Distribution of the chromatin protein DEK distinguishes active and inactive CD21/CR2 gene in pre- and mature B lymphocytes

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Abstract
DEK is an abundant and ubiquitous chromatin protein that has only recently attracted attention. DEK preferentially binds to cruciform and superhelical DNA and induces positive supercoils into closed circular DNA. It is quite likely therefore that DEK performs an important architectural function in chromatin. However, it is not known how DEK is distributed in chromatin. As the first study of its kind, we investigate the distribution of DEK at the CD21/complement receptor 2 gene regulatory regions in two B lymphocyte lines, namely Ramos, which expresses the CD21 gene, and Nalm-6, which does not. We use a chromatin immunoprecipitation approach and show that DEK appears to be distributed over various regions of the expressed and silent genes, but occurs in 2- to 3-fold higher amounts at a promoter-proximal site of the expressed gene. Moreover, induction of CD21 expression in Nalm-6 cells leads to accumulation of DEK at this site. We propose that the accumulation of DEK is functionally linked to gene expression.

Introduction
The nuclear protein DEK was discovered in a subset of patients with acute myeloid leukemia where a t(6;9) translocation has led to a fusion of DEK with the nucleoporin CAN (1). Like other nuclear proteins, DEK has been identified as a common antigen in several forms of autoimmune diseases (2–4).

In fact, DEK is an abundant (>10⁶ copies per HeLa cell nucleus) and ubiquitous chromatin protein. The human DEK protein consists of 375 amino acids with a central DNA-binding domain of the SAP/SAF type and several stretches rich in acidic residues. It preferentially binds to cruciform or superhelical DNA and shows little, if any, sequence specificity. A remarkable feature of DEK is its ability to induce positive superhelical turns into SV40 minichromosomes and closed circular DNA (review: 5). It is thus likely that DEK functions as an architectural protein in chromatin. Indeed, over-expressed DEK has been detected in a complex with the co-repressor Daxx and an associated histone deacetylase (6) and with other transcription factors such as AP-2 α (7) indicating that it may modulate the structure of chromatin around active genes.

To better understand its in vivo function on chromatin, we decided to investigate the distribution of DEK around the developmentally regulated promoter of the gene encoding the human CD21/complement receptor 2 (CR2). This gene is primarily expressed in B lymphocytes and follicular dendritic cells as well as in subsets of thymocytes and epithelial cells. During B cell development, CD21 is present in early activated and mature B cells, but not in pro- and pre-B cells, and is absent in lymphocytes at the plasma cell stage. CD21 in a complex with CD19 and CD81 forms the receptor for activating fragments of the complement component C3 and amplifies the B cell response to antigens by several orders of magnitude. The CD21 receptor is also involved in negative regulation during the selection of autoreactive B cells and absence or de-regulated expression is linked to autoimmunity (review: 8–10).

The expression of the CD21/CR2 gene is regulated by signal transduction pathways that target proximal promoter elements (within ~120 bp around the transcriptional start) as well as regulatory sequences in the first intron (11–13). These sequence elements are highly conserved between mouse and man and serve as binding sites for a host of negatively and positively acting transcription factors including SP1, AP-1/ AP-2, nuclear factor-κB, NFAT-4, YY1 and others (14–18).

Furthermore, gene activity is controlled by the conformation of chromatin since both the promoter-proximal and the intronic region contain DNase-I-hypersensitive sites in CD21-expressing cells but not in non-expressing cells (13, 19); potent inhibitors
of histone deacetylases stimulate gene expression in a cellular background where the CD21 gene is normally silent (20). In fact, the intronic regulatory region, termed complement receptor 2 silent (CRS) (13), includes a binding site for the C promoter-binding factor 1 protein, a component of the Notch signaling pathway, that is known to attract a histone deacetylase and may therefore be responsible for the silencing of the CD21 gene in non-expressing cells (21).

Thus, the regulatory region of the CD21 gene constitutes an array of adjacent positive and negative sequence elements conferring cell type specificity and developmental regulation and is therefore well suited to investigate whether the DEK protein associates with particular elements, and, if yes, whether this is associated with CD21 expression.

