Dynamic Regulation of Nucleosome for Detection of CD80 Gene Expression in Murine

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ABSTRACT

The basic building blocks of chromatin are referred to as the nucleosome. Nucleosomes play major role in transcriptional regulation, ranging from repression through occlusion of binding sites for transcription factors, to activation through spatial juxtaposition of transcription factor-binding sites. Examining the position of nucleosome in CD80 promoter is essential to detect the mechanism of tissue specific transcriptional regulation at chromatin level. The CD80 is the most potent of all of the co-stimulatory molecule, expressed on the surface of B cells upon antigen stimulation. The expression of CD80 is tightly regulated, normally limited to only a few cell types including B cells. Therefore this study is important, how nucleosomes become positioned during gene regulation in diverse murine tissues and cell lines. The upstream region of CD80 promoter was examined for its nucleosomal organization by low resolution analysis of MNase-southern blot assay. Four nucleosomes were detected in all murine tissues and cells screened for this analysis. Curiously, similar pattern of nucleosome positioning was detected in all the tissues and cell lines screened. This results conclude that there is no regulation at the level of nucleosome positioning in murine CD80 promoter. However, there must be a regulatory mechanism involved in these tissues either at the molecular level or chromatin level or both as the CD80 gene expression was detected in organs rich in antigen presenting cells, spleen, thymus and lung. This was addressed by possible remodeling mechanism through covalent modification of histone proteins which mark for active transcription and repression of gene regulation.

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INTRODUCTION

The position of nucleosomes plays an important role in regulating gene expression in addition to transcription factors, enhancer-binding proteins and chromatin remodeling factors. The positioning of nucleosomes is probably most critical in promoter and enhancer regions as it regulates gene expression (Mellor, 2005). The dissociation or displacement of nucleosomes from DNA by the action of other protein factors, such as DNA- and histone-modifying enzymes (methylases, kinases, acetylases and deacetylases) likely gives the basal transcription apparatus access to the promoter regions of genes. Therefore, the positioning of nucleosomes and how the remodeling of chromatin alter the position of nucleosomes is important in the regulation of transcription. Nucleosome positioning at genetic regulatory sequences is not well understood. Chromatin has a different higher order structure in different tissues, with the number of nucleosomes packaged into each superbead being related to the transcriptional activity of the tissue.

However, the regulatory mechanism of CD80, especially cell-type specific mechanism is less well understood. The CD80 (B7-1) is the most potent co-stimulatory molecules expressed on the surface of B cells upon antigen stimulation. Co-stimulation is required for the productive T cell activation. T cells are critical immune system cells that help to destroy the infected cells and co-ordinate the overall immune response. It is an essential task to know whether the CD80 is

regulated in all cell types to detect the cell type specific mechanism.

Formation of a permissive chromatin environment is a prerequisite for active transcription of eukaryotic genes. Understanding the purpose of nucleosome positioning in CD80 promoter is essential to detect the mechanism involved in tissue-specific chromatin regions for identifying the expression of CD80 gene in a tissue-specific manner. The important role played by chromatin in the regulation of gene expression has become increasingly apparent and the complete understanding of the regulation of gene of interest requires studies of nucleosome positioning and remodeling at the endogenous locus. A variety of covalent histone and DNA modifications can influence whether a chromatin template is programmed to be transcriptionally active or silent (Strahl and Allis 2000; Berger 2002; Geiman and Robertson, 2002).

The present study focused to examine the pattern of positioning of nucleosome and also how the nucleosome is structured to detect the tissue specific mechanism of CD80 promoter in various murine tissues and cell lines.

MATERIALS AND METHODS

Isolation of Tissues from Murine

Female BALB/C mice, 4-6 weeks of age were sacrificed. Mice were wiped with alcohol to sterilize them, pinned onto a thermacol board. The skin was cut from the lower abdomen towards upwards of the body and pinned. Then the organs such as kidney, heart, lung, spleen, brain and liver were removed and washed thoroughly either with HBSS or RPMI. Then the tissues were powdered with liquid N_2 and resuspended in RPMI.

Cell Culture

A20 cells (ATCC, Rocksville, MD, USA) were maintained in RPMI 1640 supplemented with 10 % fetal bovine serum and 1 X penicillin/streptomycin while NIH-3T3 cells were maintained in DMEM supplemented with 10 % fetal bovine serum and 1 X penicillin/streptomycin and incubated at 370C in 5 % CO_2 atmosphere.

