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Gene-specific factors determine mitotic expression and bookmarking via alternate regulatory elements

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ABSTRACT

Transcriptional silencing during mitosis is caused by inactivation of critical transcriptional regulators and/or chromatin condensation. Inheritance of gene expression patterns through cell division involves various bookmarking mechanisms. In this report, we have examined the mitotic and post-mitotic expression of the *DRA* major histocompatibility class II (*MHCII*) gene in different cell types. During mitosis the constitutively *MHCII*-expressing B lymphoblastoid cells showed sustained occupancy of the proximal promoter by the cognate enhanceosome and general transcription factors. In contrast, although mitotic epithelial cells were depleted of these proteins irrespectively of their *MHCII* transcriptional activity, a distal enhancer selectively recruited the PP2A phosphatase via NFY and maintained chromatin accessibility. Based on our data, we propose a novel chromatin anti-condensation role for this element in mitotic bookmarking and timing of post-mitotic transcriptional reactivation.

INTRODUCTION

Major histocompatibility complex class II (MHCII) proteins, expressed on antigen-presenting cells (APCs), are essential determinants of immune regulation: antigen presentation to the $CD4^+$ T helper lymphocytes (Th) requires the interaction of the T cell receptor with the MHC–antigenic peptide complex that results in T cell activation and proliferation. Consequently, defective *MHCII* expression results in a rare severe primary immunodeficiency (Bare Lymphocyte Syndrome, BLS), causing patients failure to generate Th-mediated immune responses (1,2). The MHCII molecules are encoded by the major histocompatibility complex class II (*MHCII*) gene locus that provides an excellent system to study both the molecular processes underlying the regulation of gene expression and their functional effects in the immune response.

Transcriptional regulation of *MHCII* genes requires formation of a multi-protein complex called MHC class II enhanceosome (MCE), which has been studied extensively in a prototypical *MHCII* gene, *DRA* (3–6). MCE is formed by regulatory factor X (RFX) complex (RFX5, RFXAP and RFXANK), nuclear transcription factor Y (NFY) complex (NFYA, NFYB and NFYC) and cyclic-AMP responsive element binding protein (CREB), which bind cooperatively to the conserved elements on the proximal gene promoter known as S/W, X and Y (1). Additional XY-like elements have been identified that are dispersed within the *MHCII* locus. Such an element located 2 kb upstream of the *DRA* (*DRA*-LCR/XL4) with enhancer and/or locus control region-like properties has been shown to mediate regulatory long-range chromatin interactions (7–10). The MCE is essential, but not sufficient, for *MHCII* expression that requires the presence of yet another factor, the class II transactivator (CIITA). CIITA is constitutively expressed in professional antigen-presenting cells and is induced by interferon gamma (IFNγ) in other cell types. It does not bind DNA directly, but is recruited by the MCE and activates transcription via various mechanisms involving association with co-activators, and the basal transcriptional machinery to promote chromatin changes and RNA PolII activation (11–17).

Contrary to the above, little is known about the maintenance and/or re-establishment of *MHCII* expression through the cell cycle that is necessary for sustained immunological functions in antigen presentation.

Mitosis causes a disruption of the transcriptional machinery (18) manifested by chromatin condensation, and dissociation of bound transcription factors (19,20). These changes are reversed upon mitotic exit when the cell enters telophase by ordered factor recruitment, reassembly of nuclear structure and functional recovery (21). Recent studies suggest that various mechanisms act as gene bookmarks to dictate propagation of past gene activity to daughter cells (22,23). Some studies have focused on the mitotic bookmarking by gene-specific transcription

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factors (24–32) or general transcription factors like TATA box binding protein (TBP). The latter was shown to protect from chromatin condensation by recruiting protein phosphatase 2A (PP2A), a S/T phosphatase that dephosphorylates condensin subunits and inhibits promoter compaction during mitosis (33). Other reported bookmarking processes involve the chromatin remodeler mixed-lineage leukemia (MLL) (34), persistence of histone post-translational modifications (PTMs) (35) and deposition of the histone variant H3.3 at the promoter of active genes even in metaphase (36). We have recently shown that IFN gamma (IFNγ)-mediated *MHCII* gene induction generates a spatial epigenetic memory that involves the locus relocalization close to promyelocytic leukemia nuclear bodies. This permits accelerated induction upon restimulation that is maintained through several cell generations (37) and has important implications for the APC function and immune response.

To investigate how the short-term *MHCII* expression is regulated through mitosis, we studied the promoter architecture along with transcriptional activity of the prototype *DRA* gene across the cell cycle. Here we show that the MCE components, RFX5 and CREB, are dynamically associated with mitotic chromatin. Maintenance of the MCE correlates with transcriptional activity and an open chromatin state that is fully or weakly maintained in mitotic lymphoblastoid or non-lymphoblastoid cells, respectively. In the former, mitotic transcription can be rescued by exogenously added CIITA, but not in the latter. Conversely, reduced abundance of activation-associated histone PTMs during mitosis does not support a specific bookmarking role. We provide evidence for a new role of the upstream *DRA*-LCR/XL4 in the selective mitotic occupancy by PP2A via NFYA interaction to bookmark the *DRA* gene and control the proper temporal regulation of its transcription in the next cell cycle. Overall, we show that cell- and gene-specific processes determine the mitotic and post-mitotic expression of the *DRA* gene. In B lymphoblastoid cells and potentially other professional APCs, sustained mitotic occupancy by regulatory complexes allows uninterrupted expression contrary to the epithelial non-professional APCs. In spite of mitotic disruption of *MHC* expression, a gene bookmarking process that involves the alternative use of an upstream regulatory region ensures proper gene reactivation after mitosis.

MATERIALS AND METHODS

Cell culture

Raji, a Burkitt lymphoma derived cell line, which constitutively expresses *MHCII* (*MHCII*$^+$) and RJ2.2.5, its CIITA-defective derivative (*MHCII*$^-$) cell line, were grown in RPMI with 20% fetal bovine serum (FBS). HeLa cervical carcinoma epithelial cells and HEK293T cells were grown in DMEM with 10% FBS. Synchronization of Raji cells was done by double Hydroxurea (HU) (Sigma) block (200 μM for 12 h, 8 h release, block for 14 h before final release) or 1 μM nocodazole (Sigma) for 20 h for prometaphase-arrested cells. Samples were processed for DNA analysis by propidium iodide-PI (Sigma) and flow cytometry for DNA content (FACS Calibur). Mitotic cell content was assessed by DNA staining with Hoechst 33442 (Sigma) and direct fluorescent microscopy or double α-H3S10-Phos–Propidium Iodide staining followed by flow cytometry as described in (34). Similarly treated epithelial cells were further enriched for mitotic cells by shaking off. When necessary, apoptotic cells were removed by a magnetic bead-streptavidin and biotin-AnnexinV depletion.

Reagents

α-RFX5 was from Rockland, α-NFY-A (H-209) (sc-10779), α-NFYA (G-2) (sc-17753), α-NFY-B (FL-207) (sc-13045), α-CREB (C-21) (sc-186), α-RNA Polymerase II (N-20) (sc-899), normal rabbit IgG (sc-2027), normal mouse IgG (sc-2025) were from Santa Cruz Biotechnology, α-TBP (ab28175) and α-H3 (ab1791) were from Abcam, α-acetyl H3 (06-599), α-acetyl H4 (06-866), α-H3K4me2 (07-030), α-H3K4me3 (07-473), α-H3S10-Phos (06-570), α-PP2Ac (Cat.# 05-421) and α-CREB (17-600) were from Millipore, α-PP2Ac (#2028) was from Cell Signaling, α-β-tubulin (T4026) was from Sigma-Aldrich, α-GFP was from Minotech, α-JellyRed (α-KillerRed, Cat.# AB962) was from Evrogen, mouse monoclonal α-myc antibody was secreted from the hybridoma cell line Myc-9E10.2 (38) and anti-CIITA antibody was described earlier (39). PP2A-Cα (sc-43509) and PP2A-Cβ (sc-36301) siRNAs were from Santa Cruz Biotechnology and siSCR (scrambled) was from CUREVAC. siRNAs were used at 140 nM final concentration. RNA transcription was blocked with 100 μM DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole) (Sigma) treatment for 1 h.

Microscopy

Cells were grown in Lab-Teck chambers or regular tissue culture plates and fixed with 2% paraformaldehyde/0.1% Triton X-100/20 mM HEPES or 70% acetone in 1× PBS for 5–10 min, 24–48 h post-transfection, and washed with 3% FCS in 1× PBS prior to observation or antibody staining. DNA staining was done by Nuclear-ID Red from Enzo (Cat. No.51008-100). Live microscopy was performed with adherent cells growing on Lab-Teck coverslips or suspended cells mounted on glass slides and were examined within 30 min at room temperature. Confocal microscopy was carried out on a Zeiss Axioscope 2 plus microscope equipped with a Bio-Rad Radiance 2100 laser scanning system and LaserSharp 2000 imaging software. Fluorescence recovery after photobleaching (FRAP) analysis was done using a standard region of interest (ROI) and monitoring close to saturation of recovery. After subtracting the background, fluorescent intensities were normalized against a companion unbleached ROI in the same cell.

