Gamma Interferon-Dependent Transcriptional Memory via Relocalization of a Gene Locus to PML Nuclear Bodies†‡

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Memory of past cellular responses is an essential adaptation to repeating environmental stimuli. We addressed the question of whether gamma interferon (IFN-γ)-inducible transcription generates memory that sensitizes cells to a second stimulus. We have found that the major histocompatibility complex class II gene DRA is relocated to promyelocytic leukemia (PML) nuclear bodies upon induction with IFN-γ, and this topology is maintained long after transcription shut off. Concurrent interaction of PML protein with mixed-lineage leukemia generates a prolonged permissive chromatin state on the DRA gene characterized by high promoter histone H3 K4 dimethylation that facilitates rapid expression upon restimulation. We propose that the primary signal-induced transcription generates spatial and epigenetic memory that is maintained through several cell generations and endows the cell with increased responsiveness to future activation signals.

Antigen presentation is a central process for the development and function of the adaptive immune system. It is mediated by the major histocompatibility complex (MHC) molecules that present peptides to effector cells. MHC class II (MHC-II) proteins are the major antigen-presenting determinants of exogenous peptides to helper T cells to initiate the immune response against pathogens such as bacteria and fungi. MHC-II molecules loaded with the peptide that is being presented are recognized by the T-cell receptor, along with the CD4 coreceptor on the surface of helper T cells (49). This triggers their activation with concomitant cytokine secretion to assist the activation of effector cells such as B cells to initiate antibody production (34).

Loss of MHC-II expression leads to severe immunodeficiency with both cellular and humoral immunity being affected. The syndrome called the bare lymphocyte syndrome (BLS) is characterized by recurrent bacterial and fungal infections and CD4+ lymphopenia (38). BLS patients have been classified into four genetic complementation groups, each characterized by the absence of a particular transcription factor, necessary for MHC-II transcription. These factors, three DNA-binding transcription factors (RFX5, RFXAP, and RFXANK) and the class II transactivator (CIITA), have been isolated by using genetic approaches (14, 30, 46, 47). The DNA-binding factors are recruited to the MHC-II enhancerosome but are not sufficient to drive transcription. The regulatory region of MHC-II genes forming the MHC-II enhancerosome is constantly required for transcription through interactions with the general transcription machinery (15, 19). Several PTMs are enriched throughout the gene during the active transcription process (39). It has been shown that altering the histone modification equilibrium with the use of deacetylase inhibitors in the absence of CIITA, results in the induction of MHCII transcription (18).

Apart from epigenetic changes that occur at the promoter during transcription, MHCII gene activity has been shown to correlate with long-range chromatin reorganization. IFN-γ treatment results in locus relocalization to the exterior of the human chromosome 6 territory (50). Such effects may be linked to the binding of activated STAT1 protein (9) or the reorganization of matrix attachment sites across the locus via SATB1-PML complexes (22). The role of PML nuclear bodies (PML-NBs) remains controversial, although they have been described to be rich in transcriptional coactivators (5), and many active genes—including the MHC genes—are found in their vicinity (7, 22, 42). This controversy may be due to the dynamic character of the underlying interactions or the heterogeneous nature of the PML-NBs conferred by the various PML isoforms (5).

Although the local or long range cellular events that mark activation of the MHCII genes have been well studied, very little is known about the postinduction processes. In other systems, long-range chromosomal movements along with histone variants (6, 32), epigenetic modifications (29, 31), chromatin remodelers (17, 23) and catabolic enzymes (54) have been shown to characterize not only transcriptional activation but also transcriptional memory. We show here that restimu-
lation of cells with IFN-γ leads to earlier and stronger MHCII transcriptional induction. This adaptive response is caused by earlier CIITA recruitment facilitated by altered chromatin architecture and increased accessibility on the DRA promoter that are maintained in IFN-γ-primed cells for several cell cycles after removal of the primary stimulus and transcription shut off. These features are attributed to persistent high levels of dimethylation on lysine 4 of histone H3 (H3K4me2). We also show here for the first time that persistence of this epigenetic mark correlates with a change of the subnuclear localization of the MHC locus that approaches PML-NBs upon activation and remains in their proximity for several cell generations after cease of transcription. Importantly, we show that MLL, the H3K4-methyltransferase complex, and PML-NBs interact via the MLL-WDR5 core subunit and the highly IFNγ-inducible isofom PML-IV, respectively. Importantly, we show that PML has a direct role in the persistence of memory and the H3K4me2 levels in the promoter.