MNase Digestion

Ten million cells were harvested and the cell pellet was washed with 10 ml ice-cold 1 X PBS. The pellet was resuspended in 5 ml of ice-cold NP-40 lysis buffer and incubated on ice for 5 mins. Nuclei were pelleted at 4°C followed by a wash with ice cold MNase digestion buffer. Pelleted nuclei were resuspended in 1 ml of MNase digestion buffer containing 1 mM $CaCl_2$ and divided into 100 μl aliquots. Micrococcal nuclease was added at the concentration 200 u and incubated at 37°C for 1 hr. To stop the reaction, 80 μl of MNase digestion buffer and 20 μl of MNase stop buffer were added to each sample, followed by 3 μl of proteinase K (25 mg/ml) and 10 μl of 20 % SDS and then incubated overnight at 37°C. Then the samples were extracted with phenol/chloroform, treated with RNase A (10 mg/ml), followed by a second round of phenol/chloroform and ethanol precipitation with 3 M sodium acetate. The pellet was resuspended in 100 μl of TE.

Southern blot Hybridization

10 μg DNA obtained from MNase digestion was run in a 1 % agarose gel. The gel was treated with denaturing solution, followed by neutralizing solution with gentle shaking. Then the DNA was transferred to nylon membrane. Transferred DNA was cross-linked to the nylon membrane by UV (254 nm). The radiolabled probe was prepared by Nick Translation as per manfacturer's instructions, followed by purification by QIAGEN column. Prehybridizaton and Hybridization were carried out, followed by washings at 65°C with increasing stringency progressively. Blots were exposed to Kodak film and developed.

Chromatin Immuno-precipitation

Nuclei isolated from formaldehyde fixed cells and followed digestion with MNase was processed for mononucleosome-ChIP. Cells were maintained at 1.5×106 million cells per ml and stimulated with the H202. Proteins were crosslinked to DNA by adding formaldehyde directly to the culture medium to a final concentration of 1 % and incubated for 10 mins at 37°C. To quench excess formaldehyde glycine was added to the medium at a final concentration of 0.125 M and incubated at room temperature for 10 mins. Cells were pelleted and washed with PBS supplemented with protease inhibitor cocktail. Cells were resuspended in 200 μl of SDS lysis buffer and incubated on ice for 10 mins. Samples were sonicated at 30 % amplitude for 30 secs followed by centrifugation at 13,000 rpm for 10 mins at 4°C. The clear supernatant was diluted 10 fold with ChIP dilution buffer. 10 % of supernatant was set aside as input. Samples were precleared for 2 hrs with Protein A Sepharose beads at 4^0C. The antibody for immuno-precipitaion was added at 2 μg/ml and incubated overnight with rotation at 4°C. Immune complexes were collected with Protein A Sepharose blocked with salmon sperm DNA (200 μg/ml) and BSA (10 mg/ml) for 1 hour with rotation. Washes were done with 1 ml of each of wash buffer, first with MNase digestion buffer and followed MNase stop buffer. 500 μl elution buffer (1 % SDS, 0.1 M NaHCO$_3$) was added to elute the antibody-protein-DNA complexes. 20 μl of 5 M NaCl was added to the eluate and incubated at 65°C overnight. The next day, 10 μl of 0.5 M EDTA, 20 μl 1M Tris-HCl, pH 6.5, and 2 μl of 10mg/ml proteinase K was added to the eluate and incubated for 1 hour at 45°C. DNA was recovered by phenol/chloroform and ethanol precipitation. 20 μg of yeast tRNA was added as an inert carrier. PCR was performed with 10 % of the resuspended sample. Antibodies α- dimetH3K4 and α-dimetH3K9 (Upstate Biotechnology, Lake Placid, NY, USA) were used for immuno-precipitation.

