Binding of CCCTC-binding factor in vivo to the region located between Rep* and the C promoter of Epstein–Barr virus is unaffected by CpG methylation and does not correlate with Cp activity

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In this study, the binding of the insulator protein CCCTC-binding factor (CTCF) to the region located between Rep* and the C promoter (Cp) of Epstein–Barr virus (EBV) was analysed using chromatin immunoprecipitation and in vivo footprinting. CTCF binding was found to be independent of Cp usage in cell lines corresponding to the major EBV latency types. Bisulfite sequencing and an electrophoretic mobility-shift assay (using methylated and unmethylated probes) revealed that CTCF binding was insufficient to induce local CpG demethylation in certain cell lines and was unaffected by CpG methylation in the region between Rep* and Cp. In addition, CTCF binding to the latency promoter, Qp, did not correlate with Qp activity.

CCCTC-binding factor (CTCF) is an evolutionarily conserved zinc-finger nuclear phosphoprotein that binds to target sites of approximately 50 bp that have remarkable sequence variation. The formation of different CTCF–DNA complexes results in distinct functions, including gene activation, repression and chromatin insulation (Ohlsson et al., 2001). DNA methylation prevents CTCF binding to many CpG-containing CTCF target sites. However, certain sites do not contain CpG dinucleotides and cannot be regulated by DNA methylation. Thus, CTCF sites can be divided into two functional categories based on their susceptibility to epigenetic modulation of binding through DNA methylation. However, CTCF has been shown to prevent spreading of CpG methylation and therefore to protect nearby promoters from silencing by keeping them free of DNA methylation (Filippova, 2008).

The C promoter (Cp) of Epstein–Barr virus (EBV) is a lymphoid-specific promoter active only in type III cell lines, whereas the Q promoter (Qp) is active in type I and type II latency (Liebowitz, 1998). Two recent reports from the same laboratory (Chau et al., 2006; Day et al., 2007) reported strong CTCF binding in type I Burkitt’s lymphoma (BL) cell lines and weak CTCF binding in a lymphoblastoid cell line (LCL) at a region located between Rep* and Cp (Rep*–Cp; nt 10515–10560; Chau et al., 2006) of EBV. However, these two reports are at variance with each other with regard to in vivo binding of CTCF at this region in Raji cells, which is an unusual type III BL cell line with inactive C and W promoters (Woisetschlaeger et al., 1989; Walls & Perricaudet, 1991; Table 1). Chau et al. (2006) reported weak in vivo CTCF binding, whilst Day et al. (2007) showed strong in vivo CTCF binding at Rep*–Cp in Raji cells. A further difference between the reports is the function of CTCF binding at Rep*–Cp as either a repressor (Chau et al., 2006) or an activator (Day et al., 2007). As a comparative study including Raji and latency type II cells may help to distinguish whether in vivo binding of CTCF at Rep*–Cp correlates with latency type (Chau et al., 2006) or with the inactivity of Cp (Day et al., 2007), we analysed the in vivo CTCF binding at Rep*–Cp and at the upstream region of Qp and the EBV-encoded RNA 1 (EBER1) promoter (5’EBER1p) in six well-characterized EBV-positive cell lines covering all latency types [Mutu-BL-I-Cl-216 and Mutu-BL-III-Cl-99 (subclones of the BL line Mutu; Gregory et al., 1990), Rael,
Table 1. Promoter utilization of cell lines

