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The 5′ regulatory sequences of active *miR-146a* promoters are hypomethylated and associated with euchromatic histone modification marks in B lymphoid cells

Kalman Szenthe [a,b], Anita Koroknai [a], Ferenc Banati [a,b], Zoltan Bathori [a], Rita Lozsa [c], Jozsef Burgyan [c], Hans Wolf [d], Daniel Salamon [e], Katalin Nagy [f], Hans Helmut Niller [d], Janos Minarovits [a,g,*]

[a] Microbiological Research Group, National Center for Epidemiology, Piheno ut 1, H-1529 Budapest, Hungary
[b] RT-Europe Nonprofit Research Center, Var 2, Szigetkoz Building, H-9200 Mosonmagyarovar, Hungary
[c] Agricultural Biotechnology Center, Szent-Györgyi Albert u. 4, H-2100 Gödöllö, Hungary
[d] Institute for Medical Microbiology, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany
[e] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Nobels väg 16, S-17177 Stockholm, Sweden
[f] Department of Oral Surgery, Faculty of Dentistry, University of Szeged, Tisza Lajos krt 64, H-6720 Szeged, Hungary
[g] Department of Oral Biology and Experimental Dental Research, Faculty of Dentistry, University of Szeged, Tisza Lajos krt. 64, H-6720 Szeged, Hungary

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ABSTRACT

Although the microRNA miR-146a is an important regulator of immunological processes and contributes to the pathogenesis of certain B cell lymphoma types, in B cells the epigenetic regulation of miR-146a expresion has not been studied yet. To elucidate the mechanisms controlling miR-146a expression in B lymphoid cells we analysed epigenetic marks, including CpG methylation and histone modifications, at the *miR-146a* promoter in well characterized Epstein-Barr virus (EBV) positive and EBV negative B cell lines. In addition, EBV positive epithelial cell lines were also studied as controls. In cells with a silent miR-146a promoter the 5′ regulatory sequences comprising a CpG island were devoid of activating histone modifications, independently of the methylation pattern of the regulatory region. The regulatory sequences flanking the inactive *miR-146* promoter were hypermethylated at CpG dinucleotides in the EBV positive Burkitt's lymphoma (BL) cell lines of memory B cell phenotype (Rael and Akata), partially methylated in the mammary carcinoma cell lines C2G6 and C4A3, and completely unmethylated in the nasopharyngeal carcinoma cell line C666-1. In contrast, in EBV positive cell lines of activated B cell phenotype, and EBV negative BL cell lines the invariably unmethylated 5′ regulatory sequences of active *miR-146a* promoters were enriched in the euchromatic histone modification marks acetylated histone H3, acetylated histone H4, and histone H3 dimethylated at lysine 4. The euchromatic histone modification marks extended over the immediate vicinity of the transcriptional initiation site to the 3′ intron, too. We concluded that similarly to the promoters of protein coding genes, both DNA methylation and histone modifications contribute to the host cell dependent expression of miR-146a.

1. Introduction

In mammals, microRNAs (miRNAs) modulate messenger RNA (mRNA) and protein levels by binding to and destabilizing their target mRNAs or suppressing target mRNA translation [1,2]. Primary miRNA transcripts (pri-miRNAs) are typically transcribed by RNA polymerase II. and processed in the nucleus to precursor miRNAs (pre-miRNAs) by the RNase III enzyme Drosha. In the cytoplasm, pre-miRNAs are converted into small RNA duplexes and the mature, single stranded miRNA (the guide strand) is loaded to the RNA induced silencing complex (RISC). miRNAs usually target the 3′ untranslated region (3′ UTR) of mRNAs reviewed in [3].