RESULTS AND DISCUSSION

Nucleosomal Organization of Murine CD80 Promoter

The overall chromatin structure of the upstream region of the CD80 gene was analyzed by MNase digestion followed by gel electrophoresis and blotting. Cell nuclei was isolated from various murine tissues (kidney, liver, lung, spleen and heart) and also from murine cell lines (A20 and NIH-3T3). Micrococcus nuclease (MNase) enzyme was added to cell nuclei which cleaves the DNA regions that are devoid of nucleosomes resulting the mononucleosomal DNA. Mononucleosomal DNA indicated that the digestion is complete (Figure 1).
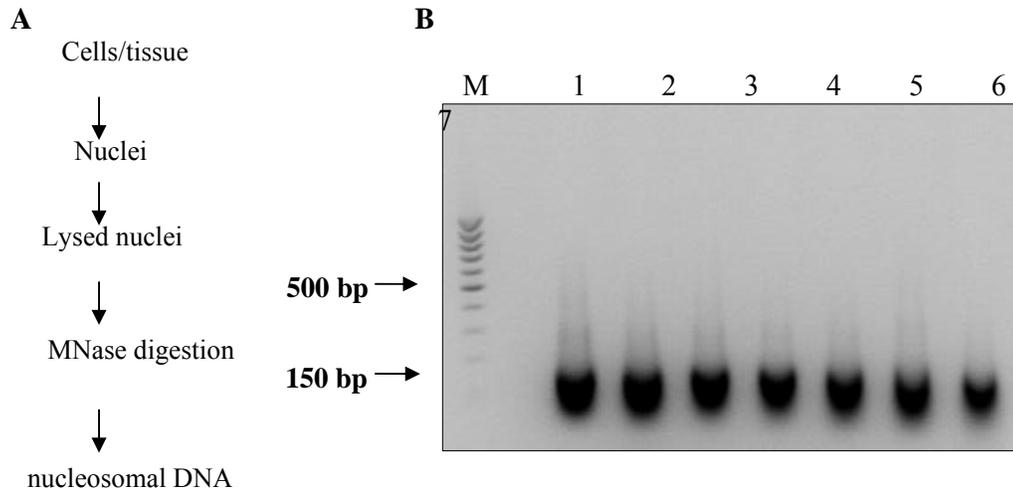
Cells/tissue

↓

Nuclei

↓

Lysed nuclei

↓

MNase digestion

↓

nucleosomal DNA

B

M   1   2   3   4   5   6

500 bp →

150 bp →

Figure 1: Isolation of Mononucleosomal DNA by using MNase digestion
A. The Biochemical Isolation Scheme
B. Isolation of Mononucleosomal DNA treated with Micrococcal nuclease
10-12 µg of murine DNA obtained from cells of A20, NIH-3T3 and kidney, liver, lung, spleen and heart tissue of mice (BALB/c) was completely digested with micrococcal nuclease to obtain a mononucleosome, electrophoresed in 1.4 % agarose shown in Lane 1-7 respectively. M is DNA size marker. MNase treated sample resulted in bands of 150 bp (indicated by an arrow)

Further, digestion with 200 u MNase for 1 hr resulted a prominent band of DNA centered at approximately 150 bp. In addition, this pattern was consistent in all the tissues and cell lines used in this analysis.

Further, radiolabelled probe selected from the proximal region of the sequences of CD80 promoter in relation to transcription site (Selvakumar et al., 1993), spanning from +155 to –427, -546 to –683, -771 to –1121 and –1141 to –1303 and also from the distal region of the CD80 promoter sequences in relation to transcription site spanning from –1276 to –1623, -1726 to –2047, -2109 to –2334 and –2521 to –3004 were generated and tested to hybridize the blotted mononucleosomal DNA to position the nucleosome along the CD80 promoter region to identify the chromatin-specific regulatory mechanism.