**MATERIALS AND METHODS**

**Cell culture.** HeLa cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. IFN-γ was used at a concentration of 100 U/ml unless otherwise stated. Cells were left untreated (control) or stimulated with IFN-γ for 24 h, washed three times with phosphate-buffered saline (PBS), trypsinized, and plated on new plates with fresh medium to allow for subsequent cell divisions to occur (primed). During this time period, untreated and IFN-γ-treated cells went through more than four successive replicates as calculated by direct cell counting. Cell doubling times (in hours ± the standard deviation) for untreated and IFN-γ-treated cells were 20.3 ± 2.1 and 21.0 ± 1.8, respectively. At 96 h post-release, IFN-γ was added again to monitor re-stimulation kinetics in comparison to primarily stimulated cells.

**Reagents.** IFN-γ was purchased from R&D Systems (catalog no. 285-IF), Antibodies against the phosphorylated form of STAT1 (Tyr701; catalog no. 9171) from Cell Signaling Technology while total STAT1 (E-23; catalog no. sc-346), RNA pol II promoter as a control, which is not affected by IFN-γ treatment. Restriction enzyme accessibility assay. A total of 2 × 10^6 HeLa cells were washed with ice-cold PBS and nuclei were isolated with the use of lysis buffer (10 mM Tris-HCl [pH 7.5], 60 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.1% SDS and NaCl up to 0.5 M). Immunoprecipitated chromatin was reverse cross-linked by 0.5% SDS and 0.2 mg of protease K/ml at 55°C for 3 h, followed by overnight incubation at 65°C. DNA was phenol-chloroform extracted and precipitated with ethanol and glycogen. Enrichment of specific sequences in the immunoprecipitated DNA was measured by real-time PCR with SYBR green I and expressed as a percentage of input DNA. PCR was carried out for DNA, as well as GAPDH promoter as a control, which is not affected by IFN-γ treatment.

**Restriction enzyme accessibility assay.** A total of 2 × 10^6 HeLa cells were washed with ice-cold PBS and nuclei were isolated with the use of lysis buffer (10 mM Tris-HCl [pH 7.5], 60 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.1% SDS and NaCl up to 0.5 M). Immunoprecipitated chromatin was reverse cross-linked by 0.5% SDS and 0.2 mg of protease K/ml at 55°C for 3 h, followed by overnight incubation at 65°C. DNA was phenol-chloroform extracted and precipitated with ethanol and glycogen. Enrichment of specific sequences in the immunoprecipitated DNA was measured by real-time PCR with primers flanking the Sp1 site (DRA promoter upper and lower) with increased accessibility yielding less PCR product. PCR at the 3’ end of DRA gene with primers (DRA RT, upper and lower) that did not flank any restriction site was carried out in every restriction reaction to correct for equal loading of the samples.

**Plasmids and transfection.** DNA was delivered to cells by using the calcium phosphate DNA precipitation method. Delivery of siRNA was carried out according to the manufacturer’s instructions. PML isoform III and IV expression vectors were cloned on pCDNA3 plasmid (Invivogen) in frame with the myc epitope cloned N terminally.

**RNA extraction and quantitative reverse transcription-PCR (qRT-PCR).** Total RNA was isolated by using the TRIzol reagent according to manufacturer’s instructions (Invitrogen). Reverse transcription was carried out using M-murine leukemia virus reverse transcriptase (Finnzymes) and random hexamers using the manufacturer’s protocol. Relative abundance of transcripts was measured by quantitative real-time PCR with SYBR green I. The data were corrected with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels for equal loading.

**Protein and chromatin immunoprecipitation (ChIP).** Immunoprecipitations were done with HeLa cell extracts prepared by lysis buffer (EB) containing 50 mM Tris 8.0, 170 mM NaCl, 50 mM NaF, 0.5% NP-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein extracts were precelared by adding EBC-washed 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 EBC, and reactions were incubated for 3 additional hours. Reactions were next washed extensively with NETN buffer (10 mM Tris [pH 8.0], 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF). Laemmli loading buffer was added, and the samples were boiled prior to SDS-PAGE analysis. Input lanes represent 10% of the lysate used for the immunoprecipitation.