Post-transcriptional fine-tuning of mRNA and protein levels by miRNAs plays an important role in developmental and immune regulatory processes [3–6]. Accordingly, dysregulation of miRNA expression may have pathological consequences resulting in tumorigenesis and the onset of autoimmune disorders [7–11]. The microRNA miR-146a, a regulator of both innate and adaptive immune responses [4,12] was implicated both in lymphomagenesis and tumor suppression. An oncogenic function was attributed to miR-146a due to its overexpression in splenic marginal zone lymphomas [13] and in T cell lines infected with human T-cell leukemia virus (HTLV-1) [14]. In addition, in formalin fixed/paraffin embedded lymphoma samples from patients with diffuse large B-cell lymphoma (DLBCL) the level of miR-146a had a prognostic value: low levels were associated with a higher complete remission rate [15]. In contrast, in extranodal NK/T cell lymphoma (NKTL) miR-146 acted like a tumor suppressor: low miR-146a

* Corresponding author at: Department of Oral Biology and Experimental Dental Research, Faculty of Dentistry, University of Szeged, Tisza Lajos krt. 64, H-6720 Szeged, Hungary. Fax: +36 62 545 301.
E-mail address: minimicrobi@hotmail.com (J. Minarovits).

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expression was associated with a poor prognosis and overexpression of miR-146a inhibited the proliferation of NKTL cell lines *in vitro* [16]. Downregulation of miR-146a expression in NKTLs and NKTL cell lines was associated with hypermethylation of the miR-146a promoter [16]. Similarly to vertebrate genomes, the gemomes of certain vertebrate viruses, including DNA tumour viruses, also encode miRNAs modulating the level of both viral and cellular mRNAs reviewed in [17]. In addition, DNA tumour viruses may affect the miRNA profile of their host cells *via* viral oncoproteins that up-regulate or down-regulate distinct cellular miRNAs [18–22]. Latent membrane protein 1 (LMP1), a transmembrane oncoprotein encoded by the genome of the human gammaherpesvirus Epstein-Barr virus (EBV), induced the expression of a series of cellular microRNAs [18,23–28] including miR-146a, and down-regulated miR-203, a miRNA expressed specifically in epithelial cells [29]. All of these changes were implicated in the genesis or progression of EBV-associated neoplasms.

In spite of the physiological role of miR-146a in immunological processes and its potential contribution to the development of B cell lymphomas the epigenetic changes associated with switching off or switching on of *miR-146a* gene promoter have not been extensively studied in B lymphoid cells. We analysed therefore the epigenetic marks including CpG methylation and histone modifications at the *miR-146a* promoter in well characterised EBV positive and EBV negative lymphoid and epithelial cell lines expressing various levels of pri- and mature miR-146a. The epigenetic marks at active *miR-146a* promoters were compared to those associated with silent *miR-146a* promoters. We observed that hypermethylated *miR-146a* promoters were completely silent and devoid of activating histone modifications. In contrast, active *miR-146a* promoters were unmethylated and enriched in the euchromatic histone modification marks acetylated histone H3, acetylated histone H4 and histone H3 dimethylated at lysine 4. We concluded that similarly to the promoters of protein coding genes, both DNA methylation and histone modifications contribute to the host cell dependent expression of miR-146a.

2. Materials and methods

2.1. Cell lines and tissue culture

Well characterized B cell and epithelial cell lines were analysed (Table 1.). Cells were maintained *in vitro* as described earlier [30,31].

2.2. Nuclear run-on assay

The level of pri-miR-146a was assessed using a nuclear run-on assay [32]. Briefly, nuclear extracts were prepared from isolated nuclei of the cell lines and transcription was initiated using a mixture of ATP, CTP, GTP and biotin-labeled-UTP. RNA was isolated, and the RNA containing incorporated biotin-labeled UTP was purified using streptavidin-coated magnetic beads. After reverse transcription with random hexamers, real-time PCR was performed using miR-146a specific primers (Metabion, Germany) (Table 2.). The levels of pri-miR-146a were normalized to GAPDH mRNA levels.

Table 1
Characteristics of the cell lines analysed.