The detectable autoradiography signal was observed for only with 4 different probes (Figs: 2 and 3) spanning from -546 to –683, –1141 to –1303, -1726 to –2047, -2109 to –2334 for all the cell lines and tissues which have been screened for this analysis. No detectable signal was observed with the probes spanning from +155 to -427, 771 to -1121, -1276 to -1623 and -2521 to -3004 for any murine cell lines or tissues. However, the MNase- blot analysis revealed that the detection of signal with four different probes indicating the existence of four nucleosomes in the CD80 promoter region. Since the positioned nucleosomes were observed in different region of promoter illustrating the nucleosomes which are inconsistently present along the CD80 promoter. Surprisingly, similar pattern of nucleosome array was observed for all the murine tissues and cell lines which were used for this analysis.
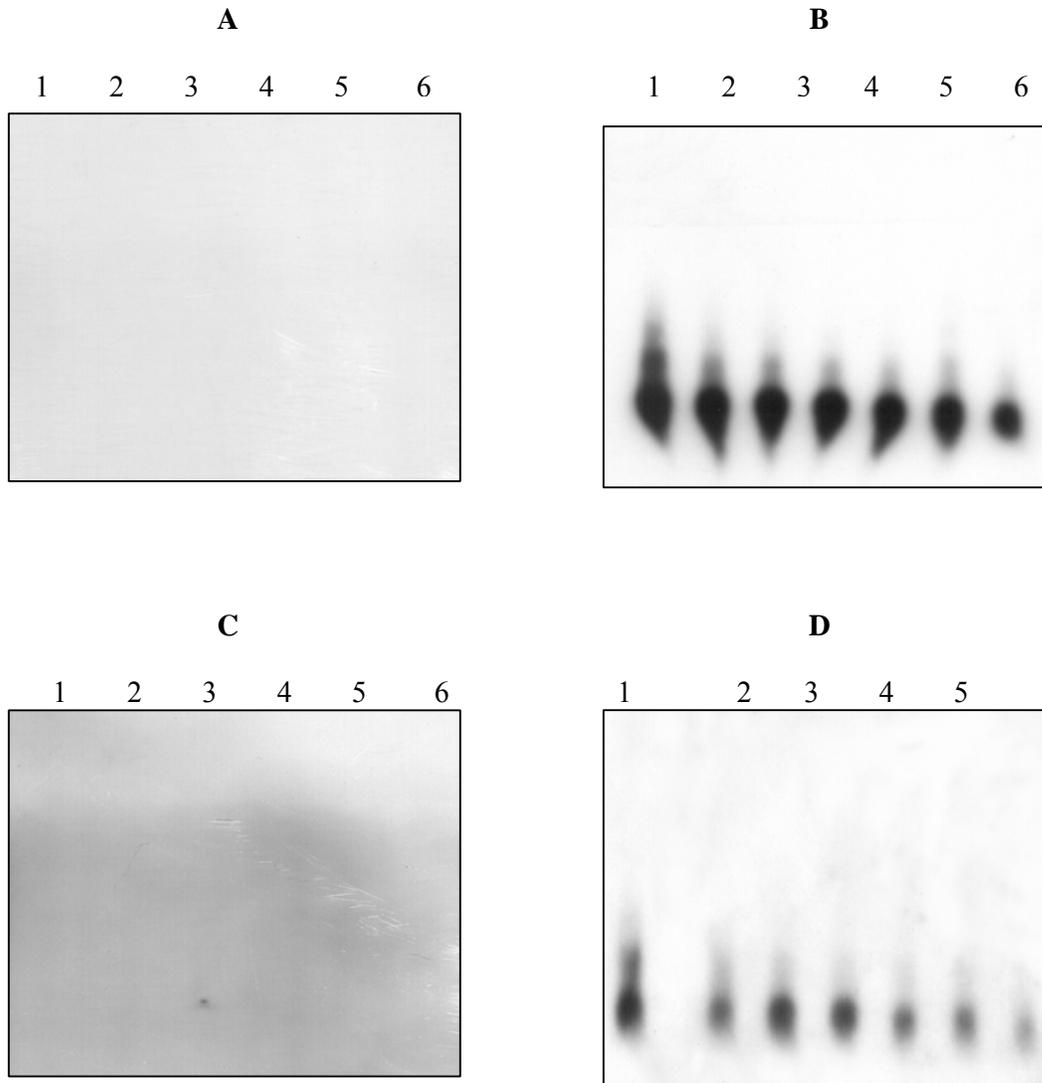
Figure 2: MNase - Blot Assay to analyze the Nucleosome Positioning in the Proximal region of the Upstream sequences of CD80 promoter in relation to Transcription Start Site

The MNase digested DNA obtained from cells (shown in figure:1) of A20, NIH-3T3 and also from murine tissues such as kidney, liver, lung, spleen and heart was hybridized with the ^{32}P-labelled probe, indicated in lane 1 to 7 respectively. The probes extending from +155 to -427, -546 to -683, -771 to -1121 and -1141 to -1303 in relation to transcription start site were used for the MNase-southern blots A, B, C and D respectively.

Since there are no differences observed in the pattern of nucleosome distribution among murine cell lines (A20 and NIH-3T3) and tissues (kidney, liver, lung, spleen and heart), it clearly indicated that there is no regulatory mechanism involved at the level of nucleosome positioning in murine CD80 promoter.

