides DNA methylation, acetylation of histones also plays an important role in the regulation of promoter activity since it leads to chromatin relaxation and/or provides unique signals for transcription factor binding [22]. CBF1 in the absence of EBNA-2 (or the proteolytically cleaved intracellular domain of Notch) represses transcription partially through histone deacetylases [23,24], while binding of EBNA-2 to CBF1 directs histone acetyltransferases to the LMP-1 promoter [25]. Furthermore, the levels of acetylated histones correlate with the activity of the CBF1-regulated Cp and LMP2Ap of EBV [19,20,26,27]. Although *in vitro* activation of CD23a by EBNA-2 was reported to be mediated through a CBF1 site at the core promoter [12], the results of our ChIP analysis (Fig. 3) suggests only a minor role for AcH3 in the regulation of CD23a core promoter and Cp, but surprisingly a major role in the regulation of CD23b promoter.

Previous *in vitro* experiments suggested a central role for a CBF1 binding site at the CD23a core promoter [12] and an intronic enhancer sequence [13–15] in the upregulation of CD23a by EBV. According to current models, CBF1 constitutively represses target promoters, and transcription occurs when EBNA-2 binds to the already promoter-bound CBF1 [24,28]. In line with this model, both in our previous and current analysis we detected two different, but characteristic footprint patterns at the central core sequences of CBF1 binding sites at the CBF1-regulated Cp [18] and LMP2Ap ([17] and Fig. 4C) of EBV. These patterns were specific either for the active or inactive promoters and were not affected by the differences in the flanking sequences. Interestingly, the DMS *in vivo* footprint patterns of the CBF1 sites we observed at the active promoters were similar to the methylation interference analysis patterns obtained previously with nuclear extracts from an LCL [29], validating further the specificity of DMS *in vivo* footprinting. Because the core sequences (GTGGGAA) of the CBF1 sites at the CD23a core promoter, Cp and LMP2Ap are identical, the lack of characteristic footprints at the intermediate-affinity CBF1 site of CD23a core promoter (Fig. 4A), despite their presence at the CBF1 sites of LMP2Ap (Fig. 4C) in our experiments, indicate that *in vivo* EBNA-2 does not bind to, and induce CD23a through the intermediate-affinity CBF1 site. This conclusion is in contrast to previous *in vitro* results [12], but is in line with the study of Spender et al. [30], which did not identify CD23 as a simple direct target of EBNA-2, and with the results of Wang et al. [6], which suggested that EBNA-2 may transactivate a regulatory element common to both CD23 promoters. A candidate for such a function is the intronic EBV-responsive enhancer. However, we could not find any significant sign of *in vivo* protein binding at this intronic sequence either. Therefore we propose that this intronic element may not play a role in the transactivation of CD23a by EBV, although we cannot rule out the possibility, that proteins binding to this region may be invisible to DMS *in vivo* footprinting. In summary, our *in vivo* results support the complex model of CD23 induction by EBV [6,30], rather than a simple model with direct transactivation of CD23 by EBNA-2 [12].

Nucleotide sequence accession numbers: Sequences of CD23 regulatory regions were deposited in GeneBank/EMBL/DDJB with accession numbers FN597065-110.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.127.

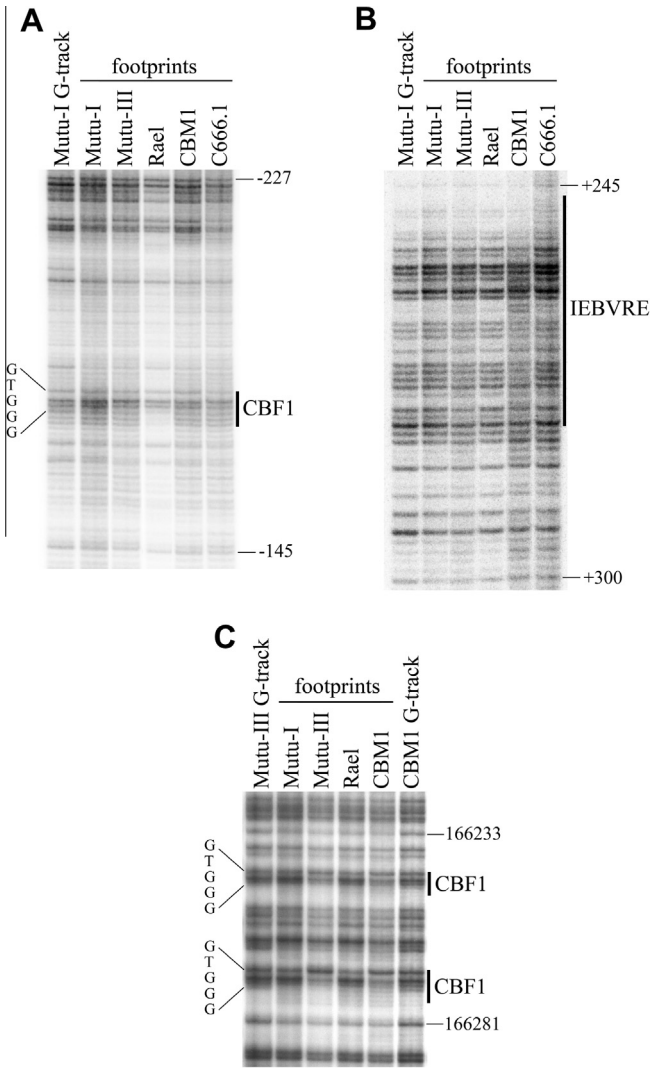


**Fig. 4.** DMS *in vivo* footprinting analyses of CD23a regulatory regions and LMP2Ap. DMS *in vivo* footprinting analyses at the intermediate-affinity CBF1 binding site of the CD23a core promoter (A), intronic EBV-responsive enhancer sequence of CD23a (B), and CBF1 sites of LMP2Ap (C). Numbers at the right of panel A and B indicate positions of nucleotides relative to the transcriptional start site of CD23a. Numbers at the right of panel C are given according to the B95-8 sequence [31]. Published regulatory elements are indicated by vertical bars. Each footprinting reaction was repeated twice with different batches of cells. Representative autoradiograms are shown. Abbreviations: CBF1, CBF1 binding site; CBM1, CB-M1-Ral-STO; IEBVRE, intronic EBV-responsive enhancer; Mutu-I, Mutu-BL-I-cl-216; Mutu-III, Mutu-BL-III-cl-99.

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