Designation	Cell type	EBV	Expression of LMP1 protein	Ref.
BL41	BL	–	–	[45]
BJAB	BL	–	–	[46]
DG75	BL	–	–	[47]
CBM1-Ral-STO	LCL	+	+	[48,49]
BL41-E95B	BL	+	+	[45]
Raji	BL	+	+	[50]
Rael	BL	+	–	[48,49]
Akata	BL	+	–	[51]
C666.1	NPC	+	–	[52]
C2G6	Breast cc	+	–	[53]
C4A3	Breast cc.	+	–	[53]

EBV: Epstein-Barr virus; LMP1: latent membrane protein 1; BL: Burkitt's lymphoma; LCL: lymphoblastoid cell line; NPC: nasophyryngeal carcinoma; Breast cc.: breast carcinoma.

Table 2
Oligonucleotides used in this study.

PCR primers, control DNA sequencing:	
Regulatory region and first exon	F1: 5′-GGCAGAGTAATGCAGTGTACCAG-3′ R2: 5′-CCCTAATCCCACAGCAATCATC-3′ F10: 5′-CCTCAATTCAAATCCTGGCTCTG-3′ F3: 5′-GGCACCCATGGAAACACTTAC-3′ R4: 5′-CCAGGCTTCCTGTCTGTACTCTC-3′ F6: 5′-GGTGCTCAGGAAGATTTCTCAGT-3′ R5: 5′-CCCTCTTGCAGCACGTGTCAGG-3′ F8: 5′-CCTGACACGTGCTGCAAGAG-3′ R7: 5′-CAGCTAAGGCCTATTTGCATATG-3′ R9: 5′-GTGCCATTTACTCTGCAATAATC-3′
Second exon	F11: 5′-GGACAGGCCTGGACTGCAAC-3′ R12: 5′-TAGCTACTTGGAACCCTGCTTAGC-3′
Primers for nuclear run-on assay:	
miR-146a	F: 5′-CGATGTGTATCCTCAGCTTTG-3′ R: 5′-GGATCTACTCTCTCCAGGTCCTC-3′
GAPDH	F: 5′-GGAAGGTGAAGGTCGGAGTCA-3′ R: 5′-ATGGGTGGAATCATATTGGAACA-3′
Primers for PCR amplification of bisulfite-modified DNA:	
miR-146a outer	F: 5′-GTAGAGTGTTTGGTGTTTAGTAGGTG-3′ R: 5′-CCATTTACTCTACAATAATCCACTATAC-3′
miR-146a inner	F1: 5′-Uni-GAGAAAGGTGTTTAGGAAGATTTTTTAG-3′ F2: 5′-Uni-GAGAGTATAGATAGGAAGTTTGG-3′ F3: 5′-Uni-GAGGTTTTGGTTGAAATTTAGTTTG-3′ F4: 5′-Uni-GGAATAAAAGTATATGTAAATAGG-3′ F5: 5′-Uni-GAGAAGTTGATATTGTTAGGTTGG-3′ R6: 5′-Biotin-CCACTATACAAAAACCTACTACCTCTC-3′
Primers for ChIP assay:	
miR-146a regulatory region (covering nucleotides −1160 to −1010)	F: 5′-GTGACTACATCTGCCTGGAAGC-3′ R: 5′-CAAACTGGAAAGAACGTACAAGG-3′
miR-146a initiation site and first exon (covering nucleotides −55 to +129)	F: 5′-CATATGCAAATAGGCCTTAGCTG-3′ R: 5′-TGTCTCCTGTCCATCCTGTCC-3′
miR-146a first intron (covering nucleotides +414 to +588)	F: 5′-GTGACTTGGAGATATTTCCAGTGC-3′ R: 5′-CAGTCTACAACTCCAGGTTTAAAGC-3′

Oligonucleotides were purchased from Metabion (Martinsried, Germany). Uni: M13 universal primer sequence (5′-GTAAAACGACGGCCAGT-3′).

2.3. Northern blotting

Northern blot analysis was performed as described earlier [33,34], using the following P^{32} labeled LNA (locked nucleic acid) probes (Exiqon, Denmark): for miR-146a, 5′-AACCCATGGAATT-

CAGTTCTCA-3′; for U6 RNA, 5′-GCAGGGGCCATGCTAATCTTCTCTG-TATCG-3′). The sequences of control RNAs (Metabion, Germany) used were 5′-CUUUGAGAACUGAAUUCCAUGGGUU-3′ for miR-146a, and 5′-CUGAGAACUGAAUUCCAUAGGCUG-3′ for miR-146b.