A total of 2 × 10^7 HeLa cells were fixed in 1% formaldehyde for 10 min at room temperature. Formaldehyde was subsequently quenched with 0.125 M glycine for 5 min, and the cells were washed three times with ice-cold PBS. Cells were lysed in buffer A (10 mM Tris [pH 8.0], 1 mM EDTA, 0.5% NP-40, and 1 mM PMSF) on ice for 10 min. Nuclei were pelleted and resuspended in buffer B (10 mM Tris [pH 8.0], 1 mM EDTA, 0.5 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.5% sarcosyl) for 10 min on ice. Chromatin was pelleted and resuspended in buffer C (10 mM Tris [pH 8.0], 1 mM EDTA, and 0.1 M NaCl) and sonicated to 500-bp to 1-kb size fragments, as verified by agarose gel electrophoresis. Fragmented chromatin was centrifuged to remove the insoluble fraction and was precleared with protein G beads. Then, 20-μg portions of chromatin were immunoprecipitated with the respective antibodies in immunoprecipitation assay buffer (1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl, 1 mM PMSF) overnight rotating at 4°C. The antibody-chromatin reactions were precipitated with protein G beads for 3 h by rotation. Unbound chromatin was removed by seven washes in RIPA buffer supplemented with 0.1% SDS and NaCl up to 0.5 M. Immunoprecipitated chromatin was reverse cross-linked by 0.5% SDS and 0.2 mg of protease K/ml at 55°C for 3 h, followed by overnight incubation at 65°C. DNA was phenol-chloroform extracted and precipitated with ethanol and glycogen. Enrichment of specific sequences in the immunoprecipitated DNA was measured by real-time PCR with SYBR green I and expressed as a percentage of input DNA. PCR was carried out for DNA, as well as GAPDH promoter as a control, which is not affected by IFN-γ treatment.

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**Three-dimensional immunofluorescence in situ hybridization (immuno-FISH) and microscopy.** HeLa cells were cultured on glass coverslips coated with 0.5% gelatin. Cells were treated with CSK buffer (0.1 M NaCl, 0.3 M sucrose, 3 mM MgCl₂, 10 mM PIPES, 0.5% Triton X-100 [pH 6.8]) for 5 min on ice and fixed with 4% paraformaldehyde in 1× PBS for 12 min at room temperature to preserve the three-dimensional structure of the nucleus. Paraformaldehyde was subsequently washed three times with PBS, and the cells were treated with 0.1 N HCl for 10 min at room temperature to remove histones. The cells were subsequently dehydrated with 70, 80, 95, and 100% ethanol. Heat denaturation was carried out in 50% formamide, 10% dextran sulfate, and 25 mM PO4 in 2× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate) with nick-translated probe directly labeled with fluorescent nucleotides for 5 min at 80°C, followed by hybridization at 37°C for 16 h. Coverslips were washed three times with 2× SSC (at room temperature and at 37 and 50°C) and blocked with 1% normal goat serum in 1× PBS. Immunostaining was then performed, with α-PML antibody for 1 h, followed by three washes with PBS and then another hour of incubation with secondary antibody labeled with fluorescein. Secondary antibody was washed again three times with PBS, and the samples were mounted on microscope slides. For green fluorescent protein (GFP)/red fluorescent protein (RFP) fusion analysis, cells grown on chambers (Lab-Tek) were transiently transfected and were analyzed alive or after fixation. 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. Stacks of 0.3 μm were captured and processed. Euclidean distances in three-dimensional space between the genomic loci and the nearest PML body were measured by using Velocity software (Improvision).

**Immunostaining.** Cells for immunostaining were fixed essentially as described for three-dimensional immunofISH to preserve the three-dimensional structure and directly stained with the respective antibodies. Samples were analyzed by confocal microscopy.

**Primers.** The primers used here were as follows. For RT-PCR, we used DRA RT Primer (5’-GAAAGGACGTCATCTTFCAGGTG-3’) and DRA RT Lower...
(5′-AGAGGCTTGGATGTTTAAT-3′) and CIITA RT Lower (5′-CCACAGCTGTGGTGTT-3′) as well as HLA-DRA and GAPDH RT Lower (5′-AGGGCGCTGCTGATTTTCGACCT-3′) and GAPDH RT Lower (5′-CCTTGGATCAGTGCCCACT-3′). For ChIP, we used DRA promoter Upper (5′-GTTGTCCTGTTTGTTTAAGAAC-3′) and GAPDH promoter Lower (5′-GTTGTCCTGTTTGTTTAAGAAC-3′) and GAPDH promoter Upper (5′-GGAGCTGCTCGCACTCAAAGC-3′); and GAPDH RT Upper (5′-GCCTGGATCAGTGCCCACT-3′) and GAPDH RT Lower (5′-GTTGTCCTGTTTGTTTAAGAAC-3′). GAPDH RT Upper (5′-GCCTGGATCAGTGCCCACT-3′) and GAPDH RT Lower (5′-GTTGTCCTGTTTGTTTAAGAAC-3′).

Statistical analysis. Frequencies at various PML locus distance intervals were compared by chi-square (χ²) analysis. All distributions had abnormal or uncertain distributions (goodness-of-fit analysis by Kolmogorov-Smirnov (KS) and Anderson-Darling methods [data not shown]). The data were compared by the Mann-Whitney (MW) test for differences in their median values and by the KS method for differences in their overall frequency distributions.