To address this point, we compare B cell lines representing two developmental stages, namely Ramos, a line of mature B cells shown to express the CD21 gene (see below), and Nalm-6, a pre-B cell line which does not express CD21.

The procedure that we have chosen is the immunoprecipitation of cross-linked chromatin by DEK-specific antibodies [chromatin immunoprecipitation (ChIP)] and the identification of specific cross-linked DNA sequences by quantitative PCR. We present evidence showing that DEK occurs at many sites in the CD21 gene regulatory region with a clear preference for a promoter-proximal site in active genes.

**Methods**

**Cell lines and culture conditions**

The human pre-B cell line Nalm-6 and the human mature B cell line Ramos were obtained from the American Type Culture Collection. Nalm-6 cells were maintained at 37°C in 5% CO₂ in Iscove’s modified DMEM with 10% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. The Ramos cells were maintained at 37°C in 5% CO₂ in RPMI 1640 with 10% FBS, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin.

For 5-aza-2’-deoxycytidine (aza-dc) treatment, Nalm-6 cells were diluted to 3.5 × 10⁵ ml⁻¹ and allowed to grow overnight. Freshly prepared aza-dc was added to a final concentration of 4 μM and the cells were allowed to grow for 24, 48 or 72 h.

**Reverse transcription–PCR for endogenous CD21 expression**

Total RNA from Nalm-6 and Ramos cells were isolated using reagents and protocols of the RNaseasy RNA isolation kit (Qiagen). The reverse transcription (RT) reaction was performed using 2 μg total RNA primed with oligo(dT) according to the protocol of ThermoScript™ RT-PCR System (Invitrogen). Reverse transcripts were used in the PCR reaction with the following primers: CD21, 5’-ATCACCTCCCTATTTCTCA-3’ and 5’-GTCTATCTCTTTTGCCATT-3’ (corresponding to positions 900–920 and 1321–1342 of CD21 cDNA sequence) (22) and GAPDH, 5’-CATCATCTGCCCCTCTC-3’ and 5’-GCCTGTTCCACACCTC-3’ (corresponding to positions 432–450 and 851–869 of GAPDH cDNA sequence) (23). The cycling protocol used was 90 s at 94°C, then 30 s at 94°C, 30 s at 55°C and 60 s at 72°C for 30 cycles and finally 7 min at 72°C. PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

**In vivo cross-linking and nucleoprotein preparation**

Formaldehyde was added to the Nalm-6 and Ramos cells at a final concentration of 1% and incubated at 37°C for 2 min if not otherwise indicated (24). The reaction was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were collected by centrifugation and washed three times with cold PBS. All centrifugation steps were carried out at 670 × g for 5 min at 4°C. The cells were re-suspended in hypotonic RSB buffer (10 mM Tris–HCl, 3 mM MgCl₂, pH 8.0). All buffers contained 10 mM sodium bisulfite (pH 8.0) as a protease inhibitor. After 10 min incubation with RSB buffer on ice, the cells were disrupted by Dounce homogenization. The nuclear material was collected by centrifugation and washed twice in RSB buffer and once in high-salt SNSB buffer (1 M NaCl, 10 mM Tris–HCl, 0.1% NP-40, 1 mM EDTA, pH 8.0) and then incubated on ice for 5 min. Finally, the nuclear material was re-suspended in low-salt NSB buffer (0.1 M NaCl, 10 mM Tris–HCl, 0.1% NP-40, 1 mM EDTA, pH 8.0) and loaded onto a step gradient consisting of 1.3, 1.5 and 1.75 mg ml⁻¹ CsCl in gradient buffer (20 mM Tris–HCl, 1 mM EDTA, 0.5% sarcosyl; pH 8.0). Nucleoprotein complexes were collected after ultracentrifugation (37 000 revolutions per minute, 24 h and 18°C) and dialyzed against TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) overnight at 4°C. Nucleoprotein was then sonicated by a total number of 100 pulses on ice. Nucleoprotein fragments <1 kb were obtained by digestion with 7 U micrococcal nuclease mg⁻¹ nucleoprotein in the presence of 3 mM CaCl₂ for 15 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 20 mM. The nucleoprotein fragments were analyzed on a 1% agarose gel (25).