Further, since the CD80 gene expression was detected in organs rich in APC; spleen, thymus and lung (Zhang et al., 2000), there must be a regulatory mechanism involved in these tissues either at the molecular level or chromatin level or both.
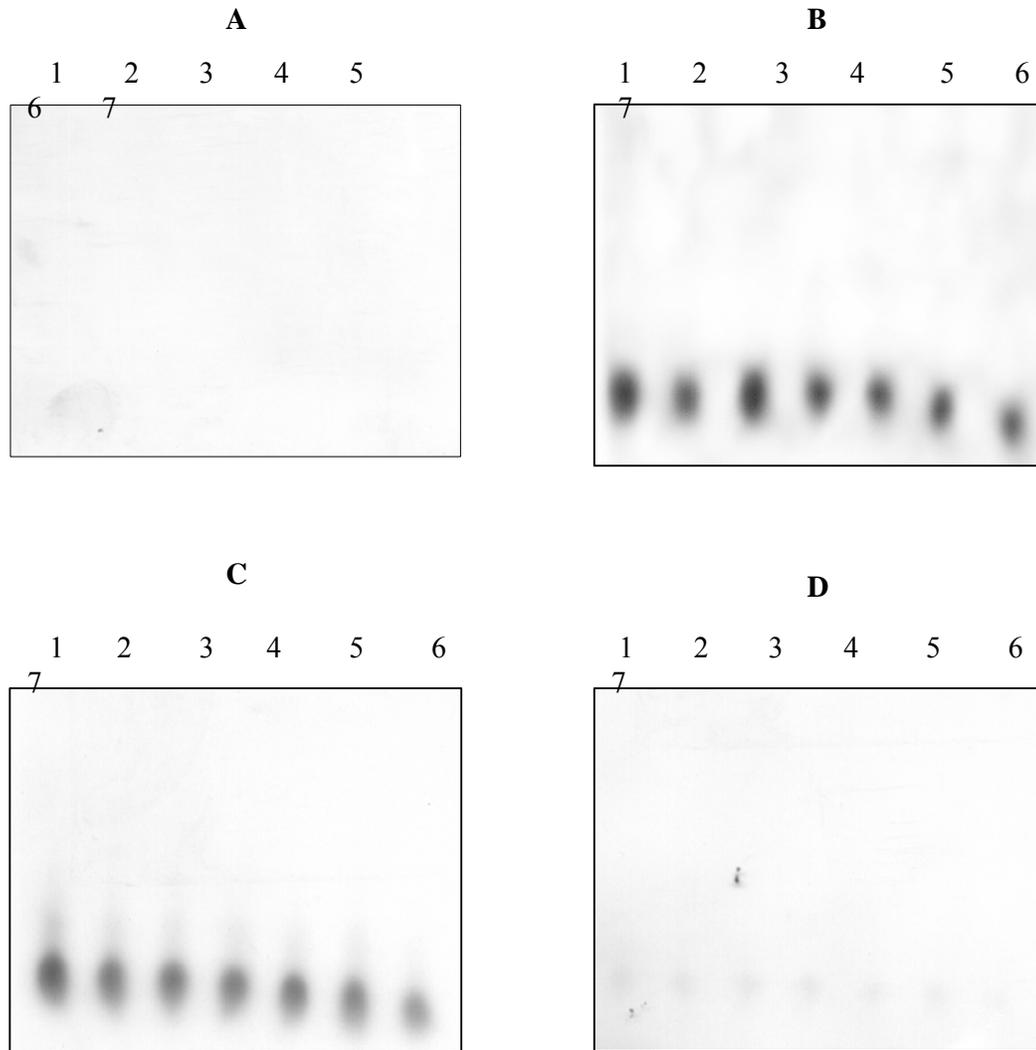
Figure 3: MNase - Blot Assay to analyze the Nucleosome Positioning in the Distal region of Upstream sequences of CD80 promoter in relation to Transcription Start Site

The MNase digested DNA obtained from cells (shown in figure:1) of A20, NIH-3T3 and also from murine tissues such as kidney, liver, lung, spleen and heart was hybridized with the ^{32}P-labelled probe, indicated in lane 1 to 7 respectively. The probes extending from -1276 to -1623, -1726 to -2047, -2109 to -2334 and -2521 to -3004 in relation to transcription start site were used for the MNase-southern blots A, B, C and D respectively.