The corresponding cumulative distributions and their median values were compared by the KS and MW methods to test the null hypothesis that the experimental data belong to the same distribution. To analyze the spatial PML locus proximity patterns, we used the neighbor-neighbor introduced by Clark and Evans for a three-dimensional space (10). Spherically modeled nuclear volumes of untreated, IFN-treated, or primed cells were calculated from their threshold pixel areas at equatorial confocal planes. Values of nuclear volume and PML abundance were fed into the statistical parameters specified by the above approach to generate estimates of particle (PML + loci) densities in the nuclear volume, their theoretically expected mean minimal distances (Re), their associated standard deviations, and their probability distributions under the different experimental conditions. The ratio R of experimentally calculated mean minimal distance (Ro) over Re is a measure of departure from randomness. Pairs of experimental and their matched theoretical were compared to calculate their associated standard variant Z and its associated probability under the normal curve. Simulated and experimental distributions were further compared by using the KS test.

RESULTS

Memory of previous transcription leads to enhanced MHC-II induction by IFN-γ. To examine the transcriptional status following stimulus withdrawal, we studied the IFN-γ-inducible HLA-DRA gene, a well-studied MHCII model gene, in epithelial cells. After induction with IFN-γ, cells were subsequently released and left to grow in parallel with untreated cells for ~96 h (primed) to allow return of the mRNA back to its uninduced levels (see Fig. S1 in the supplemental material). Primed cells were then restimulated with IFN-γ, and the kinetics of induction were monitored in comparison to the primarily stimulated cells (Fig. 1A). The RNA levels of HLA-DRA and its activator CIITA were examined in control and primed cells by qRT-PCR after stimulation with the cytokine. Production of DRA mRNA in primed cells appeared earlier (4 h or less versus 6 h in control cells) and reached 2.5-fold higher levels relative to similarly treated untreated cells (Fig. 1B). This was also found for other IFN-γ-inducible MHCII genes examined but not the constitutively expressed MHC class I HLA-A gene (see Fig. S2 in the supplemental material), suggesting the involvement of an MHC-II locus-wide mechanism.

To exclude the possibility that this effect was due to faster accumulation of the master regulator CIITA, we measured in parallel the CIITA mRNA levels by qRT-PCR and found no significant difference in induction kinetics between primed and control cells. We next examined IFN-γ signaling by STAT1 phosphorylation, the major transducer of IFN-γ and found identical kinetics of tyrosine 701 phosphorylation between control and primed cells (Fig. 1D). Thus, changes in the IFN-γ/CIITA signaling pathway activity did not account for the observed enhanced transcriptional response.

To examine whether priming could stimulate responses to limiting doses of IFN-γ, we stimulated control and primed cells for 12 h with various concentrations of the cytokine ranging from 1 to 100 U/ml. We observed that HLA-DRA responded to even 1 U of IFN-γ/ml in primed as opposed to control cells, whereas CIITA transcription was almost identical in both situations (Fig. 1E and F). Collectively, these data indicate that primary IFN-γ-mediated expression generates a memory state that lasts for several cell divisions. This process enables the
MHC-II promoter to respond more efficiently even to low IFN-γ levels.

Past transcriptional activity generates a prolonged permissive chromatin state that facilitates expression upon restimulation. Since IFN-γ signaling and CIITA transcription were identical in control and primed cells, we sought to determine whether the local chromatin state of the DRA promoter was involved in the molecular mechanism responsible for the facilitated transcription of primed cells. Earlier results have shown that after IFN-γ, CIITA recruitment coincides with increasing promoter occupancy by chromatin remodelers, coactivators, Pol II kinases, and activatory histone modifications (39, 43, 57).

Therefore, we compared the chromatin of primed versus unprimed control cells after IFN-γ stimulation under matched parallel conditions regarding the promoter occupancy by two activatory posttranslational modifications (PTMs), namely, the histone H3 acetylation (H3ac) and lysine 4 trimethylation (H3K4me3) and the coactivator CIITA (Fig. 2A, B, and C, respectively). ChIP results showed minimal H3ac but accelerated and enhanced H3K4me3 and CIITA occupancy, detectable at even 2 h after IFN-γ. In all cases, excess occupancy slowly decreased over 24 h of IFN-γ treatment.

To investigate the mechanism of enhanced CIITA recruitment, we studied the promoter chromatin architecture by the restriction enzyme accessibility assay. It has been previously shown that an SspI restriction site at ~80 bp from the transcription start site of the DRA gene (outlined in Fig. 2D) becomes accessible to digestion upon transcriptional activation (28). Digestion with increasing amounts of SspI enzyme and detection of the undigested DNA with qPCR revealed that the DRA promoter of IFN-γ-treated but not untreated cells was sensitive to the enzyme. Increased accessibility of the locus persisted throughout subsequent generations long after transcriptional shutoff in primed cells (Fig. 2E).