**ChIP**

Immunoprecipitations were performed in NET buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA: 0.5% NP-40). Nucleosome-digested nucleoprotein was centrifuged for 10 min at 15 000 × g at 4°C. The supernatants from 1 mg of digested nucleoprotein were incubated with 10 μg of affinity-purified DEK antibodies, 6 μg of AP-2α antibodies (SantaCruz Biotechnology) from rabbit or 10 μg of control rabbit IgG overnight at 4°C on a rolling platform. Then 50 μl of 50% protein A-Sepharose was added and further incubated for 4 h at 4°C. Immunocomplexes were washed eight times with RIPA (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS), three times with LiCl buffer (10 mM Tris–HCl, pH 8.0; 250 mM LiCl; 0.5% NP-40; 0.5% sodium deoxycholate; 1 mM EDTA) and five times with TE buffer. Beads were transferred to new tubes after each change of buffer in order to reduce contamination of unspecific DNA sticking to the tube walls. The washed precipitates were divided for protein and DNA extraction.

For western blotting experiments, proteins were eluted with 2% SDS in H₂O twice for 15 min at 37°C. Cross-links were reversed by incubation at 65°C for 4 h and proteins were extracted with methanol/chloroform (26). Input and supernatant were treated accordingly. Proteins were separated by...
SDS-PAGE, transferred onto nitrocellulose membranes and treated with monoclonal DEK antibodies from mouse.

For DNA extraction, nucleoproteins were eluted with 1% SDS in TE twice at 37°C for 15 min. Proteins were digested with 200 μg ml⁻¹ protease K overnight at 37°C. Cross-links were reversed by incubation at 65°C for 4 h and DNA was purified by the standard phenol–chloroform extraction, ethanol precipitation and dissolved in 40 μl TE. One-twentieth of the sample was used for quantitative real-time PCR.

Quantitative real-time PCR

Real-time PCR was performed with the Light Cycler instrument (Roche Diagnostics) using a ready-to-use ‘hot start’ reaction mix (FastStart DNA Master SYBR Green I, Roche Diagnostics). The mix contains Taq DNA polymerase and a fluorescent dye, SYBR Green I, for real-time detection of double-stranded DNA. Reactions were set up in 10 μl volumes including 0.5 mM of each primer. PCR reactions were performed at 45 cycles routinely, using the standard settings recommended by the manufacturer. Annealing temperatures of individual primer pairs are indicated in Table 1. Standard DNA samples (human genomic DNA) were serially diluted to 30 and 3 ng and 300, 30 and 3 pg. After PCR, the x-axis crossing point of each standard sample was plotted against the logarithm of concentration to produce a standard curve. Genomic equivalents of DNA samples were determined by extrapolation from the standard curve (25).

Results

DEK in lymphocytes

We first confirmed by a RT-PCR procedure that the CD21 gene was expressed in Ramos cells but not in Nalm-6 cells. As shown in Fig. 1A, CD21 mRNA was well detected in Ramos, was expressed in Ramos cells but not in Nalm-6 cells. As expected (27). We next shown in Fig. 1(A), CD21 mRNA was well detected in Ramos, was expressed in Ramos cells but not in Nalm-6 cells. As

