Inducible Deposition of the Histone Variant H3.3 in Interferon-stimulated Genes*[5]

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The H3.3 histone variant is synthesized throughout cell cycle and deposited onto chromatin in a replication-independent manner. It is enriched in transcriptionally active regions of chromatin and is implicated in epigenetic memory. The dynamics of H3.3 deposition during transcriptional activation, however, have not been fully studied so far. Here we examined H3.3 incorporation into interferon (IFN)-stimulated genes in confluent mouse NIH3T3 cells expressing H3.3 fused to the yellow fluorescent protein (YFP). Following IFN stimulation, H3.3-YFP was rapidly incorporated into all four IFN-activated genes tested, with the highest enrichment seen in the distal end of the coding region. Surprisingly, H3.3 enrichment in the coding region continued for an extended period of time, long after transcription ceased. The promoter region, although constitutively enriched with H3.3-YFP, did not show an increase in its deposition in response to IFN stimulation. Further, although H3.3-YFP deposition stably remained in non-dividing cells for days after IFN stimulation, it was rapidly diminished in dividing cells. Lastly, we examined the role of H3.3 in IFN-stimulated transcription by a short hairpin RNA approach and found that IFN-stimulated transcription was significantly impaired in H3.3 knockdown cells. Results indicate that H3.3 plays a role in IFN-mediated transcription, and its deposition leaves a prolonged post-transcriptional mark in these genes.

Recent studies of the histone H3.3 variant indicate that it is incorporated into nucleosomal chromatin in association with active gene expression (1–4). Although other H3 variants, H3.1 and H3.2, are synthesized predominantly in S phase and are deposited onto newly replicated DNA, H3.3 is synthesized throughout the cell cycle, independent of DNA replication. Replication-independent incorporation of H3.3 is mediated by the HIRA complex, through a mechanism distinct from that of replication-dependent deposition of H3.1 mediated by the CAF1 complex (5). In Drosophila as well as in mammalian cells, H3.3 is enriched in nucleosomes carrying post-translational modification patterns characteristic of active transcription (6, 7). On the other hand, H3K9 dimethylation, typically seen in transcriptionally repressed chromatin, is scarce in H3.3. Histone H3.3 is accumulated in the transcriptionally active ribosomal DNA array in the Drosophila nucleus (8). A genome-wide analysis of H3.3 distribution patterns showed that H3.3 is distributed predominantly over regions of active genes and is enriched in the promoter regions coinciding with methylated Lys-4 in H3 and abundant RNA polymerase II binding (9). These studies led to a proposition that H3.3 marks active chromatin and may be involved in the epigenetic maintenance of chromatin status (3, 10–12). Evidence supporting the role of H3.3 in the inheritance of activated gene status was recently presented by nuclear transplantation experiments in Xenopus (13).

It has been shown that H3.3 replacement is triggered upon transcriptional activation of the HSP 70 genes in Drosophila (14). In that study, H3.3 deposition began within minutes of heat shock stimulation. This induced deposition coinciding with chromosomal puffs, providing an immediate link between transcription and H3.3 enrichment. Further supporting transcription-coupled H3.3 deposition, Janicki et al. (15) visualized H3.3 accumulation on the multicopy transgene array in human cells immediately after hormone induction.

Despite these studies, there are questions that have remained uncertain regarding H3.3 incorporation. For example, it is unclear whether transcriptional activation is a prerequisite of H3.3 deposition. Also unclear is the stability of transcription-induced H3.3 deposition as well as the spatial patterns of H3.3 incorporation within induced genes. In addition, the biological significance of transcription-coupled H3.3 enrichment has remained elusive.

With respect to the sites of H3.3 enrichment within a gene, widely varied results are reported for vertebrate cells, ranging from promoter-biased H3.3 incorporation to broader distribution patterns that include coding regions (16, 17). A study by Jin and Felsenfeld (18) on chicken erythroid cells concluded that there is no straightforward correlation between gene expression and H3.3 enrichment.

In this report, we have studied interferon (IFN)-dependent transcription as a model to address signal-induced H3.3...
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replacement. A special attention has been placed on detailed time course and sites of H3.3 deposition within a transcribed gene. Type I IFNs\(^6\) stimulate transcription of numerous genes that collectively confer anti-viral and anti-microbial states upon cells (19–21). Many of these genes begin transcription immediately after IFN stimulation, without requiring new protein synthesis, followed by rapid cessation of transcription afterward (21, 22).