Plasmids and short hairpin RNA vectors

A CMV-driven myc epitope-tagged CIITA construct was used to isolate G418-resistant RJ2.2.5 and HeLa cells that express high levels of MHC class II genes. Co-transfection of RJ2.2.5 cells with a tetracyclin-regulated plasmid driving *CIITA* expression and

rtTA-GBD (reverse tTA fused to the glucocorticoid receptor-binding domain) (40) allowed for isolation of a bulk cell population that was highly inducible by the combined action of 0.5 μg/ml doxycycline (Sigma) and 0.5 μM dexamethasone (Sigma). HeLa and HEK293T were transfected by the calcium–phosphate method or by lipofectamin according to the manufacturer's instructions. Lymphoblastoid cells were transfected by electroporation or the Amaxa method according to the manufacturer's protocols. NFYA fragments were constructed on pJRed-C plasmid (Evrogen), CREB on pECFP-C, RFX5 and NFYB on pEGFP-C (Clontech) and PP2Ac on pCDNA3 plasmid in frame with the myc epitope cloned N-terminally. The lentiviral pLKO.1 base plasmids carrying a short hairpin (sh) scrambled (kindly provided by Dr R. Everret) and the shRNA-NFYA (TRCN0000014930, Thermo scientific) were used. For lentivirus production, the shRNA vector and second-generation packaging plasmids (from Dr. Trono lab) pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) were co-transfected into HEK293T cells. Lentivirus-containing supernatants was collected 48 and 72 h after transfection, centrifuged to remove cell debris, 0.45 μm filtered, concentrated by PEG precipitation and stored at -80°C until use. Viral titers were determined by infecting HeLa cell monolayers in the presence of 5 μg/ml polybrene with serial dilutions of viral stocks and measuring fractions of puromycin resistance over a 2-day period. For efficient knock down, cells were infected at 4–8 MOI.

Restriction endonuclease and DNase I hypersensitivity assay

For the restriction endonuclease accessibility assay, 10^7 cells were collected and washed three times with 1× PBS containing 0.1 mM PMSF, 0.5 μg/ml leupeptin and 2 μg/ml aprotinin. The cells were lysed in 5 ml cell lysis buffer (10 mM Tris–HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.1% NP-40, 300 mM sucrose). The extent of cell lysis and the intactness of the nuclei were confirmed by Trypan blue staining and light microscopy. Nuclei were collected by centrifugation and resuspended in 1 ml digestion buffer (50 mM Tris–HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT). 2×10^6 nuclei were digested to a volume of 100 μl in each reaction, with increasing concentrations of *Ssp*I restriction endonuclease (Minotech) (0, 10, 50, 100 and 200 units) or (0, 1, 5, 10 and 20 units) of DNase I (Sigma). Digestion was carried out for 30 min at 37°C. Following digestion, nuclei were lysed by addition of 200 μl nuclear lysis buffer (300 mM sodium acetate, 5 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K) followed by overnight incubation at 55°C. DNA was purified twice by phenol/chloroform extraction followed by ethanol precipitation. The amount of purified DNA was quantified by Nanodrop spectrophotometer, and 50 ng was used in every real-time PCR reaction. Primer sequences that were used for real-time PCR are provided below. For DNase I hypersensitivity assay, a 3-hour treatment with 50 nM okadaic acid, OA (Sigma), was performed after nocodazole blockage for 20 h.

Protein co-immunoprecipitation assay

Co-immunoprecipitations were performed with HEK293T cell extracts prepared by RIPA cell lysis buffer containing 25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% Deoxycholate, 0.1% SDS, 1 mM PMSF. Protein extracts were diluted at least three times with IP buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl) and then were pre-cleared by adding protein G beads for 1 h at 4°C. Beads were removed by centrifugation. The antibody was added to the supernatant, and the reactions were incubated overnight at 4°C. Protein G beads were added after washing with IP buffer, and reactions were incubated for three additional hours. Non-specific proteins were washed away three times with NETN buffer (10 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF). Laemmli loading buffer was added, and samples were boiled prior to SDS-PAGE analysis. Input represents 10% of the lysate used for the immunoprecipitation. In the case of the endogenous protein co-immunoprecipitation, the above protocol was modified as follows: HeLa cell extracts were pre-cleared with IgG-covered protein G beads, the incubation with the antibody was reduced to 2 h followed by addition of protein G beads for 1 h. Input represents the 5% of the lysate used for immunoprecipitation.

Chromatin immunoprecipitation assay (ChIP)

Chromatin, from 10^7 cells, was prepared by fixation of the cell culture with 1% formaldehyde and incubation for 10 min at RT. The cross-linking reaction was quenched by addition of glycine to 0.125 M. Three successive washes with ice cold 1× PBS (with 1 mM PMSF) followed, and cells were subsequently lysed by addition of 100 μl per 10^6 cells lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.0) and incubation for 15 min at 4°C. Upon centrifugation, chromatin was sonicated to an average length of 500 bp. The efficiency of DNA fragmentation from asynchronous and mitotic cells was examined by agarose gel electrophoresis. Immunoprecipitation was performed with the equivalent of 10^6 cells per sample, diluted 5 times with ChIP dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.0, 167 mM NaCl supplemented with protease inhibitors) to which 2–10 μg of each antibody were added and rotation followed at 4°C overnight. Twenty microlitre protein G agarose beads per sample were blocked with 1 mg/ml BSA, 100 μg/ml salmon sperm DNA in ChIP dilution buffer and rotated separately at 4°C overnight. Twenty microlitre were kept for input DNA (20%). Each sample was mixed with the blocked beads and rotated for 3 h at 4°C. Immunoprecipitated material was washed twice with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl and protease inhibitors), twice with high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.8, 500 mM NaCl and protease inhibitors) and

once with TE, pH 8.0. Following the last wash, samples were incubated with proteinase K 200 μg/ml, SDS 0.5% in TE for 3 h at 55°C and then formaldehyde cross-links were reversed by overnight incubation at 65°C. DNA was purified twice with phenol/chloroform extraction, and was ethanol precipitated. Quantitative analysis was performed on an MJ-Chromo 4 real-time PCR instrument and the primer sets that were used are provided below.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total cell RNA was extracted by the addition of Trizol (Cat.# 15596-018, Invitrogen) directly on the pellet. DNase I (Takara) treatment took place according to the manufacturer's protocol. 2 μg RNA was reverse transcribed with M-MuLV (F-572S, Finnzymes) supplemented with RNase inhibitor (HT Biotechnology). Primer sets used for real time PCR analysis are shown below. Relative abundance of each transcript was measured by quantitative real time PCR with SYBR Green I (Invitrogen).

Primers

The sequences of primers that were used for PCR are as follows:

DRA promoter:	5′-GTTGTCCTGTTTGTTTAAGAA-3′
	5′-TCTTTTGGGAGTCAGTAGAGC-3′
DRA–2000:	5′-CAGAGAAAGGGAACTGAAAGTCATTT-3′
	5′-GGGCCAGATGAGAGCCTATGGTATC-3′
DRA–1800:	5′-AAGCCCTTTGATGCGATCAG-3′
	5′-TTCCATACTGTTGTGCTGGG-3′
DRA LCR/XL4:	5′-CAGAGAAAGGGAACTGAAAGTCATTT-3′
	5′-TTATGACACTGTTTAGTCCTAGAACACTGA-3′
DRA exon1:	5′-GCCTCACTCCCGAGCTCT-3′
	5′-CGCTCATCAGCACAGCTATG-3′
DRA exon5:	5′-GAAAGCAGTCATCTTCAGCGTT-3′
	5′-AGAGGCATTGCATGGTGATAAT-3′
DRA exon–intron:	5′-GCCTCACTCCCGAGCTCT-3′
	5′-ATGCTTCGTAGTCTATCGTCCC-3′
CIITA exon:	5′-CACAGCCACAGCCCTACTTT-3′
	5′-CGACATAGAGTCCCGTGAGC-3′
PP2A exon:	5′-ATGGACGAGAAGGTGTTCA-3′
	5′-TTTGCCACCAATTCTAAAC-3′
H4 promoter:	5′-GAGAGGGCGGGGACAATTGA-3′
	5′-CTTTCCACAGCCGGACATGACC-3′
CD4 promoter:	5′-TGTGCTCTGCCCAGTTGTCT-3′
	5′-GCTCATGACCAGTTCCAAGAGAA-3′
IFNb promoter:	5′-CCCAAGTCTTGTTTTACAATTT-3′
	5′-GGGTATGGCCTATTTATATGAG-3′
GAPDH promoter:	5′-TGAGCAGACCGGTGTCACTA-3′
	5′-AGGACTTTGGGAACGACTGA-3′
GAPDH exon:	5′-CCTTCCGTGTCCCCACTGCCAAC-3′
	5′-GTGTCGCTGTTGAAGTCAGAGGAG-3′
GAPDH exon–intron:	5′-GGTCGTATTGGGCGCCTG-3′
	5′-GCTGCCCATCAGCCAGGT-3′
CyclinB1 promoter:	5′-GGAGCAGTGCGGGGTTTA-3′
	5′-CGACCAGCCAAGGACCTACA-3′
CyclinB1 exon:	5′-GCAAGCAGTCAGACCAAAAT-3′
	5′-CATGAACCATCAATAATGG-3′
CyclinB1 exon–intron:	5′-GCAAGCAGTCAGACCAAAAT-3′
	5′-CCTGGAAGGGAAAACTCTTA-3′
MYC exon–intron:	5′-CACCACCAGCAGCGACTCT-3′
	5′-CAATGAAAATGGGAAAGGTATCCA-3′
DRB1 promoter:	5′-AAAGTGTTTTACATGCAACTGG-3′
	5′-TCCTGGATTGGGTAATCTAGTTG-3′
XL7:	5′-GAGGAGGCAAGTCTTACTCAGGA-3′
	5′-CCGACCCAGGAGCAAGAAAGTC-3′
XL9:	5′-TTGTGGTACCAATCTTGCTGTAA-3′
	5′-CAGCTACTGACTCAAGAGCAATGT-3′