Dynamic regulation of dimet H3K4, dimet H3K9 at the endogenous murine CD80 locus

Since the position of the nucleosome remained identical in all the murine tissues and cell lines studied, dynamic properties of nucleosomes were investigated to understand the remodeling mechanism which led to detect the CD80 tissue specific expression at chromatin level. Indeed, these findings led to realize the pivotal roles of histone proteins in regulating chromatin structures where reconfiguration of the nucleosome was examined as a consequence of methylation. The methylation of histone H3 correlates with either gene expression or silencing depending on the residues modified. Many of the lysine and arginine residues are methylated in vivo (Zhang et al., 2003, 2004; Margueron et al., 2005) and the

degree of lysine methylation on nucleosomes and their relative locations throughout the genome are linked to chromatin and transcriptional regulation.

Therefore, the present study focused to detect the histone methylation which marks at proximal promoter of murine CD80 gene in various murine tissues, following stimulation with H_2O_2. Therefore, mononucleosome-ChIP assay was performed to detect the distinctive site- specific H3 methylation in murine tissues (spleen, kidney, brain, liver and heart) with antibodies specific to dimethyl-H3K4 and dimethyl-H3K9 individually. Experiments revealed that the regulatory nucleosome was specifically subjected to dynamic changes in the methylation status of lysine residues upon stimulation. Amplified signal detected for immuno-precipitated DNA with indicated antibodies and with primers specific only for regulatory nucleosome. No change in the methylation of the H3 histones detected for the rest of the nucleosomes in the CD80 promoter with any of the antibodies tested for any of the tissues screened (data not shown). Immuno-precipitated DNAs from both ChIPs for all the murine tissues screened were compared individually with total genomic DNA input. Positive signal was detected in the spleen only with antibodies for dimethyl-H3K4 and also in kidney, brain, liver and heart with antibodies for dimethyl-H3K9 (Figure 4), indicating an enrichment of specific histone modifications at H3, providing a rich view of distinctive histone modification on lysine residues demonstrating the distinct downstream events in different murine tissues. Further, level of dimethylated histone H3-lysine 4 was observed following stimulation in spleen only not in rest of the tissues screened suggesting this modification serve as a mark for active transcription in spleen. This could be true statement as spleen is known to be rich in APC where endogenous CD80 gene expression was detected (Zhang et al., 2000). In contrast, methylation of lysine 9 of H3 was detected in kidney, brain, liver and heart

demonstrating that the dimethyl-H3K9 may serve as a mark for repression of transcription. The molecular mechanism of how these different methylation states help to generate potentially different functional chromatin states is presently unclear.

Given the presence of positioned nucleosomes in diverse regulatory contexts, how nucleosome particles become positioned during gene regulation was investigated. Basically, the present study focused on positioning of nucleosomes at CD80 promoter sequences. In addition, the remodeling of chromatin which alter the position of nucleosomes resulting to either activation or repression of CD80 transcription was also confirmed. By examining the proximal as well as distal region of CD80 promoter for its nucleosomal organization by low resolution analysis of MNase-southern blot assay on *in vivo* MNase digested nuclear DNA, positive signal was detected with four different probes for all the murine tissues and cell lines screened for this analysis. These results indicated that the corresponding regions, *in vivo*, where the DNA fragment is assembled into nucleosomes, were resistant to MNase digestion. Also, the data revealed that there was no change in the pattern of nucleosome positioning in all the cell lines and tissues screened, leading to further analyze the remodeling mechanism involved in murine cells and tissues.

Studies of the mouse mammary tumor virus (MMTV) and human immunodeficiency virus (HIV) promoters have revealed that nucleosomes positioned over essential transcription factor binding sites in both the active and silent states (Fragoso et al., 1995; Truss et al., 1995; Verdin et al., 1993). Other studies showed that the nucleosomes become positioned at certain yeast promoters when they are silent, with the octamers occluding basal transcription components from the DNA (Han et al., 1988; Shimizu et al., 1991).

Remarkably, important aspect of this study was the detection of consistent pattern of nucleosome positioning among

murine cell lines and tissues. Apparently, there might be several reasons why do nucleosomes in different murine tissues locate the same position. In conclusion, tissue specific regulatory mechanism at chromatin level could not be demonstrated by positioning of nucleosomes in murine CD80 promoter. However, possibility of contributions from the dynamic properties of nucleosome will lead to explain the distinct downstream events in different murine tissues. Therefore the studies were performed to view how chromatin structure is regulated in CD80 and then how chromatin exerts effects on transcription were analyzed. The exact role of chromatin structure in regulating any particular promoter in a natural context remains to be completely defined (Svaren and Horz, 1995, 1996).