Table 1. Sequences and amplification conditions for primers

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Map positionsa (bp)</th>
<th>Length (bp)</th>
<th>Annealing temperature (°C)</th>
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<tr>
<td>P-F</td>
<td>TGACCGACCCACAGAAACT</td>
<td>1089664–1089682</td>
<td>204</td>
<td>62</td>
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<tr>
<td>P-R</td>
<td>CCCACCCCAGTTCTTATTTT</td>
<td>1089848–1089867</td>
<td>204</td>
<td>58</td>
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<tr>
<td>P-F</td>
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<td>1090893–1090911</td>
<td>204</td>
<td>58</td>
</tr>
<tr>
<td>P-R</td>
<td>CAGGACCAGGGCCTTATT</td>
<td>1091078–1091096</td>
<td>204</td>
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<tr>
<td>In1-F</td>
<td>GGGTCCCTCCCTCCTAAATAAA</td>
<td>1091386–1091405</td>
<td>288</td>
<td>55</td>
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<tr>
<td>In1-R</td>
<td>CAGCCATCTCTGAGCCAAGA</td>
<td>1091654–1091673</td>
<td>288</td>
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<tr>
<td>RS1-F</td>
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<td>1095179–1095191</td>
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<td>55</td>
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<tr>
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<td>1106365–1106384</td>
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The extracted proteins were analyzed by immuno (western) blotting. The data indicated that DEK is several folds less abundant in the lymphocytes compared with HeLa cells. Since the western blot signal of 5 × 10⁴ HeLa cells corresponds to the western blot signal of 15 × 10⁴ lymphocytes (Fig. 1B), we estimate that the lymphocyte cell lines express approximately one-third of the amount of DEK found in HeLa cells (~4 × 10⁶ copies per nucleus) (28). While this may be a rough estimate, it is, however, more important for the present purpose that DEK was found to be equally abundant in Ramos cells, which express the CD21 gene, and in Nalm-6 cells, which do not (Fig. 1B).

DEK on chromatin

Cell fractionation and immunofluorescence showed that DEK in lymphocytes is nuclear (not shown) exactly as in all other cell types examined. However, it had to be shown whether the contacts between DEK and DNA are close enough for a formaldehyde-mediated covalent linkage as required for ChiP. To address this point, we exposed exponentially growing Ramos and Nalm-6 cells to formaldehyde (1%) for various lengths of time. Chromatin was then extracted, mechanically sheared and subjected to equilibrium centrifugation in cesium chloride gradients to separate cross-linked nucleoprotein from other material (24). Aliquots with equal amounts of DNA were then de-cross-linked and investigated by denaturing polyacrylamide gel electrophoresis. According to Coomassie staining, core histones became cross-linked to DNA already after formaldehyde treatment of 1 min, whereas maximal cross-linking of linker histones and of various non-histone chromatin proteins required longer treatment (Fig. 2A, upper panel). Western blotting of the same gel showed that a formaldehyde treatment of only 1 min was sufficient to cross-link most detectable DEK to DNA (Fig. 2A, lower panel), suggesting that DEK is most probably in close contact with DNA in lymphocytes. The experiment in Fig. 2(A) was performed with Ramos cells, but identical results were obtained with Nalm-6 cells (not shown).

In the experiments, described below, we used a cross-linking time of 2 min and digested the sheared chromatin with micrococcal nuclease to produce chromatin fragments with DNA of <1 kb lengths (Fig. 2B). Nuclease digestion has been
shown to increase the resolution of the PCR analysis of immunoprecipitated chromatin (25).

**Distribution of DEK**

One condition for the determination of DEK at and around the CD21 gene promoter is that the available antibodies react well with and precipitate DEK on cross-linked chromatin. To investigate this point, we compared DEK-specific antibodies with unspecific antibodies in immunoprecipitations. As shown in Fig. 3(A), DEK antibodies, but not control antibodies, recognized and precipitated DEK, covalently linked to chromatin, although a substantial fraction of cross-linked DEK remained in the supernatant. The immunoprecipitable fraction could not be increased using higher antibody concentrations (not shown), suggesting that DEK in the supernatant fraction of cross-linked chromatin is either not accessible to antibody or altered by formaldehyde treatment such that it is unable to react with the antibody. In either case, we have extracted and then analyzed the DNA in immunoprecipitated chromatin by quantitative PCR in a Light Cycler system.