RESULTS

MHCII enhanceosome (MCE) components are dynamically associated with mitotic chromatin

Earlier biochemical and subcellular localization studies (15,41) have defined an intricate hierarchy of interactions between the MCE subunits required for enhanceosome formation and subsequent recruitment of CIITA. The MCE is thought to form in a step-wise manner involving the independent assembly of the NFY and RFX subcomplexes first, followed by synergistic binding to chromatin. In spite of abundant biochemical and functional information, little is known about the dynamics of MCE–CIITA and its stability in living cells, especially during the cell cycle. Because during mitosis transcription is generally disrupted, we chose to study whether MHCII factors are retained on chromatin in this stage of the cell cycle. Initially, we performed immunostaining experiments in HeLa cells with antibodies specific for RFX5 and CREB. CREB and to a lesser extend RFX5 proteins were found to be associated with mitotic chromatin (Figure 1A). To further investigate this, fluorescent protein fusions of RFX5 and CREB were expressed and their subcellular localization was studied. Both proteins were observed to be associated with chromatin in various stages of mitosis such as in anaphase as shown in Figure 1B. To demonstrate the specificity of chromatin association, various deletion derivatives were tested. In Supplementary Figure S1 is shown such an example of RFX5 and CREB truncations that harbour the N-terminal regions of these proteins that lack chromatin-binding ability.

To examine the dynamics of the observed mitotic association of these proteins with chromatin, we used photobleaching experiments. Results in Figure 1C and D show that both RFX5 and CREB are recruited on chromatin in a dynamic manner, although with variable kinetics in different stages of the cell cycle. The lower mobility of both proteins observed in telophase has also been reported for the Brd4 transcriptional co-activator that specifically bookmarks M/G1 genes (25), and may reflect a common process important for G1 gene re-expression. As a control, histone H2A was used, which is strongly associated with DNA. This showed much lower mobility both in interphase (Figure 1D) and mitosis. These results suggest that MCE components are dynamically recruited to chromatin throughout the cell cycle.

Uninterrupted occupancy by *DRA* promoter factors throughout the cell cycle

Recent studies indicate that some DNA-binding factors such as TBP (33), Runx (32), HSF2 (24) as well as histone PTMs persist through mitosis regardless of gene expression (35,42). These events could have a memory or bookmarking function for fast transcriptional recovery to the pre-mitotic state. To examine the occupancy of the *MHCII* promoter by MCE components throughout cell cycle transition, we performed ChIP and quantitative PCR analysis in Raji lymphoblastoid cells, which constitutively express *MHCII*, after synchronization/release

from double hydroxyurea block as described in Supplementary Figure S2. This analysis showed occupancy by variable amounts of regulatory factors and histone PTMs at distinct phases of the cell cycle. Parallel analysis of newly synthesized RNA showed that RNA PolII recruitment was the best indicator of gene expression in the *DRA* as well as the control *Cyclin B1* that is specifically expressed at the G2/M and beginning of mitosis (43) and *GAPDH* genes.

To examine the mentioned changes in a better defined mitotic environment, we used 1 μM nocodazole to arrest Raji and their CIITA-negative derivatives (RJ2.2.5) cells in prometaphase. Mitotic arrest was monitored by flow cytometry using Propidium Iodide (PI) analysis (Figure 2A left). Mitotic cell content was further assessed by double α-H3S10Phos–PI staining (representative results are shown in Supplementary Figure S3). ChIP analysis of MCE components showed that CREB, RFX5 and NFYB remained generally unchanged in mitosis, irrespective of *CIITA* expression (Figure 2B). Mitotic recruitment of CIITA itself was slightly reduced. To monitor the transcriptional machinery components, antibodies against TBP and RNA PolII were used and results showed that in arrested cells *DRA* occupancy was reduced ~50% and 30% the recruitment observed in asynchronous cells, respectively (Figure 2B). As expected, recruitment of these factors was CIITA-dependent, as CIITA-deficient cells were devoid of TBP and RNA PolII whether prior or after the mitotic arrest (Figure 2B). For comparison, a similar analysis performed in RFX5-deficient ($RFX5^-$) cells and its wild type-like derivative generated by exogenous RFX5 expression that rescues *MHCII* transcription showed that RFX5 was essential for factor occupancy both in asynchronous and mitotic cells (Supplementary Figure S4).

In agreement with the results from the synchronization/release studies (Supplementary Figure S2), histone PTMs at the *DRA* promoter of Raji cells arrested in mitosis showed that histone H3 and H4 acetylation as well as di- and tri-methylation of lysine K4 at histone H3 (Figure 2C) were retained at high levels relative to asynchronous cultures (acetylation: 50 and 66%, methylation: 75 and 70%, respectively). As expected, H3 acetylation and H3K4me3 were recruited in low levels in their CIITA-negative counterparts and did not change significantly during mitosis (Figure 2C). Interestingly, high H3K4me2 levels were observed in both cell lines, irrespective of CIITA expression and were maintained in mitosis at ~50–80% of the asynchronous levels (Figure 2C).

To examine whether these properties also apply to non-lymphoblastoid cells, we used HeLa cells that are physiologically induced by IFNγ. To overcome the problem of transient induction and decay in monitoring expression through the cell cycle, we generated CIITA transfectants that express *MHCII* genes at levels similar to Raji as measured by qRT-PCR (Supplementary Figure S5). Mitotically arrested HeLa or HeLa CIITA transfected ($CIITA^+$) cells (Figure 2A right) were monitored as before. Figure 2D shows that the integrity of the enhanceosome is compromised during mitosis regardless of CIITA expression. Thus the observed global chromatin association of RFX5 or CREB in mitosis may reflect weak interactions that do not predict locus-specific factor occupancy. Recruitment of CIITA, TBP and RNA PolII was also reduced in arrested cells. Overall, these data show reduced mitotic occupancy of the *DRA* promoter in non-lymphoblastoid cells (HeLa) that cannot be counteracted by *CIITA* expression.

Examination of activating histone PTMs in HeLa cells showed a lack of histone H3 acetylation and methylation marks in either asynchronous or mitotic cells (Figure 2E). On the contrary, when *CIITA* was ectopically expressed in HeLa cells, H3K4 di/tri-methylation was maintained during mitosis at high levels (80% of the asynchronous—similar to Raji), whereas acetylation dropped to 30–40% of the asynchronous levels (Figure 2E). Strikingly, although CIITA-deficient B cells (RJ2.2.5) retain high H3K4me2 promoter levels, HeLa cells are devoid of this modification that occurs only after transcriptional activation of the *DRA* gene (Figure 2E). Thus, although this modification does not directly correlate with active transcription, it may be related to a highly accessible chromatin state as we have shown previously (37).

To study whether mitotic retention of MCE components correlates with the chromatin state of the *DRA* gene, we used a restriction endonuclease assay on asynchronous or nocodazole-arrested cells. To this end, we used an SspI site, located 80 bp upstream of the *DRA* transcription start site (TSS) that was previously shown to be differentially sensitive to digestion correlating with transcription or transcription potential (Figure 3A) (37). Digestion efficiency was quantified by qPCR with primers flanking the SspI site and expressed as percentage of undigested DNA relative to that found in non-digested mock-treated sample. This approach revealed that B lymphoblastoid cell hypersensitivity of this particular site to SspI was retained during mitosis to levels similar to those observed in asynchronous cultures in a CIITA-independent manner (Figure 3B and C) over a range of enzyme concentration. As a control, RFX5-deficient SJO cells were also examined and this particular region was found to be inaccessible to digestion irrespectively of the amount of restriction enzyme used.

In asynchronous HeLa cells, the *DRA* promoter was accessible to SspI cleavage (Figure 3D and E), although to a significantly lesser extent relative to Raji cells, but sensitivity was abolished in mitosis. CIITA-expressing HeLa cells, however, exhibited a higher degree of restriction enzyme accessibility that was partly maintained during mitosis. Thus, chromatin accessibility in distinct cell settings and cell cycle stages correlates well with increased transcription factor occupancy and gene activation-associated histone modifications.