Nucleosome depletion has been observed close to transcription start sites of active or transcriptionally poised genes by ChIP of histone H3 (25, 33, 35). Monitoring histone H3 abundance by ChIP showed that DRA gene activation results in reduced total histone H3 levels that remain low in the promoter of primed cells 4 days after release from the primary stimulus (Fig. 2D). For comparison, we also checked the H3 occupancy of the IFN-γ-inducible CIITA promoter IV. We also found that there was no depletion of H3 in any of the experimental conditions used (see Fig. S3A in the supplemental material).

We conclude that IFN-γ-mediated transcriptional activation results in a promoter-specific chromatin reorganization manifested by high accessibility and nucleosomal depletion that persists long after removal of the stimulus and transcriptional shutoff. This open chromatin environment in primed cells may facilitate CIITA recruitment at lower protein abundance during restimulation and earlier transcription onset.

Persistence of H3 lysine 4 dimethylation for several cell generations as a stable epigenetic mark. To examine potential epigenetic determinants of promoter sensitization, we monitored the time course of acetylation and histone H3 lysine 4 (K4) methylation of the HLA-DRA promoter after release from IFN-γ (approximately every cell population doubling) as outlined in Fig. 3A. CIITA recruitment was diminished rapidly after removal of the stimulus as found by ChIP, in accordance with the very short half-life of the protein, which is ~30 min (40) (Fig. 3B). Acetylation of histones H3 and H4 correlated
studies have shown that MHCII PML expression is also induced by IFN-
scriptionally active parts of the genome (51). Interestingly, PML-NBs have been previously found to associate with tran-
tations that affect the expression of individual MHC-I genes (22).

due to differential anchoring of SATB1-PML protein interac-
tion. of the territorial locus topology (50). Altered loop formation
accompanied by long-range processes manifested by changes
upon gene activation is maintained in primed cells.

FIG. 3. Stability of epigenetic modifications after transcription shut off. (A) HeLa cells were treated with IFN-γ for 24 h and then washed free of cytokine and further cultured. Chromatin was isolated from the cells every 24 h to study histone modifications on the HLA-DRA promoter. (B, C, D, E, and F) qPCR on chromatin samples immunoprecipitated with antibodies against CIITA (B), acetylated H3 (C) and H4 (D), trimethylation (E), and dimethylation (F) of H3 lysine 4 (H3K4me3 and H3K4me2, respectively). con, untreated control cells. Shown are the time points (in hours) after release from the cytokine. The mean precipitate as a percentage of input chromatin and the standard deviations are shown.

well with CIITA recruitment and returned to basal levels quickly after release (Fig. 3C and D) (4). We next studied histone lysine methylation that has been reported to correlate with the DNase I sensitivity of chromatin (41). Trimethylation of histone H3 K4 (Fig. 3E) was also found to correlate with CIITA recruitment and active transcription, as has been shown in other experimental systems (31). In contrast, increased H3K4 dimethylation was maintained for at least four cell generations after removal of IFN-γ at the DRA promoter (Fig. 3F) but not at the CIITA promoter IV (see Fig. S3B in the supplemental material).

Thus, the initial transcriptional activity causes both short-
and long-lasting epigenetic alterations of the DRA promoter chromatin architecture. In particular, the memory state corre-
lates with maintenance of high H3K4me2, increased enzyme accessibility of the chromatin, and fast transcriptional induction.

Relocalization of the MHCII locus closer to PML bodies
upon gene activation is maintained in primed cells. Earlier studies have shown that MHCII gene induction by IFN-γ is accompanied by long-range processes manifested by changes of the territorial locus topology (50). Altered loop formation due to differential anchoring of SATB1-PML protein interactions that affect the expression of individual MHC-I genes (22). PML-NBs have been previously found to associate with transcri-
tionally active parts of the genome (51). Interestingly, PML expression is also induced by IFN-γ (24, 45). Based on these findings, we tested whether the PML protein could participate in the transcriptional memory process. The PML protein abundance after IFN-γ treatment was found to increase and remain high even 96 h later (Fig. 4A), that is, long after MHC-II or CIITA transcription ceases. The number of PML bodies per nucleus in three-dimensionally preserved nuclei was measured by immunofluorescence and confocal microscopy and was also found to significantly increase in IFN-γ and primed cells in accordance with the protein levels (Fig. 4B).