The cell lines shown in the table contain only tightly latent episonal EBV genomes as tested by terminal-repeat analysis (Takacs et al., 2001; Bakos et al., 2007; J. Minorovits, unpublished data). Furthermore, Western blot analysis revealed that early antigens associated with productive EBV replication could not be detected in the cell lines or in clones and tumour tissues throughout our experiments. For Cp, + indicates that the relative amount of Cp-initiated transcript (normalized to β-actin) was >90 % and – indicates that it was <0.5 % of that observed in CB-M1-Ral-STO cells as detected by real-time RT-PCR (Bakos et al., 2007; unpublished observation). For Wp, – indicates that the relative amount of Wp-initiated transcript (normalized to β-actin) was <0.5 % of that observed in Daudi cells (carrying a virus strain that has a deletion of the EBNA2 gene; Jones et al., 1984) as detected by real-time RT-PCR (unpublished observation). For Qp, + indicates that the relative amount of Qp-initiated transcript (normalized to β-actin) was >75 % and – indicates that it was <0.5 % of that observed in Mutu-Bl-1-CI-216 cells detected by real-time RT-PCR (Bakos et al., 2007; unpublished observation). Numbers show the amount of EBER1 and EBER2 RNAs normalized to β-actin relative to their level observed in C666-1 cells detected by real-time RT-PCR as described previously (Banati et al., 2008). LCL-721 and IARC-171 are immortalized by the B95-8 EBV strain (Kavathas et al., 1980; Lenoir et al., 1985); NPC-C15 and NPC-C18 were derived from a primary nasopharyngeal carcinoma (NPC) tumour and metastatic NPC tissue, respectively, and propagated in nude mice (Busson et al., 1988). ND, Not determined.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cp (Qp)</th>
<th>Wp (Qp)</th>
<th>EBER1</th>
<th>EBER2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I BL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutu-Bl-1-CI-216</td>
<td>–</td>
<td>–</td>
<td>12</td>
<td>36.7</td>
</tr>
<tr>
<td>Rael</td>
<td>–</td>
<td>–</td>
<td>17.9</td>
<td>302.2</td>
</tr>
<tr>
<td><strong>Group III BL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutu-Bl-III-CI-99</td>
<td>+</td>
<td>–</td>
<td>1.7</td>
<td>29</td>
</tr>
<tr>
<td>Raji</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>61.6</td>
</tr>
<tr>
<td>Daudi</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>LCL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB-M1-Ral-STO</td>
<td>+</td>
<td>–</td>
<td>1.1</td>
<td>22.5</td>
</tr>
<tr>
<td>LCL-721</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IARC-171</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KR4</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>NPC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C666-1</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NPC-C15</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NPC-C18</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Also analysed by S1 nuclease assay (Woitschlaeger et al., 1989; Walls & Perricaudet, 1991; Altiok et al., 1992).
other proteins in addition to CTCF may also bind to this region in vivo.

As the binding sites for CTCF map to methylation-free domains genome-wide (Mukhopadhyay et al., 2004) and DNA methylation prevents CTCF binding to many CpG-containing CTCF target sites (Filippova, 2008), we wished to analyse the methylation patterns of Rep*–Cp and its surrounding regions in well-characterized lymphoid and nasopharyngeal carcinoma (NPC) cell lines and tumour tissues by automated genomic sequencing of sodium bisulphite-treated DNAs and compare these methylation patterns with the results of the CTCF ChIP (Fig. 1a) and DMS in vivo footprinting (Fig. 1b) experiments. Bisulfite genomic sequencing was carried out essentially as described previously (Salamon et al., 2000, 2001, 2003) with direct sequencing of the PCR products generated by the outer primer pair 5′-GTGTGAGAATAAGAGTAAAGTTGTG-3′ and 5′-ACACTAACCTCTCAACTAATTTCTAC-3′ and the inner primer pair 5′-GTAAAACGACGGCCAGT-
CCCACCTACCACTTATCC-3' and 5'-'-biotin-GAGAA-TAAGAGTAAAGTTGGAATAG-3'. Previously, we and others showed that Qp with strong in vivo CTCF binding (Day et al., 2007; Fig. 1a) is unmethylated in all latency types, independent of its activity (Schaefer et al., 1997; Tao et al., 1998; Salamon et al., 2001; Bakos et al., 2007), and our analysis showed that, consistent with previous reports (Minarovits et al., 1992; Robertson & Ambinder, 1997), the coding and upstream regulatory regions of the constitutively active (Minarovits et al., 1992) and methylation-
sensitive (Banati et al., 2008) EBER genes contained only minimally methylated (0–25 %) or unmethylated CpGs between nt 6378 and 7032 in the Mutu-BL-I-Cl-216, Mutu-BL-III-Cl-99, Rael, CB-M1-Ral-STO, LCL-721, Raji and C666-1 cell lines detected by bisulfite genomic sequencing (data not shown). Surprisingly, our analysis of Rep*–Cp and its surrounding regions (nt 10077–10624) containing the CTCF-binding site showed a variable level of methylation in the cell lines examined (Figs 2a, b), as all lymphoid cell lines (with the exception of Raji) carried only minimally methylated or unmethylated CpGs between nt 10366 and 10584, whereas the majority of CpGs in this region were highly methylated (>50 %) in Raji, C666-1, NPC-C15 and NPC-C18.