Transcriptional activity of *MHCII* genes in mitosis

The above results show that the MCE-CIITA-GTM complex in mitosis is maintained in lymphoblastoid but not in non-lymphoblastoid cells. We next addressed the question whether transcription is sustained during mitosis. In most systems studied, association of regulatory

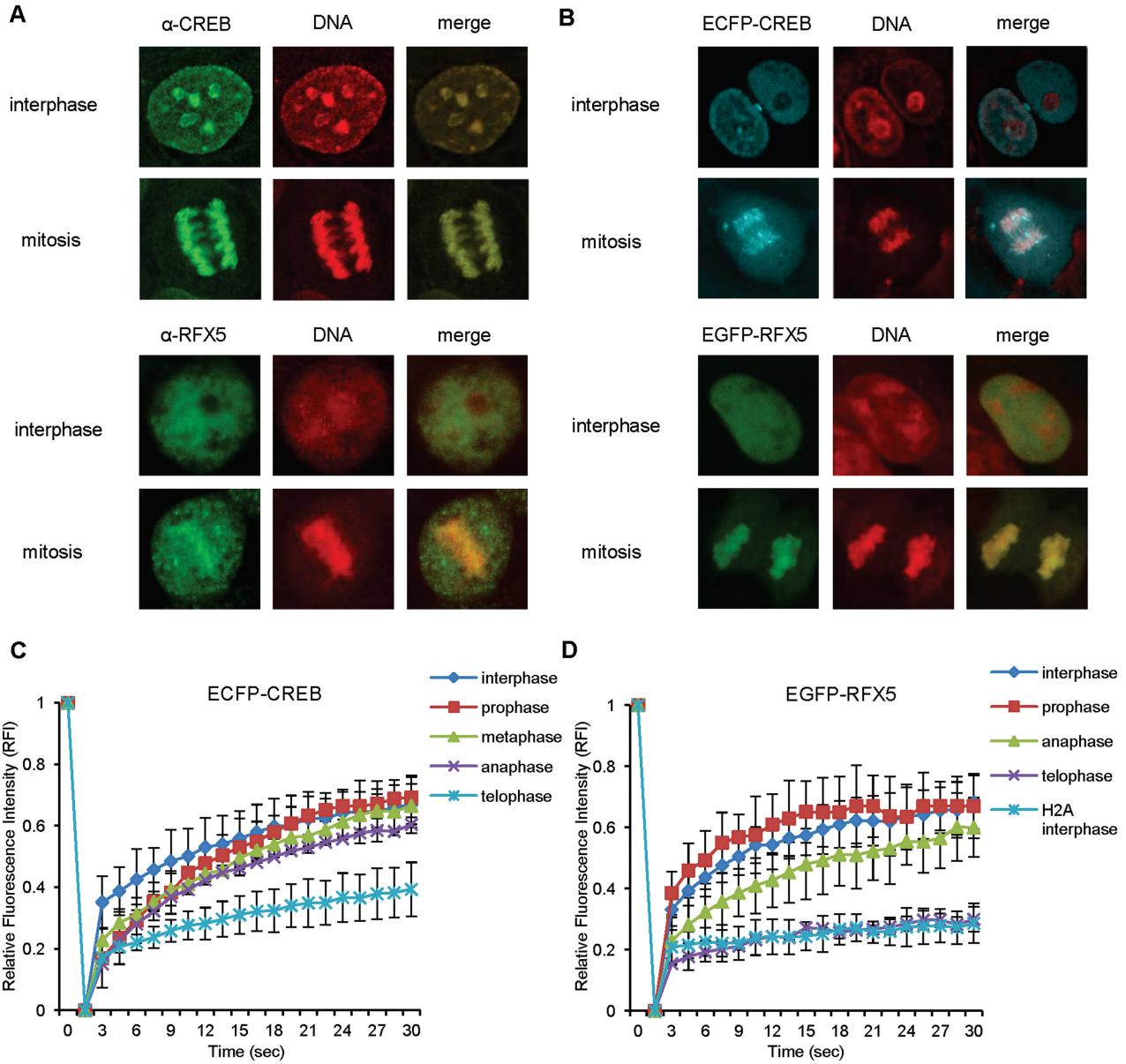

Figure 1. Enhanceosome components are dynamically associated with mitotic chromatin. (**A**) Immunostaining of HeLa cells using antibodies against CREB (upper panel) and RFX5 (lower panel). (**B**) Subnuclear localization of pECFP-CREB (upper panel) and pEGFP-RFX5 (lower panel) in interphase and mitotic HeLa cells. Nuclear-ID Red (red pseudo-colour) was used for DNA counterstaining. (**C** and **D**) FRAP experiments with transiently expressed ECFP-CREB (C), EGFP-RFX5 and Monomeric DsRed-H2A (D) in different stages of the cell cycle. Mitotic cells were chosen by confocal misroscopy. Results show mean values and standard deviation from at least six cells.

factors with DNA does not ensure active gene transcription because either not all essential factors remain bound to chromatin or PTMs such as phopshorylation may inactivate critical factors (18). To this end, we quantified total *DRA* RNA by qRT-PCR of asynchronous or nocodazole-arrested cells by using exon5–exon5 primer set and newly synthesized RNA by using exon1–intron1 primer set on the same sample in the presence or absence of the transcription elongation inhibitor DRB. To express the latter as a fraction of the mature RNA, we used genomic DNA as a standard to account for differences in amplification efficiencies between the above two primer sets. New RNA synthesis represented 4–5.5% of the total *DRA* RNA in the asynchronously growing cells shown in Figure 4A. In mitosis newly synthesized *DRA* RNA was reduced to 33% and 12% of the asynchronous levels of Raji or HeLa CIITA cells, respectively. To correlate active RNA synthesis with the recruitment of RNA PolII in Raji cells, we used ChIP. Results in Supplementary Figure S6 (A and C) show that promoter- or exon-bound (elongating) RNA PolII levels on *DRA* gene correlate well with the qRT-PCR-measured transcription in the different asynchronous or mitotic cells. Comparison of promoter and exon levels further indicates that there is direct correlation of initiation and elongation events without stalling of PolII in the different