2.4. DNA sequencing

miR-146a sequences were determined using established procedures [35] with primers (Metabion, Germany) listed in Table 2. (GenBank accession numbers: JX991284-JX991307). The sequences were analysed using a CpG island finder software (Methyl Primer Express v1.0, Applied Biosystems) with the following parameters: 300 bp minimum and 2000 bp maximum length of island, C + Gs/total bases >50%, CpG observed/ CpG expected >0,6.

2.5. Automated genomic sequencing of sodium bisulfite-treated DNA

Modification of DNA and sequencing of bisulfite-modified DNA samples was performed as described in our earlier studies [30,35].

2.6. Chromatin immunoprecipitation

Chromatin immunoprecipitation of modified histones was done as described earlier [36,37] using antibodies specific for diacetylated histone H3 (AcH3), tetraacetylated histone H4 (AcH4) and histone H3 dimethylated at lysine 4 (H3K4me2). The primers used for quantitative real-time PCR to amplify 3 different regions of *miR-146a* are shown in Table 2.

3. Results

3.1. Host cell dependent expression of primary and mature miR-146a

Cell lines carrying latent EBV genomes expressed pri- and mature miR-146a in a host cell dependent manner. We could not detect pri- or mature miR-146a in the EBV positive Burkitt's lymphoma (BL) lines (Rael, Akata) that resemble memory B cells, and EBV positive mammary carcinoma cell lines (C2G6, C4A3) (Fig. 1A and B). The nasopharyngeal carcinoma cell line C666-1