To examine the relationship of PML bodies to the MHCII locus, immunostaining was performed simultaneously with fluorescense in situ hybridization (immuno-FISH) to DNA of the HLA-DRA locus in HeLa cells. HeLa cells are polyploid and contain three copies of chromosome 6, as has been shown by karyotyping (8, 26). Visual inspection showed a closer locus-PML association in IFN-γ or primed relative to untreated cells (Fig. 4C). To quantitate this, the minimum distance of the MHCII locus to the closest PML body per nucleus was measured in untreated, IFN-γ-treated and primed cells (Fig. 4D). Only 1% of the untreated cells had at least one DRA locus in contact with a PML body (MHC-II–PML minimum distance of $d = 0$), while this percentage increased to 35% in the IFN-γ-treated cells (Fig. 4D, white bars, $P < 0.0001$ against control). In the primed state, 9% of the cells had at least one locus touching a PML body (control versus primed, $P = 0.018$). About 80% of the IFN-γ-treated cells and 45% of the primed cells but only 7% of the control cells had alleles in the vicinity of a PML body, at a distance of $<0.5 \, \mu m$ ($P < 0.001$) (Fig. 4D, black bars). The closer spacing of the MHCII loci in IFN-γ-treated and primed cells over the untreated ones were main-
tained across a wide range of distances (pairwise $\chi^2$ tests showed the highest $P$ of 0.007 up to 1.5 $\mu m$, with the exception of distance $d < 0.1 \, \mu m$, where $P = 0.023$).

To evaluate the specificity of these spatial changes, we also scored the distribution of GLULD1, an irrelevant locus located ~30 Mb away from the MHC-II cluster on the same chromo-
some (Fig. 4D). Only 4% of the control and 5% of the IFN-
γ-treated cells had at least one GLULD1 allele in contact with a PML-NB ($P > 0.9$). Similarly, no significant frequency changes were recorded at the 0- to 0.5-μm range. A total of 14% of the control cells and 19% of IFN-γ-treated cells ($P =$
the same pattern was maintained at longer range (lowest $P < 0.0001$ [KS] in all pairwise comparisons; Fig. 5B). Consequently, IFN-γ treatment or priming significantly shifted the distribution of PML-MLL contacts per cell to much higher levels ($P < 0.0001$ [KS] in all pairwise comparisons; Fig. 5B).

To examine whether PML-locus distances are randomly distributed in the nuclear space, we used a nearest-neighbor approach (10) to generate distance distributions modeling the different experimental conditions. The estimated parameters for the different simulations and statistical analysis (see Table S1 in the supplemental material) demonstrate that the experimental data depart significantly from random distributions and are compatible with a nonrandom spacing pattern that is in line with a clustered positioning. This is probably due to physical constraints within the nuclear volume, for example, the territorial organization of chromosomes, which limits the available space.

IFN-γ generates a strong locus-specific distribution shift to smaller $MHCII$-PML distances that is maintained long after gene inactivation. In contrast, both the $GLULD1$ and $CIITA$ loci show a minimal and similar decrease in distance distribution upon IFN-γ treatment that may reflect a global architectural change of the nuclear environment.

**Increased PML-MLL interaction correlates with PML-facilitated DRA transcription.** To examine the role of PML in the persistence of elevated H3K4me2 on the $DRA$ promoter, we did double immunostaining with α-PML and α-MLL antibodies in paraformaldehyde-fixed nuclei to preserve the three-dimensional structure of the nucleus (Fig. 5A). MLL is a multisubunit complex with K4 methyltransferase activity that is regulated by its WDR5 and ASH2L subunits (11), which is important for the maintenance of expression of Hox genes (27). MLL has a speckled nuclear pattern (53). We observed increased colocalization of PML bodies with the MLL speckles in IFN-γ-treated or primed cells. Quantitation (recording touching or overlapping signals) shows that 28% of the control, but 0 and 6% only of the IFN-γ-treated or primed cells, respectively, had no PML-MLL association (whereas 50% of cells had one, three, or five PML-MLL bodies in contact in control, primed, or IFN-γ-stimulated groups, respectively).

To further study the PML-MLL association, we carried out coimmunoprecipitation of endogenous proteins. The results in Fig. 5C (top) shows that MLL coprecipitated less-abundant PML isoforms, most likely type III or IV, judging by their mobility. Overexpression of the above myc-tagged PML isoforms specifically interacts with MLL (Fig. 5C, bottom). To further investigate the PML-MLL interaction, we coexpressed the GFP fusions of the WDR5 and ASH2L MLL subunits along with mRFP–PML-III or -PML-IV and examined them by confocal microscopy. The results in Fig. 5D show that the WDR5 but not ASH2L regulatory factor is recruited by PML-IV to a much greater extent than PML-III-containing PML bodies. As a control, the enhancosome factor RFX5 was found not be
recruited by either PML isoform. The results from coimmunoprecipitation confirmed the imaging experiments (results not shown). Overall, these data provide a physical basis for the increased recruitment of MLL to PML bodies after IFN-γ/H9253 treatment.

To show the functional role of PML for DRA transcriptional activation, we performed knockdown experiments of PML by siRNA prior to the treatment of cells with IFN-γ/H9253. The efficacy of the knockdown was examined by qRT-PCR analyses to detect all of the PML transcripts (Fig. 5E, right graph) and by immunostaining experiments (data not shown) showing blocking of the IFN-γ-mediated increase of PML protein. PML knockdown resulted in reduced inducibility of DRA to about half of the control at 12 h after IFN-γ stimulation (Fig. 5E, left graph) without affecting CIITA expression (Fig. 5E, middle graph).