The association of strong in vivo CTCF binding with DNA hypomethylation at Rep*–Cp and its surrounding regions in the type I BL cell line Rael is consistent with the observation that CTCF-binding sites map to methylation-free domains genome-wide (Mukhopadhyay et al., 2004). On the other hand, the presence of highly methylated CpGs in Rael is consistent with the observation that CTCF-binding sites map to methylation-free domains genome-wide (Mukhopadhyay et al., 2004). The medium level of methylation at Rep*–Cp and its surrounding regions (nt 10077–10624) containing the CTCF-binding site showed a variable level of methylation in the cell lines examined (Figs 2a, b), as all lymphoid cell lines (with the exception of Raji) carried only minimally methylated or unmethylated CpGs between nt 10366 and 10584, whereas the majority of CpGs in this region were highly methylated (>50 %) in Raji, C666-1, NPC-C15 and NPC-C18.

Until now, only a few important regulatory elements [the EBER regulatory region (Minarovits et al., 1992; Robertson & Ambinder, 1997; see above), the dyad symmetry region of the latent origin of replication (Falk et al., 1998; Salamon et al., 2000) and Qp (Schaefier et al., 1997; Tao et al., 1998; Salamon et al., 2001)] have proved to be hypomethylated or unmethylated in Rael, a type I BL cell line with highly CpG methylated latent promoters and coding sequences (Ernberg et al., 1989; Minarovits et al., 1991; Robertson et al., 1995; Schaefier et al., 1997; Falk et al., 1998; Niller et al., 2001; Salamon et al., 2001, 2003; Takacs et al., 2001). Therefore, the observation of a hypomethylated region located between the highly methylated Rep* and Cp in Rael is remarkable, and points to the importance of this region as a regulator of EBV latent gene expression.

The CTCF-binding site at Rep*–Cp contains two CpG dinucleotides and these two CpGs are highly methylated in Raji and C666-1 cells (Figs 2a, b), despite the medium level of in vivo CTCF binding observed by ChIP in C666-1 cells (Fig. 1a). To confirm that CTCF can indeed bind to this site independently of the CpG methylation status of its target, we performed an electrophoretic mobility-shift