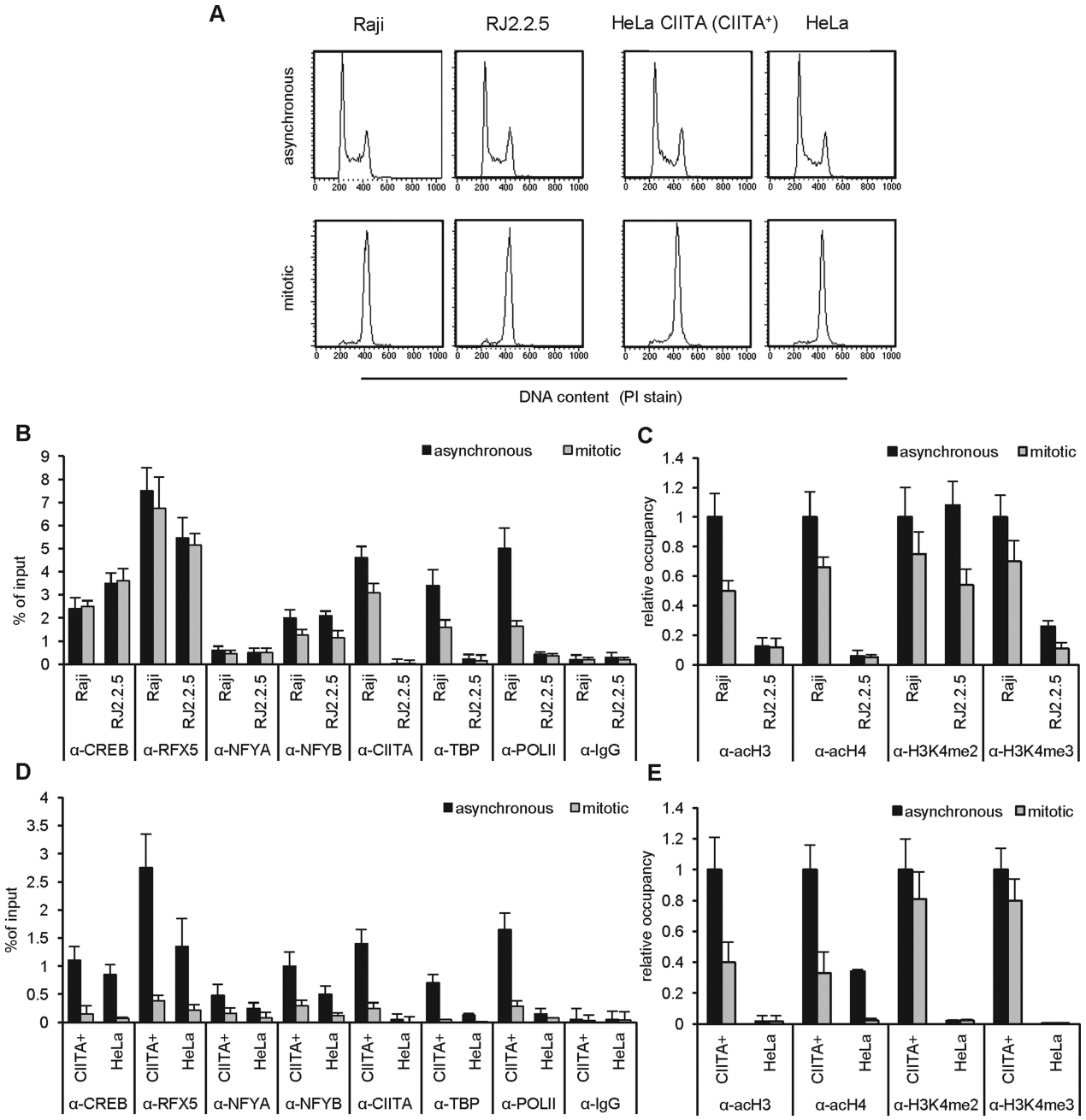

Figure 2. *MHCII* expression state-related maintenance of the enhanceosome (MCE) and activation-associated histone PTMs in asynchronous or mitotically arrested B lymphoblastoid and epithelial cells. (**A**) Flow cytometric analysis of DNA content upon propidium iodide staining of B lymphoblastoid parental Raji and its CIITA negative derivative RJ2.2.5 cell line (left panels), or epithelial parental HeLa and a CIITA transfectant HeLa (CIITA$^+$) cell line (right panels), growing asynchronously (upper panels) or mitotically arrested (lower panels) in prometaphase with nocodazole. (**B–E**) Chromatin from the above cell lines was used for ChIP-qPCR analysis with antibodies against CREB, RFX5, NFYA, NFYB, CIITA, TBP, RNA PolII and IgG (B, D) or acetyl-H3, acetyl-H4, H3K4me2, H3K4me3 (C, E) on the *DRA* promoter. Factor occupancy is expressed as % of input chromatin (B, D) or as relative occupancy of histone H3 or H4 PTMs normalized against total histone H3 or H4 (not shown) respectively (C, E). A value of 1 set for acH3/H3 in asynchronous Raji (C) or HeLa CIITA (E) cells represents 7.5%/0.93% and 11%/4% acH3 and total H3, respectively. Triplicate means and standard deviations are shown.

set ups. For specificity control, results for *CyclinB1* and *GAPDH* genes are also shown (Supplementary Figure S6B and D).

Thus, lymphoblastoid cells are more efficient in sustaining active transcription in mitosis even when expression of *CIITA* is driven by an exogenous strong promoter. To examine whether the endogenously expressed *CIITA* is the limiting factor in lymphoblastoid cells, we took advantage of the Raji-isogenic CIITA-deficient cell line RJ2.2.5. Exogenous expression of *CIITA* in the latter (RJ2.2.5 CIITA) fully rescued *MHCII* expression to levels similar to that in Raji cells (Figure 4A). Strikingly, CIITA transfectants retained 90% of the asynchronous *MHCII* expression in mitosis (Figure 4A) suggesting that an

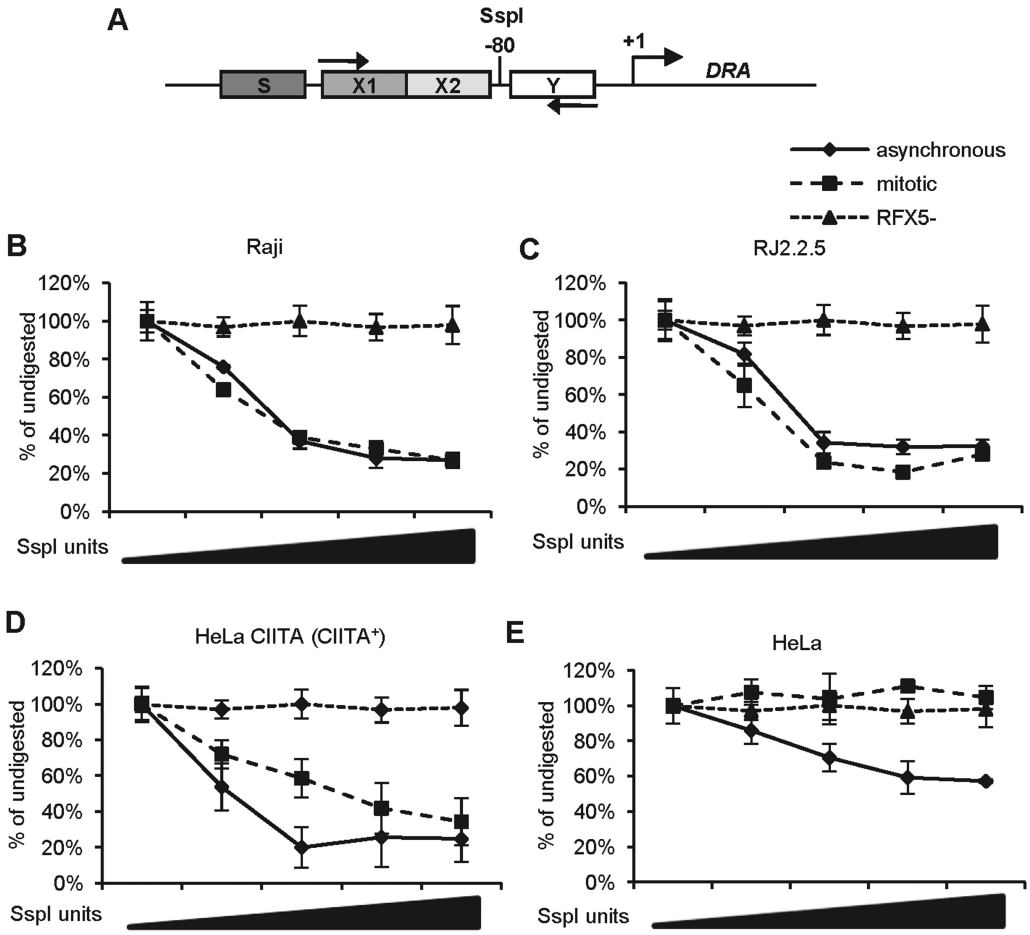

Figure 3. *DRA* locus accessibility in mitotic B lymphoblastoid cells correlates with MCE maintenance. (**A**) Schematic representation of SspI restriction enzyme site and the flanking primer target sites relative to the *DRA* gene and its regulatory elements. (**B–E**). Restriction endonuclease accessibility assay and qPCR analysis to measure the undigested DNA was performed on asynchronous growing and mitotically arrested populations of Raji (B), RJ2.2.5 (C), HeLa CIITA (CIITA^{+}) (D) and HeLa (E) cell lines. Cells were treated with increasing amounts of *SspI* enzyme (0, 10, 50, 100 and 200 units). SJO is an RFX5-deficient cell line, which is used as a negative control cell line with inaccessible SspI site on the *DRA* promoter. To normalize for template loading, a primer set spanning a region in the *DRA* exon 5 was also used that was devoid of the *SspI* recognition sequence. Map is not to scale.

exogenous CIITA supply is sufficient to rescue mitotic transcription in lymphoblastoid cells. To define whether the above reflect cell type differences in the availability of CIITA or enhanceosomal subunits we compared protein levels of asynchronous or mitotic cells untreated or after cycloheximide treatment to inhibit protein synthesis. Results in Supplementary Figure S7 show that endogenous enhanceosome subunits are equally stable regardless of cell type or cell cycle stage. CIITA is similarly short-lived in either asynchronous or mitotic lymphoblastoid cells. Exogenously expressed *CIITA* in stably transfected RJ2.2.5 cells was also short-lived but was maintained at higher levels than Raji cells and that can account for sustained mitotic transcription. Although in mitotic HeLa cells exogenous CIITA was found to be even more stable, it was not sufficient to support transcription. Thus, in lymphoblastoid cells CIITA supply determines mitotic transcription rates acting on a relatively intact enhanceosome (Supplementary Figure S8). In contrast, instability of enhanceosome seems to limit exogenous CIITA recruitment in non-lymphoblastoid cells. Taken together, these results show that mitotic transcription in the two cell types is controlled by distinct mechanisms: Availability of the transactivator and chromatin state that in turn affects enhanceosome recruitment.