Given the importance of histone methylation, the major question was addressed to identify the possible positioning mechanism of CD80 regulation in murine cells as histone methylation is now recognized as an important modification linked to both transcriptional activation and repression (Margueron et al., 2005). Early studies have shown that several lysine residues, including lysines 4, 9, 27 and 36 of H3 and lysine 20 of H4 are preferred sites of methylation (Strahl et al., 1999). Therefore, this study was focused on the H3-K4 di-methylation status and H3-K9 di-methylation status to detect the nucleosome remodeling activity of CD80. Several studies have shown that methylation at H3-K4 serves as mark for active transcription and H3-K9 methylation marks the repression of gene regulation.

In the present study, positive signals detected in response to H_2O_2 stimulation by Chromatin immunoprecipitation (ChIP) assay using antibodies specific to dimethyl H3-K4 and dimethyl H3-K9 were well correlated with the methylation programme in spleen and the rest of the murine tissues (kidney, brain, liver and heart) respectively. Studies showed that the histone methylation patterns in chromatin surrounding NMDA subunit gene promoters where the levels of methylated H3-lysine 4, H3K4me2, and H3K4me3 correlate with levels of gene expression (Stadler et al., 2005).

Based on the data, the similar pattern of nucleosome positioning of CD80 promoter sequences in murine tissues (kidney, brain, liver and heart) could be explained by repressive mark by methylation at H3-K9 leading remodeling of nucleosome.

Though the mechanism is presently unknown but it is possible that methylation at these residues can mark a gene for the recruitment of complexes involved in transcriptional repression. Since studies showed that the NF-1 involves in repression of CD80 transcription leading to gene inactivation in the unstimulated cells whereas dissociation of NF-1 results gene activation in the stimulated cells, it could be explained that binding of NF-1 functions as a repressor in this scenario, resulting transcriptional inactivation. However, methylation at H3-K4 resulted positive signal detected in spleen only, leading to transcriptional activation, it could be explained since more B cells are in spleen resulting CD80 expression. Therefore, H3-K4 may serve as a mark for active transcription via dissociation of NF-1.

The question is why the pattern of nucleosome position remains same in the spleen as like other murine tissues even though active transcription was detected in spleen. To address this issue, further covalent modifications should be analyzed as the decision to methylation is an active process which requires coordination among different covalent modifications. The modification at histones polypeptides by methylation might serve as marks for the recruitment of different proteins or protein complexes to regulate gene expression. Future studies should be needed to determine the precise order of events that led to transcriptional activation.
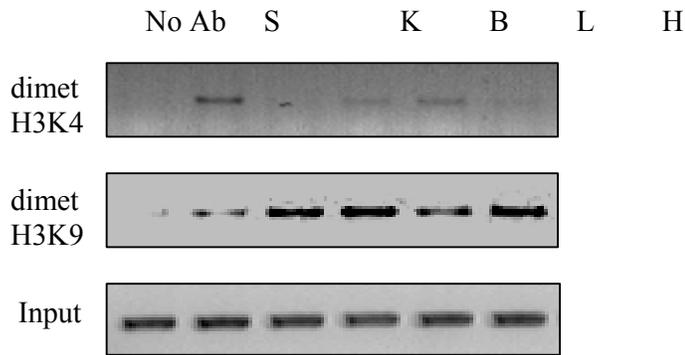
Figure 4: A selective Inducible Methylation of Regulatory Nucleosome following Stimulation
ChIP analysis with antibodies directed to the di-methylated forms of indicated lysine residues was performed in various murine tissues such as spleen, kidney, brain, liver and heart shown in lane 2 to 6 respectively. The specificity of immuno-precipitation was ensured in parallel experiment where the primary antibody was omitted (No antibody) indicated in lane 1.

CONCLUSION

Remarkably, important aspect of this study was the detection of consistent pattern of nucleosome positioning among murine cell lines and tissues. Apparently, there might be several reasons why do nucleosomes in different murine tissues locate the same position. The remodeling mechanism of the nucleosomes where, inducible methylation pattern was detected in the regulatory nucleosome. In this context, methylation at H3K9 may result nucleosome occupancy under all transcriptional states in all the murine cell lines and tissues screened for this analysis except in spleen whereas methylation at H3K4 detected only in spleen, may mark for gene expression.

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