**Fig. 2.** Methylation patterns of Rep*–Cp and its surrounding regions in latent EBV genomes and the effect of DNA methylation on CTCF binding at Rep*–Cp. (a) Typical nucleic acid sequences of bisulfite-modified DNAs at Rep*–Cp and its surrounding regions in the lymphoid and NPC cell lines indicated. Numbers adjacent to vertical dotted lines indicate the positions of cytosines within CpG dinucleotides, based on the B95-8 sequence (Baer et al., 1984). The black bar indicates the CTCF-binding site (Chau et al., 2006). Green line, adenine; blue line, cytosine; grey line, guanine; red line, thymine. (b) Summary of methylation patterns in the sequenced region between Rep* and Cp (nt 10077–10624). Numbers and sticks indicate the positions of cytosines within CpG dinucleotides, based on the B95-8 sequence (Baer et al., 1984). 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 %. The shading represents the CTCF-binding site (Chau et al., 2006). KR4 DNA was completely unmethylated in the examined area and is not represented in the figure. All cytosines outside the CpG dinucleotides were found to be unmethylated in all cell lines in the region examined. Mutu I, Mutu-BL-I-Cl-216; Mutu III, Mutu-BL-III-Cl-99; CB-M1, CB-M1-Ral-STO. (c–e) Electrophoretic mobility-shift assay: binding of nuclear proteins from Mutu-BL-I-Cl-216 cells to the unmethylated (c) or CpG-methylated (d) DNA sequence of Rep*–Cp and its closely surrounding regions (Rep*–Cp probe). Lanes: 1, labelled Rep*–Cp probe only, no protein added; 2–5, labelled Rep*–Cp probe with nuclear extract added; 3, competition with a 50-fold excess of unlabelled Rep*–Cp probe; 4, competition with a 50-fold excess of unlabelled non-specific probe (Rep*–Cp probe); 5, 1 µl anti-CTCF antibody (07-729; Upstate Biotechnology) added. Protein–DNA complexes are indicated by broken (supershift) or solid arrows. The same results were obtained with nuclear extract from Mutu-BL-III-Cl-99 (data not shown). (e) Sequence of the Rep*–Cp probe. The sequence of Rep*–Cp is shaded and the CpG dinucleotides are underlined.
assay and antibody supershift experiments with nuclear extracts prepared from Mutu-BL-I-Cl-216 and Mutu-BL-III-Cl-99 cells (Figs 2c–e and data not shown). The nuclear extract was prepared as described previously (Niller et al., 2003). Probes were generated by PCR using LCL-721 DNA and kinase-labelled primers corresponding to nt 10485–10506 and 10644–10625 (Rep*–Cp probe) and nt 103468–103492 and 103666–103641 (non-specific competitor probe). [Primer coordinates refer to the B95-8 sequence (Baer et al., 1984)]. The Rep*–Cp probe was methylated using M.SsI CpG methyltransferase (New England BioLabs). The efficiency of methylation was confirmed by using the isochromizers HpaII and MspI. Gel-retardation assays were performed as described previously (Niller et al., 2003) with the following modifications: 1 μg crude nuclear protein was incubated with 150 ng calf thymus DNA, 1 ng labelled Rep*–Cp probe and a 50-fold excess of unlabelled competitor probe. Protein–DNA complexes were resolved by electrophoresis on native 5% polyacrylamide gels (acrylamide : bis-acrylamide 69 : 1). The results showed that methylation of the CpG dinucleotides in and around the CTCF-binding site at Rep*–Cp did not inhibit CTCF binding. Furthermore, the simultaneous detection of weak in vivo CTCF binding and hypomethylation at Rep*–Cp in the Mutu clones and in CBM1-Ral-STO also suggested that DNA methylation does not play a role in the regulation of CTCF binding at Rep*–Cp.

In conclusion, we found that binding of CTCF to its target site located between Rep* and Cp is independent of Cp activity and latency type in lymphoid and epithelial cells carrying latent EBV genomes. Although CTCF binding has been suggested to prevent CpG methylation, we observed a high level of CpG methylation at Rep*–Cp and its surrounding regions in Raji and NPC cells. Thus, CTCF binding may be insufficient to induce local CpG demethylation within and around certain target sequences. We also found that in vitro CpG methylation did not affect CTCF binding to Rep*–Cp, despite the fact that this CTCF target site contains two CpG dinucleotides. We also noticed that CTCF binding to the invariably unmethylated latency promoter Qp did not correlate with activity of the promoter. Further studies are needed to clarify the exact role of CTCF and other nuclear proteins binding to the region located between Rep* and Cp in EBV latency.

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