To further examine the role of CIITA, and whether newly synthesized CIITA can gain access to the enhanceosome and initiate transcription during mitosis, an RJ2.2.5 line was generated to produce CIITA in response to doxycycline- and dexamethazone-activated rtTA promoter fused to the ligand-binding domain of the glucocorticoid receptor. Asynchronous or nocodazole-arrested cells (Figure 4B) were induced by treatment with both drugs for 6 h. *CIITA* and *DRA* RNA expression were measured by qRT-PCR. Results in Figure 4C show that treatment induced *CIITA* expression led to high levels of *DRA* transcription in both asynchronous and mitotic cells. Furthermore, ChIP showed that this was accompanied by newly recruited CIITA without any RFX5 change on the *DRA* promoter (Figure 4D). Thus

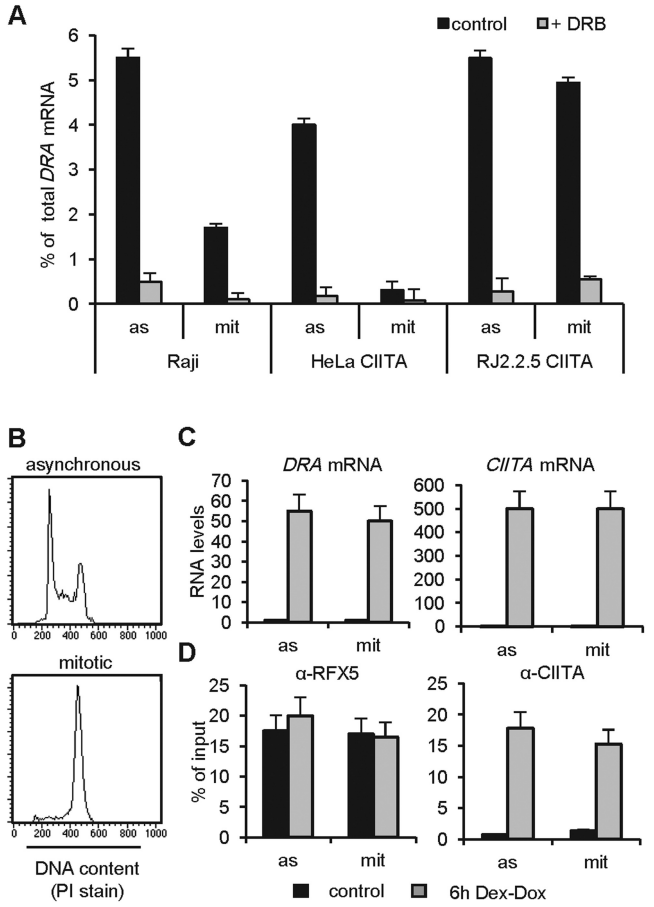

Figure 4. Cell type-specific variation of mitotic *DRA* transcriptional activity. (**A**) Total RNA was extracted from asynchronous and mitotic Raji, HeLa CIITA and RJ2.2.5 CIITA cells and active transcription of the *DRA* gene was analysed with qRT-PCR using two primer sets (*DRA* exon5–exon5 and *DRA* exon1–intron1). RNA from untreated and DRB-treated cells was used to measure net active transcription. These primer sets that monitor unspliced (nascent) or spliced (mature/total) transcripts were normalized for differences of their amplification efficiencies using a standard curve of a genomic DNA template. Newly synthesized RNA is presented as percent of mature *DRA* mRNA. (**B**) DNA content analysis by PI and flow cytometry of RJ2.2.5 cells expressing CIITA under the control of rtTA-GBD and Dex-Dox treatment (see materials and methods). Cells were blocked by nocodazole prior to CIITA induction by a 6-h treatment with Dex–Dox. (**C**) RNA was extracted from control and induced, asynchronous and mitotic RJ2.2.5 tetCIITA cells. *DRA* and *CIITA* gene expression was analysed by qRT-PCR. (**D**) Chromatin was extracted from the same samples as in (C), and ChIP assays against RFX5 and CIITA were performed.

in RJ2.2.5 cells, which maintain an intact enhanceosome on the *DRA* promoter during mitosis, the CIITA supply is the limiting factor for the uninterrupted expression of *MHCII*. Recruitment of de novo synthesized CIITA on the mitotic chromatin restored gene transcription close to asynchronous cell-levels.

NFYA–PP2A interaction on *DRA*-LCR/XL4 bookmarks *DRA* gene for rapid reactivation in G1

Our results so far establish that cell-type specific mechanisms that involve deficiency of CIITA or MCE assembly controls the transcriptional activity of the *DRA* gene promoter during mitosis. We next studied the occupation of the distal XY-like regulatory element LCR/XL4 known to mediate important gene regulatory functions (7–10). Results showed (Figure 5A) that all promoter factors studied co-occupied the upstream element but at much lower levels with the notable exception of NFYA and NFYB subunits both in interphase and in mitosis. Promoter-bound factors—especially those that remain bound to gene regulatory region in mitosis—have been implicated in bookmarking via various mechanisms including the anticondensation action of the PP2A phosphatase (24,33). To investigate the latter, we used co-immunoprecipitation of transiently expressed fluorescent protein-MHC factor fusions and myc-tagged PP2A. Results in Figure 5B showed that NFY subunits A, C and B co-immunoprecipitated with PP2A in decreasing order of strength. The interaction with NFYA was further studied and showed that the N-terminal region—between 52–158 amino acids—that include the Q-rich domain were sufficient for this interaction. Of note, NFYA and NFYC and the previously reported PP2A interacting factor TBP (33) contain Q-rich domains that may be involved in the interaction. This interaction was verified by detecting co-immunoprecipitation of the endogenous proteins (Figure 5C).

To establish the role of NFY in chromatin recruitment of PP2A, we knocked down the *NFYA* subunit by transient lentiviral infection. Of the seven different shRNAs tested, we chose one for further use, that is identical to the exon 6 targeting sh plasmid reported by Benatti *et al.* (44). Two days following infection of shSCR or shNFYA virus, cells were mock- or nocodazole-treated to obtain asynchronous and mitotically arrested cells. Efficient knock down was evaluated by Western analysis (Figure 6A). Cell cycle analysis showed that *NFYA* knock down reduced progression to the G2/M phase of the cell cycle in either untreated or nocodazole-arrested cells (Figure 6B). Chromatin from those cells was prepared and immunoprecipitated. Results in Figure 6C showed that knock down inhibits gene occupancy by NFYA in both asynchronous and mitotic cells of the *DRA*, *DRB1*, the upstream LCR/XL4 and two previously identified intergenic XL elements, XL7 and XL9 (8). Parallel immunoprecipitations showed that reduced PP2A occupancy accompanied and correlated with NFYA recruitment. In particular, even in the case of an increased mitotic occupancy of the LCR/XL4 element by PP2A, knocking down *NFYA* abrogated PP2A recruitment suggesting a yet another function of this distal *MHCII* gene regulatory region. In addition, NFYA loss also reduced CREB recruitment as expected because of the cooperative nature of the enhanceosome assembly. As a control for chromatin occupancy and quality, histone H3 was used. Results showed that NFY knock down did not significantly altered H3 recruitment. That was also the case for the XL9 element that is devoid of both MHC-related factors and PP2A, but retained high histone levels.

To extend these data to different CCAAT box-type promoters, we studied the *CyclinB1* and the *histone H4*. Results for *CyclinB1* (Supplementary Figure S9 top)