To correlate the above with the PML isoform specific interaction with MLL, we examined the effect of cotransfected WDR5 by PML-IV. Cells cotransfected with mDsRed–PML-III (left panel) or -IV (right panel) and each of GFP-ASH2L, -WDR5, and -RFX5 were examined by dual-channel confocal microscopy as indicated. (E) PML knockdown reduced MHC-II but not CIITA expression. HeLa cells were treated with 80 nM scrambled siRNA (□) or with siRNA specific to all PML isoforms (■) for 3 days prior to IFN-γ treatment for the indicated time points. qRT-PCR was used to measure the kinetics of transcriptional activation of DRA (left) in relation to its activator CIITA (middle) and the efficiency of PML knockdown (right). (F) PML enhances the CIITA-mediated induction of the DRA gene. HeLa cells were cotransfected with a 100 ng of a CIITA-expressing plasmid, along with increasing amounts of PML-expressing plasmids as indicated. qRT-PCR was used to measure the transcription fold change relative to CIITA alone (set at 1). The results show means and standard deviations from three experiments.
CIITA- and PML-expressing plasmids. Figure 5F shows a PML dose-dependent increase in the CIITA-mediated transactivation of DRA. In line with the above results, PML-III had a small effect, whereas PML-IV significantly enhanced DRA transcription and show a PML isoform-specific coactivatory potential.

Overall, these results point to a positive role of the PML body in IFN-γ/H9253-mediated activation of the MHCII locus. IFN-γ/H9253 increases the amount and isoform composition of PML bodies and facilitates recruitment of MLL components. In this line, earlier data have stressed the role of PML in transcription via its association with coactivators and other regulatory factors (13). This, in turn, may have an important role in maintaining a microenvironment that promotes expression of gene neighbors, as shown by both overexpression and knockdown experiments of PML.

Loss of memory in PML-depleted cells. Since we established the involvement of PML in DRA transcription during induction with IFN-γ, we next examined its role in the persistence of elevated levels of dimethylation on histone H3K4. HeLa cells were treated as in Fig. 1A and were subjected to PML knockdown by siRNA prior to primary stimulation and anti-H3K4me2 ChIP was carried out on DRA promoter (efficiency of PML knockdown was verified by qRT-PCR [data not shown]). Depletion of PML during priming resulted in lower induction and failure to maintain H3K4me2 levels in primed cells, as opposed to cells treated with scrambled siRNA (Fig. 6A). Significantly, trimethylation of H3K4 was not affected by siPML (Fig. 6B).

Since PML knockdown during priming affected the maintenance of the H3K4me2 mark on the promoter, we studied whether these cells could still display transcriptional memory during a second stimulation. qRT-PCR results show that the secondary response in siPML-treated cells was impaired relative to that observed in si-scrambled-treated counterparts (Fig. 6C). The magnitude of the secondary response of siPML-treated cells resembled that of a primary IFN-γ response. In contrast to the above, reinduction of either PML or CIITA transcription was not significantly affected (Fig. 6D and E). These results support a direct role of PML in the preservation of the H3K4me2 epigenetic mark on the promoter and the generation of memory in the transcription of DRA.

DISCUSSION

Although the events that are essential for the signal inducible gene expression in terms of transcriptional complex assembly and activation have been well studied in many systems, little is known about the long-term alterations that follow a transient transcriptional burst and the way they may influence response to future activation signals. Epigenetic marks and the mechanism of imposing them are good candidates for long-term effects that modulate transcriptional responses.

Accordingly, MHCII gene expression in relation to the role of the enhanceosome, various cofactors and CIITA have been studied in detail (58). In the present study we investigated long-term alterations in epigenetic marks and locus configuration that accompany past gene activation and how they affect secondary response. We show that a primary IFN induced transcriptional response generates long-term sensitization...
state (priming) characterized by a more robust secondary response. We propose that priming is an adaptive memory-like process! which may be of more general importance in various types of secondary signal regulated gene expression.

In the primed state, the MHCII promoter retains accessible chromatin and high histone H3K4 dimethylation maintained through at least four cell divisions. Persistent methyltransferase activity at the promoter may thus account for prevention of methylation decay upon DNA replication and deposition of new histone molecules. H3K4 methylation has generally been correlated with an active chromatin state. It has been shown that this methylation mark disrupts binding of the NuRD complex (55), a repressor complex containing deacetylase and nucleosome remodeling activity to suppress gene transcription (2). Thus, maintenance of the histone H3K4me2 mark correlates with increased accessibility of the locus in both actively expressing and primed cells and might prevent recruitment of remodeling complexes that would drive the promoter architecture to its original state after transcription ceases. These results are in line with previously published work demonstrating the role of histone or DNA methylation in memory of signal regulated transcription in the yeast and the human interleukin-2 systems, respectively (29, 31).