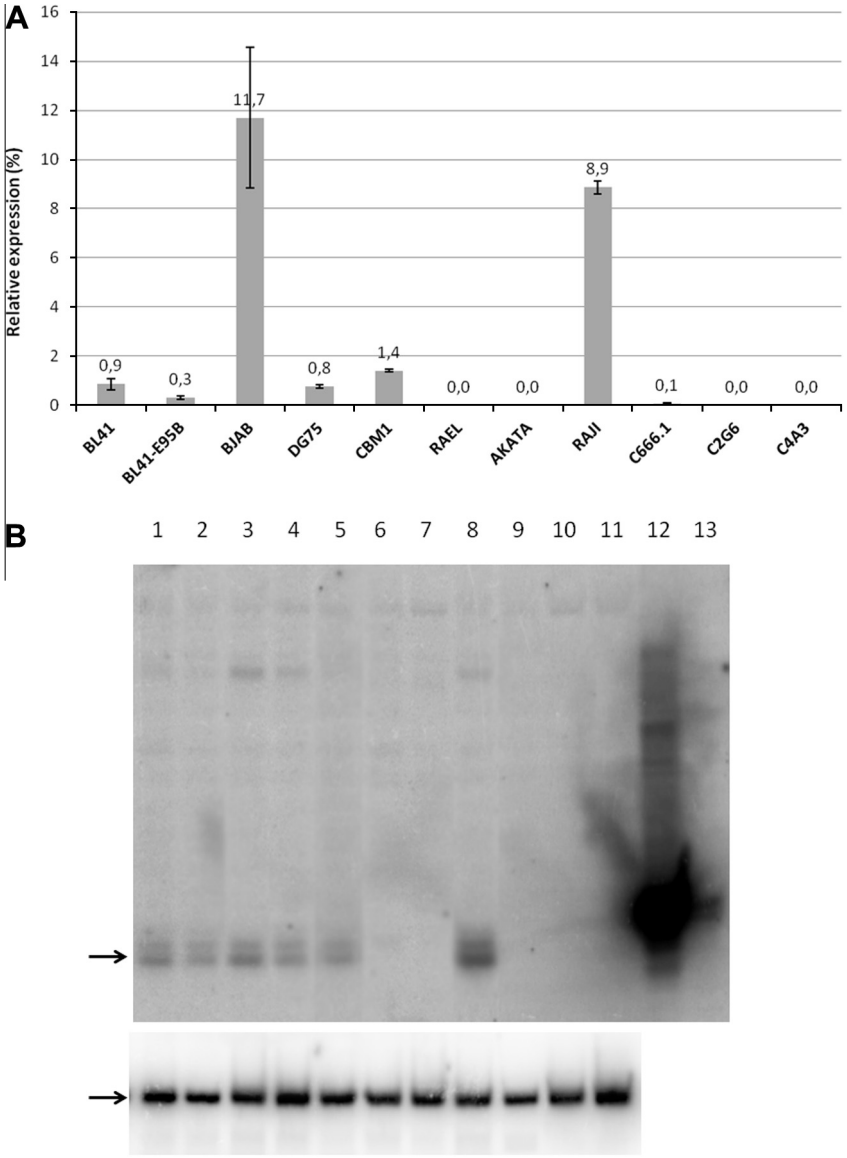

Fig. 1. Expression of pri- and mature miR-146a microRNA in B cell lines and epithelial cell lines. (A) Shows analysis of pri-miR-146a expression relative to the expression of GAPDH mRNA using a nuclear run-on assay. (B) Northern blot analysis of mature miR-146a microRNA expression. Lanes: 1: BL41; 2: BL41-E95B; 3: BJAB; 4: DG75; 5: CBM1-Ral-STO; 6: RAEL; 7: Akata; 8: Raji; 9: C666.1; 10: C2G6; 11: C4A3; 12: synthetic control miR-146a RNA; 13: synthetic control miR-146b RNA. Top: hybridisation using a P^{32}-labeled miR-146a specific probe; the position of mature miR-146a is marked by arrow. Bottom: hybridization using a P^{32}-labeled U6 RNA specific-probe.

expressed only a minimal amount of pri-miR-146a but not the mature form. In contrast, the EBV positive lymphoblastoid cell line CB-M1-Ral-STO and the BL line Raji of activated B cell phenotype expressed both the primary and the mature form of miR-146a (Fig. 1A and B). Both CB-M1-Ral-STO and Raji express LMP1, an EBV-encoded latent membrane protein capable to activate *miR-146a* expression [21,26]. Surprisingly, however, pri- and mature miR-146a expression could also be detected in the EBV negative Burkitt's lymphoma cell lines (BL41, BJAB, DG75). Comparison of BL41 with its EBV-converted derivative, BL41-E95B, indicated that the acquisition of latent EBV genomes *in vitro* did not upregulate the level of pri-miR-146a (Fig. 1).

3.2. CpG methylation analysis of the regulatory region of the miR-146a promoter

The 5′ regulatory sequences flanking silent *miR-146a* promoters were highly methylated in Rael and Akata and moderately methylated in C2G6 and C4A3 (Fig. 2). In contrast, the regulatory region that includes a 455 bp long CpG island (nucleotides −631 to −176). was completely unmethylated in lymphoid cells actively using the *miR-146a* promoter. These data suggested that CpG methylation may contribute to the silencing of *miR-146a*. We noticed, however, that the regulatory sequences of the silent promoter in C666-1 cells were also completely unmethylated (Fig. 3), indicating that epigenetic mechanisms other than CpG methylation also affect *miR-146a* promoter activity.