The human CD21 gene consists of 19 exons distributed over 35 kb of genomic DNA (Fig. 3B). Regulatory elements are in the vicinity of the transcriptional start and in the CRS region of the first intron (see Introduction). We therefore designed primer
Fig. 3. DEK at the CD21 gene. (A) Immunoprecipitation of cross-linked, micrococcal nuclease-treated chromatin from Nalm-6 and Ramos cells with DEK-specific antibodies and unspecific control antibodies (IgG) from rabbit. Input (chromatin sample before immunoprecipitation), supernatant (remaining chromatin after incubation with antibodies and protein A-sepharose) and immunoprecipitates were analyzed by western blotting with monoclonal DEK-specific antibodies from mouse. (B) Overview of the CD21 gene. The CD21 gene is shown as a gray box with scale (in kilo base pair) and the exons as black boxes. (C) Regulatory gene elements and amplified regions. Two regulatory elements, CpG island at the promoter region and CRS element within intron 1, are shown as gray boxes. Arrow indicates start and direction of transcription. Gray boxes under the gene map, regions selected for PCR amplification. (D) Enrichment for DEK-bearing DNA regions. Enrichment of CD21 gene sequences is expressed as the ratio of precipitate over input DNA in Nalm-6 (left) and Ramos cells (right). (E) Enrichment for AP-2-bearing DNA regions. Enrichment of CD21 gene sequences is expressed as the ratio of precipitate over input DNA in Nalm-6 (left) and Ramos cells (right).
pairs corresponding to promoter-proximal sequence elements and to the CRS region. The control was a primer pair corresponding to the far downstream exon 6 sequence (Fig. 3C).

We first determined the amount of amplifiable DNA in the input, i.e. in the DNA prepared from cross-linked chromatin before immunoprecipitation. These values were used as reference in comparisons with the amount of amplifiable DNA in the immunoprecipitates. As a first result, we note that unspecific antibodies precipitated very little amplifiable DNA (IgG in Fig. 3D), while DEK-specific antibodies precipitated significant amounts of all CD21 gene sequences tested. For comparisons, we expressed the results as the ratio of amplifiable specific DNA in the precipitates relative to the specific DNA in the input (Fig. 3D).

The data for the Nalm-6 cells show that all investigated CD21 gene sequences appeared in immunoprecipitated chromatin in similar amounts, namely between 5 and 10% of the input CD21-specific DNA sequences. This indicates that DEK seems to be rather uniformly distributed along the length of the non-expressed CD21 gene (Fig. 3D, left panel).

We also detected all amplifiable CD21 gene sequences in the immunoprecipitated chromatin from Ramos cells but recovered 2- to 3-fold higher amounts of the promoter-proximal DNA than of DNA from the other parts of the gene (Fig. 3D, right panel). This was found in four independent experiments and indicates that more DEK assembles at a promoter site of an active CD21 gene.

As a control, we investigated whether transcription factor AP-2, known to be involved in CD21 gene regulation (11, 17, 29), was present on the promoter sequence. For this purpose, we used AP-2-specific antibodies for the precipitation of cross-linked chromatin and analyzed the precipitated DNA as in Fig. 3(D). Our results indicate that AP-2, just as DEK, accumulated at the CD21 promoter in Ramos but not in Nalm-6 cells (Fig. 3E). This is interesting as Campillos et al. (7) had shown that DEK and AP-2 functionally interact as DEK modulates the transcriptional activation of AP-2.

Effects of aza-dc

It has been determined that the cytosine residues in a promoter-proximal CpG island are extensively methylated in the inactive CD21 gene and that a treatment with aza-dc induces demethylation and the expression of the CD21 gene in pro-B cell lines, Nalm-16 and KM3 (27). If the accumulation of DEK at the CD21 gene promoter were linked to gene expression, we would expect that more DEK appeared at the promoter of Nalm-6 cells after aza-dc treatment. In Fig. 4(A), we confirm that CD21-specific mRNA appears in Nalm-6 at 48–72 h after addition of aza-dc, although the level of mRNA did not reach the level of CD21-specific mRNA in Ramos cells.
We performed ChIP with untreated and aza-dc-treated Nalm-6 cells and performed quantitative PCR analyses exactly as described in Fig. 3. The data indicate once again that DEK was essentially uniformly distributed over the CD21 gene in untreated cells (Fig. 4B, left) and that 1.5–2.5 times more DEK appeared on promoter-proximal sequences than on promoter-distal sequences after 72 h in the presence of aza-dc (Fig. 4B, right). This enrichment for promoter sequences is somewhat less than in Ramos cells (Fig. 3) but nevertheless significant as it has been detected in two independent experiments.