Another process that may influence epigenetic state and gene activity is nuclear compartmentalization. Accumulating evidence has established the importance of locus proximity to distinct nuclear compartments in both positive and negative transcriptional control: repression associates with the nuclear or nucleolar periphery, whereas the nuclear pore complex, PML, and nuclear speckles are associated with expression (56). It is thought that nuclear compartments may interact with various cofactors such as epigenetic regulators and influence the short- or long-term activity of genes in their neighborhood. PML-NBs have been intensively studied and linked to various biological processes such as tumor suppression, growth, apoptosis, antiviral defense, protein degradation, senescence, and immune response and transcription (5). PML knockout mice have impaired retinoic acid action, are prone to tumor formation and infections (52), and show higher proliferation but lower differentiation of neural progenitors (36) and increased cycling and exhaustion of hematopoietic stem cells (18a). It has been proposed that by regulating availability or activity of regulatory factors, PML limits strong signal outputs (18b). Although part of these diverse effects may be mechanistically similar, the PML heterogeneous morphology, composition, and preferential interactions imply distinct functions of the different PML splicing isoforms (3).

PML proteins are themselves IFN inducible and, although their dynamics increase during mitosis, a subset of mitotic PML (MAPP) remain associated with mitotic chromosomes (8, 12) and may serve as nucleation sites for reestablishing PML structure and function in the next G1 phase.

Based on the findings described above, we chose to investigate whether PML can also function as a determinant of transcriptional memory in the MHC-II system. We show that IFN-γ induction, as well as priming, is accompanied by a locus-specific increased relocation closer to PML bodies. Earlier observations point to nonrandom PML locus proximity and linked PML bodies with actively transcribed loci (20, 42). The present simulation analysis also supports a clustered rather than a random PML locus spacing model. IFN-γ-induced expression is associated with enhanced and prolonged clustering. Thus, the PML action on both the ongoing transcription and the efficiency of restimulation may be mediated via long-term locus PML proximity. Increased proximity of the MHCII locus to PML bodies upon activation may bring it in close contact with a number of transcriptional coactivators to facilitate transcription. The fact that not all alleles associate with PML-NBs at any time is reminiscent of the observation that IFN activation increases the level of the MHCII loci that protrude from chromosome 6 territory from ca. 10 to 35% of the gene alleles (9, 50). It is likely that not all alleles in a cell respond to IFN-γ. Stochastic gene expression has been reported in the case of the IFNb1 gene upon virus infection (1). Alternatively, interactions may be dynamic so in fixed cells a fraction of the loci colocalize with PML at any time. We found that the H3 lysine 4 methyltransferase MLL partly colocalizes with PML bodies upon IFN-γ treatment. We also show that IFN-γ induces the expression of various PML isoforms, one of which, type IV, may recruit MLL via its interaction with one of its subunits, namely, WDR5. This interaction is maintained even after four cell divisions. These results demonstrate for the first time that two IFN-γ-induced regulators, the short-lived CIITA and the long-lived PML, act in concert to regulate both active transcription and transcriptional memory of the MHCII genes.

By overexpressing PML isoforms and by siRNA-mediated knockdown, we have shown a positive role of PML on the primary and secondary (memory type) MHCII transcriptional response to IFN-γ and provide a link between PML isoform abundance modulation, locus proximity, and high H3K4me2 levels.

We have also found that the MHCII locus subnuclear localization and chromatin state are inherited through four cell generations after termination of the IFN-γ-induced transcriptional activity. Although histone H3 K4 trimethylation marks current transcription and is probably coordinated by a still novel function of the integrator CIITA, we show that maintenance of K4 dimethylation is a signature of both the past transcription state of the gene and an indicator of priming that is manifested by enhanced responsiveness upon new exposure to the inducer. The PML-DRα locus proximity and the PML-MLL interactions through several cell generations may contribute to this phenomenon. We propose that IFN-induced PML bodies recruit MLL or its components to modulate their neighborhood in a way that favors both primary transcription and the maintenance of a poised state that enhance the response to future activation signaling. These data link the mechanism of epigenetic inheritance with the altered locus topology and show that PML bodies as sites of epigenetic regulation (48) are important in signal-mediated adaptive processes and the maintenance of poised gene states.

It is tempting to speculate that the establishment of a sensitized chromatin state of the MHCII locus could be important to the immune response under limiting amounts of cytokines.

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REFERENCES


34. Pakowski, K., and W. Reith. 1995. A novel DNA-binding regulatory factor is mutated in pri-