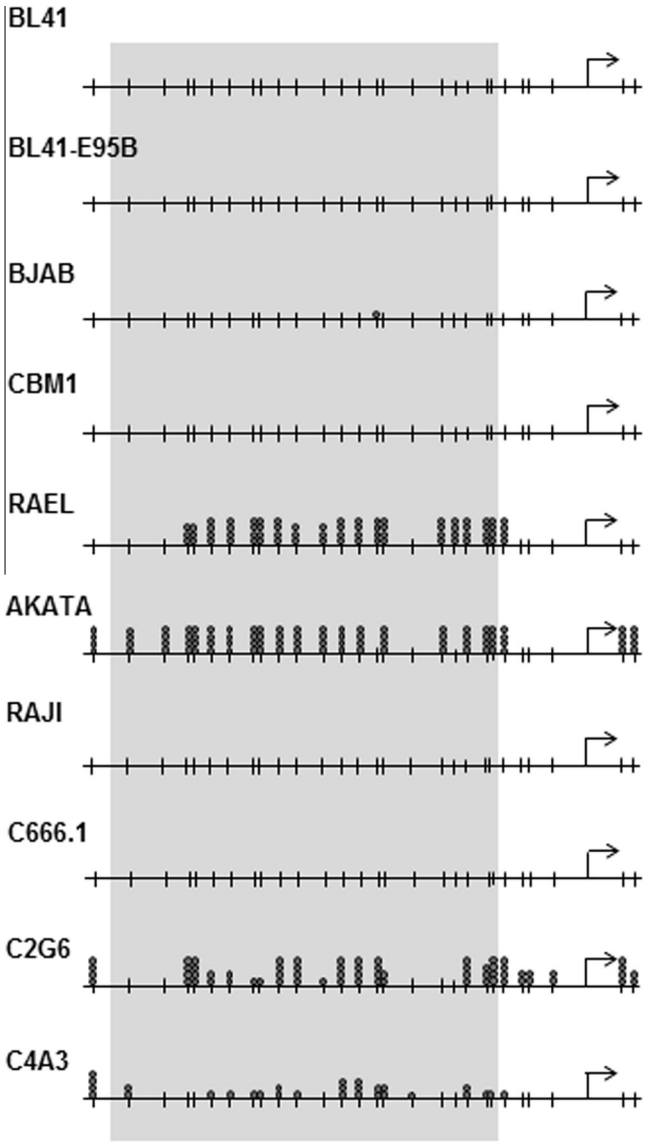

Fig. 2. CpG methylation analysis of the *miR-146a* regulatory sequences in B cell lines and epithelial cell lines. The degree of methylation of cytosines is indicated by the number of the dots as follows: stick only, 0%; one dot, 0–25%; two dots, 25–50%; three dots, 50–75%; four dots, 75–100%. A shaded area indicates a 455 bp long CpG island located in the regulatory region of miR146a (nucleotides −631 to −176).

3.3. Analysis of activating histone modifications at the miR-146a promoter

Similarly to CpG methylation, histone modifications also affect chromatin structure and regulate the activity of protein coding cellular and viral genes. We used chromatin immunoprecipitation followed with real-time PCR to assess the association of modified histones AcH3, AcH4 and H3K4me2 with *miR-146a*. We found that in Rael and Akata the silent, hypermethylated *miR-146a* promoter and its 5′ and 3′ flanking sequences were devoid of AcH3, AcH4 and H3K4me2 (Fig. 3). Activating histone modifications were detectable at low or moderate levels at the silent, moderately methylated *miR-146a* promoters in C2G6 and C4A3. Similarly, the activating histone marks were nearly absent from the silent, unmethylated *miR-146a* promoter in C666-1 cells. In contrast, in lymphoid cells actively using the *miR-146a* promoter, the 5′ regulatory region was highly or moderately enriched in H3K4me2, in combination with high or moderate levels of AcH3 and moderate levels of AcH4 (Fig. 3A). There was no strict correlation, however, between the level of individual euchromatic modifications at the regulatory region and pri-miR-146a expression, indicating that other factors may also modulate the activity of *miR-146a* promoter. The sequences located in the vicinity of the transcriptional initiation site and within the first intron of actively transcribed *miR-146a* genes were also highly enriched in H3K4me2 and AcH3 whereas AcH4 could not be detected in these regions in some of the miR-146a expressing cell lines (Fig. 3B and C). These data suggested that the region enriched in euchromatic histone modification marks at active *miR-146a* promoters extended over the immediate vicinity of the transcriptional initiation site.

4. Discussion

Euchromatic subdomains of the genome are associated with actively transcribed protein coding genes both in animal and plant cells [38,39]. In human CD4 + T cells, transcription of pri-miRNAs from "intergenic" miRNA genes that do not code for protein, as well as pri-miRNAs derived from introns of protein coding genes appeared to be regulated by chromatin modifications similar to those controlling the activity of protein coding gene promoters [40].