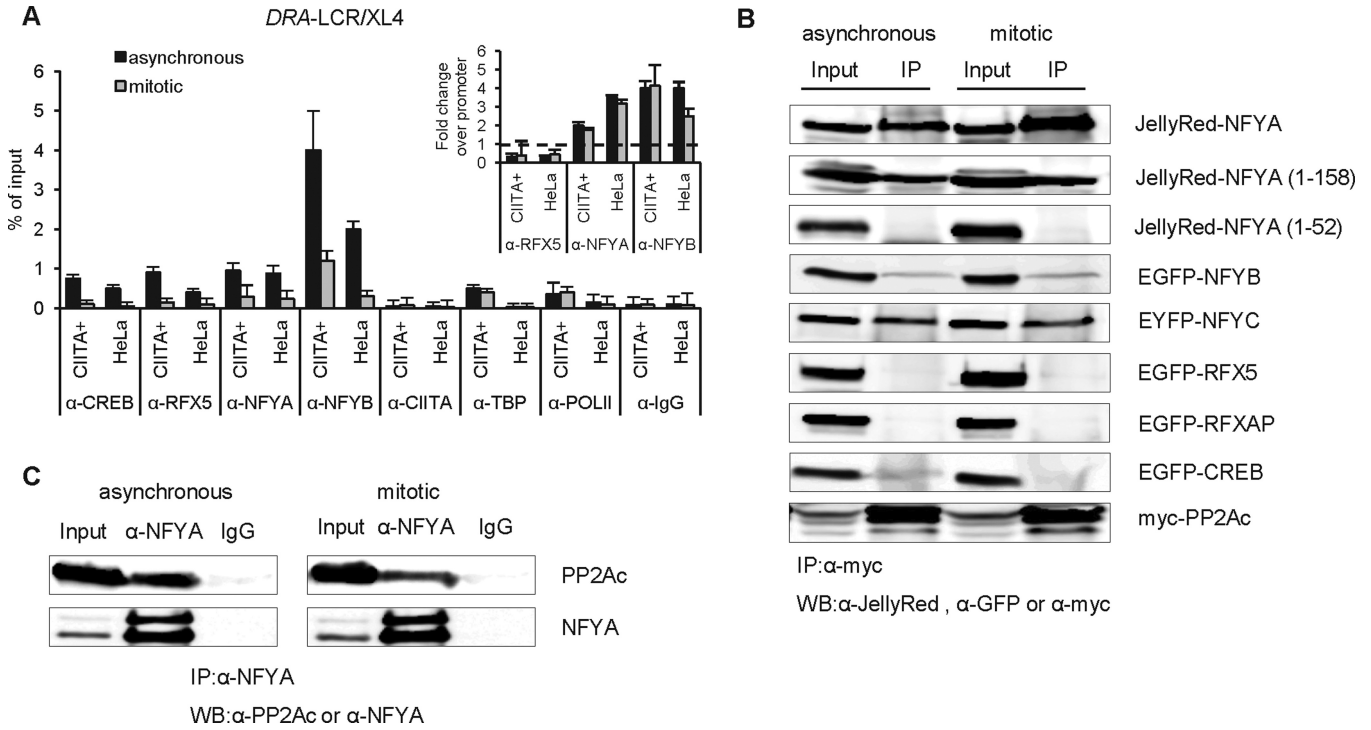

Figure 5. The *DRA*-LCR/XL4 mediates PP2A recruitment via a direct interaction with NFYA in mitosis. (**A**) *DRA*-LCR/XL4 factor occupancy using the same chromatin samples, antibodies and primer sets from asynchronous and mitotic HeLa CIITA (CIITA^{+}) and HeLa cell lines shown in Figure 2D and E. The inset shows the relative fold change of occupancy of LCR/XL4 over promoter for the indicated factors. Mean and error bars are derived as in Figure 2B. (**B**) Whole cell extracts of HEK293T cells expressing myc-tagged PP2Ac and the indicated full-length or truncated GFP- or Jelly Red-fusion proteins were used for protein immunoprecipitation experiments using an α-myc antibody. Western blotting was performed using α-JellyRed, α-GFP or α-myc antibodies. Input represents 10% of the lysate used for the immunoprecipitation. (**C**) Whole cell extracts of asynchronous or nocodazole-treated HeLa cells were subjected to immunoprercipitation by α-NFYA and immunoblotted against α-PP2Ac. Shown are the input (5% of immunoprecipitated samples), the normal IgG control and the verification of efficient NFYA immunoprecipitation.

showed that in mitotic cells, PP2A recruitment was more sensitive to NFYA levels relative to the asynchronous cells. But this was not the case for the *histone H4* gene that exhibited NFY-independent recruitment in mitosis. Parallel monitoring of CREB binding to *histone H4* and *CyclinB1* promoters showed that it's recruitment was variably depended on NFYA. The silent *interferon B* gene promoter did not show significant PP2A occupancy. Overall, these data show the *MHC* gene promoters and the LCR/XL4 distal element but also other NFY recruiting genes with distinct expression properties, recruit PP2A that is also partly maintained or increased in mitosis. Physical interaction between NFY subunits suggests a role in chromatin recruitment of PP2A as supported also by the knock down experiments. These results do not exclude the possibility of either alternative or parallel pathways for recruitment to different genes or stages of the cell cycle as exemplified above, and/or the role of local chromatin state in this process.

To determine the functional significance of PP2A mediated bookmarking on transcription, we knocked down *PP2Ac* in HeLa CIITA cells using an siRNA approach. For this, transfected cells were arrested with nocodazole and were subsequently released to proceed to the next G1 phase by removing the inhibitor. An siPP2Ac that specifically reduced *PP2Ac* mRNA levels (Figure 7A) did not affect the rate of cells entering the G1 phase (Figure 7B) or the kinetics of the *CIITA* master regulator expression levels, but caused a significant delay in *DRA* nascent RNA expression (Figure 7C). Conversely, *MYC,* which is an MLL-bookmarked gene (34), was not affected by *PP2A* knocked down. Thus, PP2A is preferentially recruited to alternative regulatory regions of the *DRA* and is required for normal timing of post-mitotic re-initiation of expression. To determine the relation of PP2A recruitment and activity with chromatin condensation, we next examined whether the phosphatase activity affects the chromatin state. For this, we measured the DNase I sensitivity of various regions across the *DRA* locus using mitotic cells in the absence or presence of the phosphatase inhibitor okadaic acid at levels that selectively inhibits the activity of PP2A (45). Figure 7D shows that both the *DRA* promoter and the *DRA*-LCR/XL4 were highly sensitive to digestion in an okadaic-dependent manner similar to the *histone H4* promoter, which has been previously shown to lose sensitivity under such conditions (33) (Figure 7E). Two regions of the *DRA* coding sequence (exons 1 and 5) were also examined and found to be sensitive. Sensitivity was also reduced in the presence of the inhibitor although to a lesser extent. Specificity was shown by the absence of sensitivity in an upstream region of *DRA* (−2000 bp from the start site) shown in Figure 7D and the silent *CD4* gene promoter (Figure 7E). Overall these results assign a novel function to the *DRA*-LCR/XL4, that of selectively retaining high amounts of factors in mitosis that associate and recruit anti-

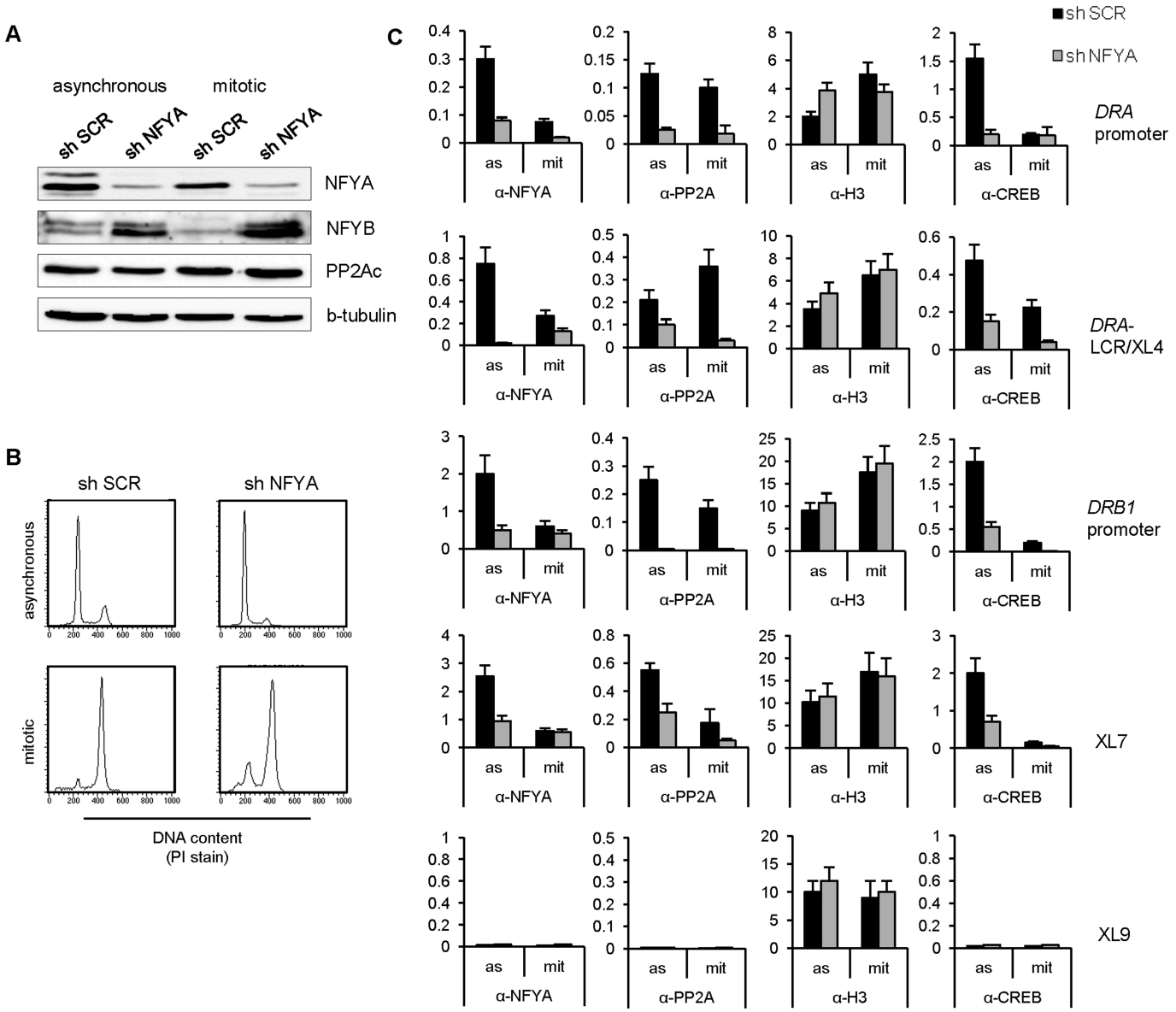

Figure 6. NFYA loss compromises recruitment of PP2A to chromatin. (**A**) Western blotting of asynchronous or mitotic HeLa cell extracts following lentiviral infection with sh-scrambled control (shSCR) or shNFYA. Equivalent protein amounts were probed with antibodies against NFYA, NFYB, PP2Ac and β-tubulin. (**B**) Flow cytometric analysis of propidium iodide-stained DNA from shSCR or shNFYA infected HeLa cells, untreated or mitotically arrested. (**C**) ChIP from shSCR or shNFYA infected, asynchronous or mitotic HeLa cells, probing the indicated regions (*DRA* promoter, *DRA*-LCR/XL4, *DRB1* promoter, XL7 and XL9) with antibodies against NFYA, PP2A, H3 and CREB. Mean and error bars are derived as in Figure 2.

condensing PP2A activity and facilitate re-initiation of gene expression upon entrance to the next G1 stage.