In conclusion, we showed that the chromatin protein DEK is more or less evenly distributed over the analyzed parts of the active and inactive CD21 gene and that 2–3 times more DEK assembles at promoter-proximal sites in lymphocytes that actively express the CD21 gene.

Discussion

DEK is known to be a major and regular constituent of mammalian chromatin occurring at an average of one DEK molecule per 4–10 nucleosomes (review: 5). However, it is not known whether DEK is uniformly distributed along the chromatin or whether it is concentrated at specific sites in genetically active or inactive chromatin regions. As a first step to address this point, we have screened a small section of human chromatin in lymphocytes using the ChIP procedure, which has recently been used in many studies on the location of specific proteins on chromatin (30).

We show here that DEK can occur at many sites of the CD21 gene in lymphocytes regardless of whether the gene is actively transcribed or not. Clearly, however, DEK accumulates 2- to 3-fold at a promoter-proximal region in cells with active CD21 genes. Thus, DEK, like many other global chromatin-modifying activities, seems to be widely distributed on chromatin but can also be recruited to specific sites such as the promoters of expressed genes. The accumulation of DEK on the active CD21 promoter is accompanied by a binding of the transcription factor AP-2 known to be involved in CD21 gene regulation. This is most likely of physiological significance as DEK has been described as being able to modulate the transcriptional activity of AP-2 (7).

The binding of DEK occurs on a sequence which includes a CpG island. This CpG island is known to be highly methylated in pro- and pre-B cells, which do not express CD21, and to be un- or hypomethylated in mature CD21-expressing B cells (27). Decreasing the methylation status by aza-dc results in an expression of the CD21 gene in pro- and pre-B cells, including Naim-6 (27), and this, as we show here, is accompanied by an enhanced binding of DEK. As a control, we searched for but failed to detect an accumulation of DEK on the promoter of the (silent) CD21 gene in HeLa cells. Conversely, however, we detected significant amounts of DEK on a genetic control region of the (active) TOP1 gene in HeLa as well as in proliferating Ramos and Nalm-6 cells (Hu et al., manuscript in preparation) excluding the possibility that Nalm-6 cells may be intrinsically unable to recruit DEK to gene promoters.

We speculate that DEK with its preference for superhelical and bent DNA may function to stabilize a higher order DNA structure as requirement for gene expression (review: 5). The precedent here is the better-characterized class of architectural chromatin proteins of the HMG-A family. Protein HMG-A1a has been shown to be an essential constituent of a complex of transcription factors (enhancosome) at the human IFN-β gene (31, 32) where it functions to bend the DNA. It is a task for the future to determine whether DEK may perform a related function at the CD21 promoter.

Finally, we like to point out that ChIP is a useful procedure for the investigation of the distribution of non-histone proteins such as DEK in the chromatin. However, it has its limitations. First, not all cross-linked chromatin was accessible or responsive to DEK-specific antibodies, either because DEK may be buried among other chromatin components or because it is so altered by formaldehyde that it no longer reacts with the antibody. We can therefore not exclude the possibility that it may be a specific subset of DEK that was detected and analyzed. Second, the screening of DNA-binding sites relied on PCR amplification of DNA in immunoprecipitated chromatin. It was impractical to use primer pairs that cover the entire genomic region under investigation. We have instead selected particular primer pairs that correspond to sequences of established or assumed regulatory function. Therefore, we could have missed additional gene regions that may or may not be enriched relative to the average immunoprecipitated chromatin.

Acknowledgements

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Abbreviations

aza-dc 5-aza-2’-deoxycytidine
CR2 complement receptor 2
ChIP chromatin immunoprecipitation
CRS complement receptor 2 silencer
FBS fetal bovine serum
RT reverse transcription

References