There were no similar studies, however, regarding the epigenetic marks of *miR-146a*, a gene implicated in B cell lymphoma development [13,15]. We found that in B cell lines and EBV positive epithelial cell lines the active *miR-146a* promoters were unmethylated and associated with the euchromatic histone modification marks H3K4me2, AcH3 and AcH4. In contrast, silent *miR-146a* promoters were associated with hypermethylated or partially methylated regulatory sequences devoid of or associated with a moderate level of activating histone modifications. Although CpG islands are usually unmethylated, cell tpe specific or tumor specific CpG island hypermethylation was also observed reviewed in [41]. We noticed that in the nasopharyngeal carcinoma cell line C666-1 the inactive *miR-146a* promoter was unmethylated, but lacked euchromatic histone modification marks. This suggesed that the absence of

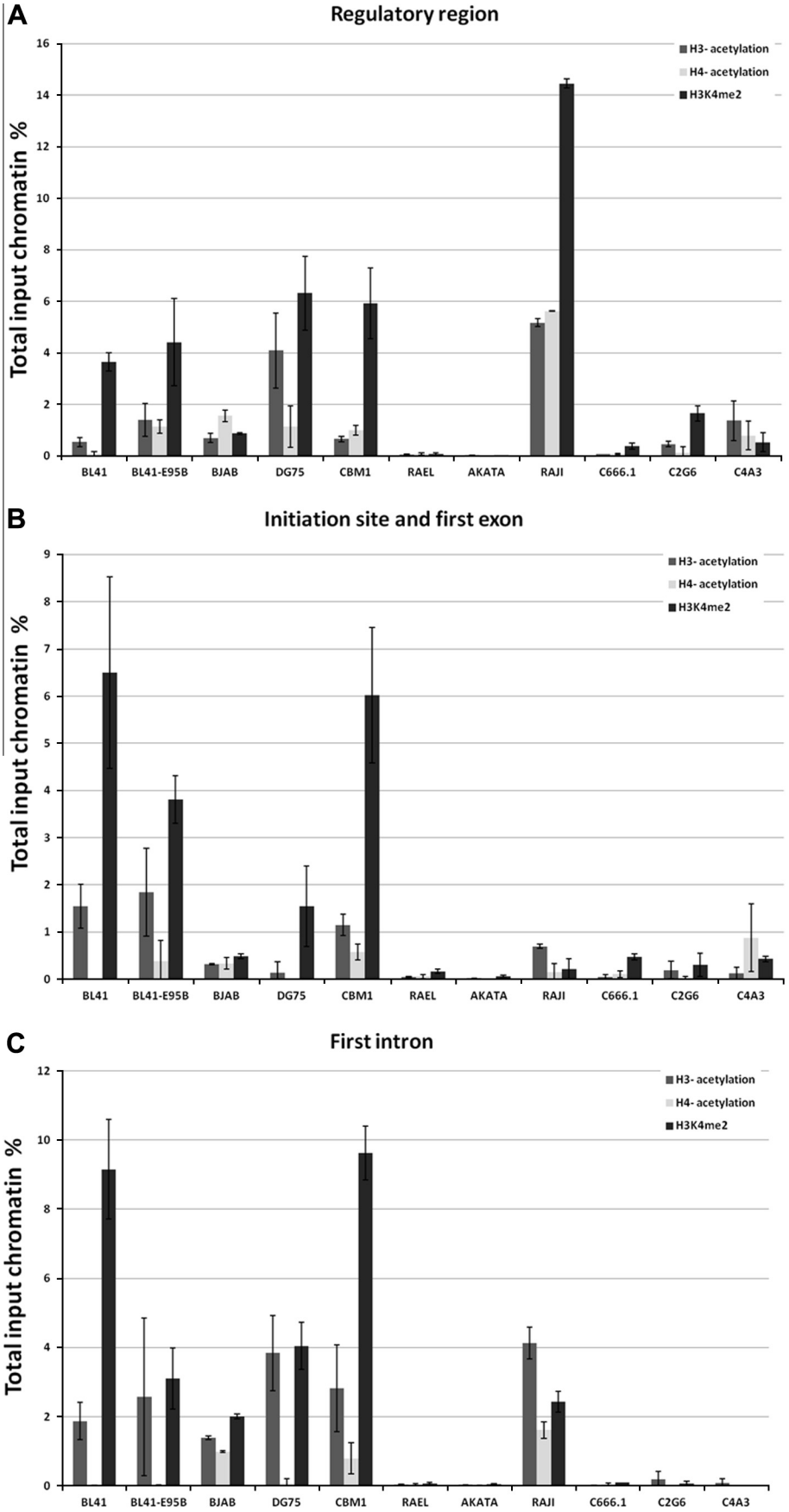

Fig. 3. Analysis of activating histone modifications at the miR-146a promoter and its flanking sequences. Chromatin immunoprecipitation by antibodies directed against diacetylated histone H3, tetraacetylated histone H4 and histone H3 dimethylated at lysine 4 (H3K4me2) was followed by real-time PCR using primers amplifying 3 regions of *miR-146a*: (A), regulatory sequences; (B), sequences covering the transcriptional initiation site and a part of exon 1; (C), a part of the first intron.

CpG methylation is insufficient in itself for switching on the miR-146a promoter.

In B cell lines carrying latent EBV genomes the pattern of viral gene expression, i.e. the so called latency type, depends on the host cell phenotype [42]. In type I latency only a single nuclear protein, EBNA1 is expressed, whereas in type III latency 6 EBNAs and transcripts for three latent membrane proteins, including the mRNA coding for the oncoprotein LMP1, could be detected. LMP1, an inducer of miR-146a expression *via* the NF-κB pathway [21,26] possibly contributed to the expression of pri-miR-146a and miR-146a in the latency type III cell lines CB-M1-Ral-STO and Raji in our experiments, and the lack of LMP1 expression in the latency type I BL lines Rael and Akata may explain the inactivity of the *miR-146a* promoter