DISCUSSION

Overall the present results show that cell type-specific mechanisms control the mitotic (in)ability for continuous active transcription of *MHCII* genes. In B lymphoblastoid cells, components of the enhanceosome and GTM as well as activating histone PTMs are maintained on mitotic chromatin of the *DRA* gene, and support low, but significant, levels of transcription. CIITA recruitment and active transcription are dispensable for enhanceosome maintenance that in turn correlates with an open chromatin state both in asynchronously growing or mitotic cells. Mitotic deficiency of *DRA* transcription is caused by CIITA insufficiency and can be rescued by its exogenous expression. Strikingly, this effect does not require continuous (interphase) gene expression, since induction of *CIITA* in mitosis can fully initiate the transcriptional process. In spite of translational deficiency in mitosis (46) the newly synthesized protein is sufficient to re-initiate transcription at full rate. Thus, in lymphoblastoid cells, *MHCII* genes maintain an active enhanceosome

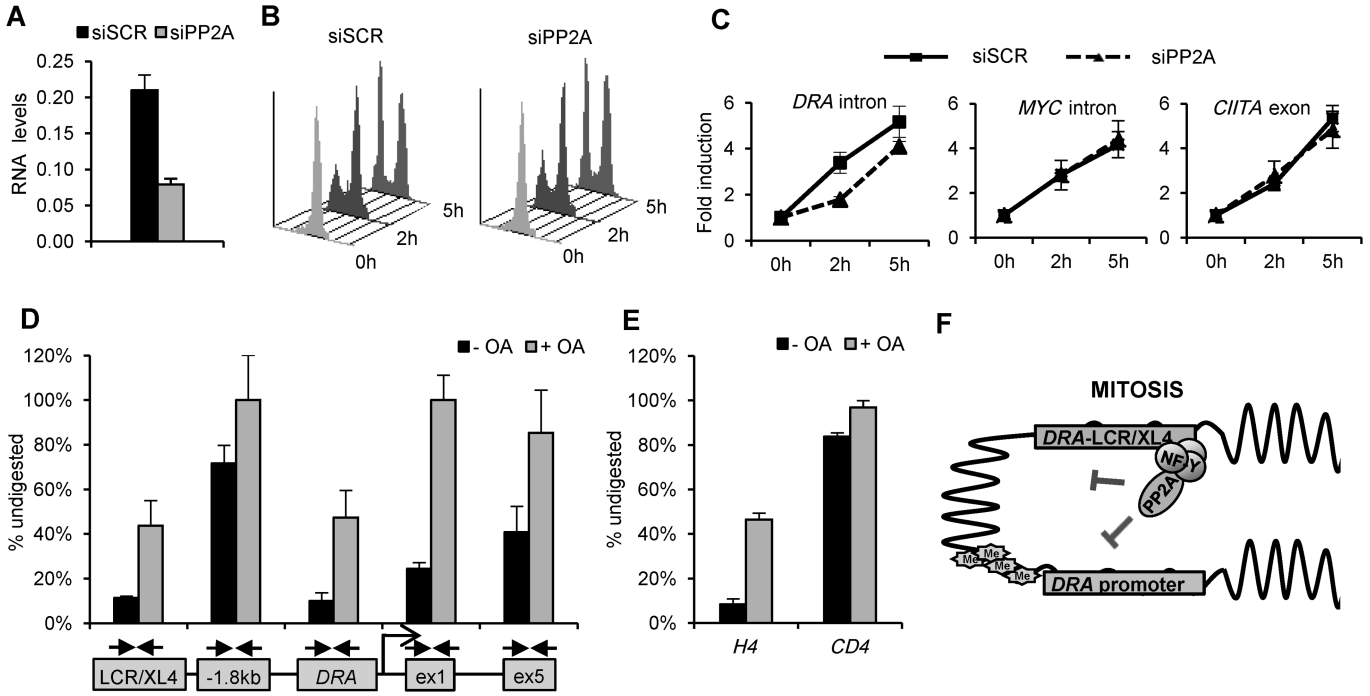

Figure 7. PP2A bookmarks the *DRA* gene for transcription timing upon entry into the G1 phase. (**A**) RNA was extracted from mitotic HeLa CIITA cells in which siRNA transfection was carried out to knock down *PP2Ac* gene expression and samples were analysed with qRT-PCR. A scrambled siRNA (siSCR) was used as a negative control. (**B**) HeLa CIITA cells transfected with siPP2Ac or siSCR blocked in mitotis with nocodazole. Upon release the samples were collected at the indicated time points (0, 2 and 5 h). Flow cytometric analysis of DNA content upon propidium iodide staining is shown on distribution plots. (**C**) RNA was extracted from the same samples as in (B) and active transcription (*DRA* and *MYC*) or total mRNA (*CIITA*) were measured by qRT-PCR analysis. (**D** and **E**) HeLa CIITA cells were mitotically arrested by nocodazole—and then treated with 50 nM okadaic acid for 3 h. DNase I hypersensitivity assay was performed and digestion was measured by qPCR with primers for five different regions on the *DRA* locus (D), *H4* and *CD4* promoters (E) in the absence or presence of okadaic acid. Results only from 20 units DNase I treatment are depicted. (**F**) Schematic representation of the *DRA*-LCR/XL4 mitotic bookmarking mechanism. Maps are not to scale.

throughout the cell cycle and their transcription is limited by the CIITA occupancy as indicated by the ability of newly synthesized CIITA to restore gene activity in mitosis.

Most studies on mitotic transcriptional shut down or bookmarking focus on a single cell type. To determine whether the same functional elements are equally important in different cell types, we also studied epithelial cells that inducibly express *CIITA* and thus *MHCII* genes. We generated a HeLa epithelial cell population that expresses *MHCII* genes at levels similar to B lymphoblastoid cells by exogenous expression of *CIITA*. The comparison of HeLa cells with the CIITA-expressing derivatives showed increased stability of the MCE, in agreement with earlier observations (15), increased chromatin sensitivity and engagement of the transcriptional machinery as expected. However in mitosis, these cells show an extensive break down of the MCE and the GTM along with significant loss of chromatin accessibility. Thus, in spite of similar structural and transcriptional properties in interphase, mitotic epithelial cells have a more pronounced break down of the transcriptional assembly and chromatin accessibility. These changes do not correlate with differences in protein availability, but they rather reflect cell type-specific chromatin accessibility that does not allow exogenous CIITA to rescue promoter assembly and transcription in mitosis. Although the basis for these differences is not established, it is tempting to evoke B lymphoblastoid cell type-specific factors that promote fast and high post mitotic levels of *MHC* expression to meet the demands of immune recognition. Histone modifications may be part of such mechanisms. For example, Valls et al. concluded that activation-linked histone methylation correlates with the mitotic expression of *Cyclin B1* and speculated that may serve as docking sites for the recruitment of effectors like BRD4, TFIID or SNF2 (42). Indeed, H4K5ac-mediated BRD4 recruitment (47) was shown recently to have a role in mitotic chromatin decompaction and post-mitotic reactivation in a real-time imaging transcriptional model. These studies, however, do not demonstrate a causal relation between promoter and histone marks. Reduced amounts of acetylation in the *DRA* locus make unlikely the possibility of a similar bookmarking mechanism. Other bookmarking factors include HSF2 (24), Runx (32) HNF1b (30) and more recently GATA1 was reported to occupy key hematopoietic regulators in mitosis (48). Such regulatory proteins and DNA methylation were termed architectural epigenetic factors that determine inheritance through mitosis (23). These and other studies have focused on the proximal TSS area for candidate bookmarking factors. TFIID/TBP has been shown to remain bound to gene promoters in mitosis (49) and play a bookmarking role (22,33). However, a recent genome-wide approach showed

bookmarking by retention of MLL binding to some gene promoters (34) and raises the possibility of distal or cryptic elements that control bookmarking. In this line, various distant enhancer-like elements have been reported in the *MHCII* locus. One such element (*DRA*-LCR/XL4), located 2 kb upstream of the *DRA* TSS, has a low MCE assembly ability, promotes extragenic transcription and histone acetylation (7). By studying MCE-component recruitment at the *DRA*-LCR/XL4 in mitosis, we suggest that NFY serves as a bookmarking factor by a mechanism similar to TBP (33). Interestingly, the NFYA subunit of NFY recruits PP2A at the *DRA*-LCR/XL4 that causes local chromatin decompaction and is responsible for the timely reactivation of post mitotic transcription (Figure 6F). Furthermore, looping of the *DRA*-LCR/XL4 to contact the proximal *DRA* promoter has been suggested to have a regulatory role in *MHCII* expression (8). Combined to our results, we suggest that among other functions, the upstream element, although devoid of most proximal regulatory factors, selectively recruits PP2A in mitosis and by its close interaction with the proximal promoter is important for the correct timing of gene expression in G1.

There are various outstanding questions regarding the factor selectivity and the dynamic position of a bookmark in a gene locus during the cell cycle. These make the analysis of transcriptome-wide bookmarking, a formidable task. NFY has more than a thousand target genes (50) that are important in cell proliferation, differentiation, apoptosis and lipid metabolism (51–53). We are currently pursuing this by genome-wide techniques aiming at the identification of new targets that may provide new tools for mitotic disruption of bookmarking of disease-associated genes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–9.

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