in the latter cells. However, moderate expression of miR-146a in the EBV negative and LMP1 negative BL cell lines BL41 and DG75 or high miR-146a expression in BJAB cells can't be explained by LMP1 mediated activation of the NF-κB pathway. The level of the RelB subunit of the NF-κB family was low in BJAB cells although it could be upregulated by transfection of the LMP1 gene [43]. Because we detected a high level of miR-146a expression in BJAB cells, comparable to that of Raji cells, we think that NF-κB is not a major factor activating miR-146a transcription BJAB cells. We suggest that in the EBV negative BL lines BL41, BJAB and DG75 that do not express LMP1, a cellular activator other than NF-κB switched on transcription of pri-miR146a. The identity of that cellular factor remains to be established.

We also observed a lower pri-miR-146a expression in BL41-E95B cells, carrying latent EBV genomes, than in the EBV negative BL line BL41. This was unexpected because the converted cells expressed LMP1, an activator of miR-146a transcription. One may consider, however, that in addition to LMP1, other latent EBV proteins may also affect miR-146a expression. The nuclear antigen EBNA2 emerged recently as a negative regulator of miR-146a [44]. Thus, the level and activity of positive and negative viral and cellular regulators may modulate miR-146a level in the individual latency type III EBV positive cell lines.

We found that activating histone modifications were practically absent from silent, hypermethylated miR-146a promoters in BL cell lines. They were present at low or moderate level at the silent *miR-146a* promoters in epithelial cell lines. In contrast, although the level of each activating histone mark varied depending on the chromatin area studied (5′ regulatory sequences; transcriptional initiation site and exon 1; intron 1), active *miR-146a* promoters were located in an extended euchromatic domain enriched in H3K4me2 and frequently also acH3.

In conclusion, our data suggest that similarly to the promoters of protein coding genes, both CpG methylation and euchromatic histone modification marks affect the host cell dependent activity of the *miR-146a* promoter.

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