Here H3.3-YFP incorporation was studied for several IFN-stimulated genes in NIH3T3 fibroblasts. To minimize the effect of replication-dependent H3.1/H3.2 deposition, H3.3 incorporation was tested in near confluent cells. We found that IFN stimulation triggered extensive and prolonged H3.3 enrichment in the coding regions of multiple IFN-response genes. In addition, IFN-induced transcription was substantially reduced when H3.3 expression is knocked down by stable H3.3 shRNAtransiently (5) and CMV-H3.3-YFP (a gift from Kami Ahmad and S. Hanakata) (5) and CMV-H3.1-YFP and MSCV-H3.3-YFP, were constructed from cDNAs from pOZ-e-H3.1 (a gift from Y. Nakatani) (5) and CMV-H3.3-YFP (a gift from Kami Ahmad and S. Henikoff) (15) as templates in the MSCV-CD8t vector (23) by PCR and other techniques. The template for the shRNA targeting both H3.3a and H3.3b was obtained by annealing nucleotides (5’-GATCCCGTGAAAGATACCAATCTGTGTTTCTGAAGACACAGATTGTTAGTCTTCTTTTTTTTTTTT3’ and 5’-AGCTTAAAAATGGAGATACCATACTGGTGTCCCTTCTTGGAAACACAGTTGTTATCTTTTCAAGGG-3’) and was ligated into BglII/HindIII sites of pSuper.retro (Oligogene) to generate the shRNA expressing retroviral vector to knockdown H3.3 genes. A control pSuper.retro carrying unrelated shRNA sequence is described previously (24). The nucleotide sequence of all inserts in the above constructs was verified by sequencing.

Retroviruses were produced by transient transfection of 293 EbnAT cells with a retrovirus vector (MSCV-CD8t or pSuper.retro), pMD.OGP (encoding gag and pol, a gift from H. Xiong), and pEco or pVSV-G (encoding ecotropic or panretroviral envelope protein, respectively, purchased from Clontech) using Lipofectamine 2000 (Invitrogen). Retroviral supernatants were collected at 48 h. NIH3T3, HeLa, and Tot2 cells were transduced with retroviruses by spinoculation (2,500 rpm) at 30 °C for 1.5 h in medium containing 3% (for transducing H3-YFP into NIH3T3 cells) or 25% (for transducing H3-YFP into HeLa and Tot2 cells or transducing shRNA) viral supernatant and 4 μg/ml Polybrene (Sigma). Transduced cells were selected by immunomagnetic cell sorting using MACS CD8 microbeads (Miltenyi Biotec) or by puromycin (4 μg/ml). For confocal microscopy, nuclei were stained with Hoechst 33342, and the cells were viewed on a TCS SP2 (Leica).

**EXPERIMENTAL PROCEDURES**

**Cells**—NIH3T3 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium containing 2 mm L-glutamine, 20 mm HEPS, penicillin, and streptomycin (complete Dulbecco’s modified Eagle’s medium) with 10% donor bovine serum (Atlantic Biologicals). Semiconfluent NIH3T3 cells were treated with mouse recombinant IFNβ (PBL Interferon Source) at 100 units/ml for the indicated periods. HeLa (ATCC) cells were grown in complete Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

**Retroviral Vectors and Transduction**—Retroviral vectors expressing H3.1 or H3.3 fused to the yellow fluorescent protein (YFP), MSCV-H3.1-YFP and MSCV-H3.3-YFP, were constructed from cDNAs from pOZ-e-H3.1 (a gift from Y. Nakatani) (5) and CMV-H3.3-YFP (a gift from Kami Ahmad and S. Henikoff) (15) as templates in the MSCV-CD8t vector (23) by PCR and other techniques. The template for the shRNA targeting both H3.3a and H3.3b was obtained by annealing nucleotides (5’-GATCCCGTGAAAGATACCAATCTGTGTTTCTGAAGACACAGATTGTTAGTCTTCTTTTTTTTTTTT3’ and 5’-AGCTTAAAAATGGAGATACCATACTGGTGTCCCTTCTTGGAAACACAGTTGTTATCTTTTCAAGGG-3’) and was ligated into BglII/HindIII sites of pSuper.retro (Oligogene) to generate the shRNA expressing retroviral vector to knockdown H3.3 genes. A control pSuper.retro carrying unrelated shRNA sequence is described previously (24). The nucleotide sequence of all inserts in the above constructs was verified by sequencing.

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**Antibodies and Chromatin Immunoprecipitation (ChIP)**

**Assay**—Antibodies used in this study were as follows: anti-GFP (Roche Applied Science), anti-H3K36me3 (ab9050; Abcam), anti-RNAAPII-2P (MMS-129R; Covance Research Products), anti-mouse IgM (Jackson ImmunoResearch Laboratories), anti-H3K4me3 (ab8580; Abcam), anti-H3K9me3 (07-442; Upstate Biotechnology), anti-H3ac (06-599; Upstate Biotechnology), normal rabbit IgG (Sigma), normal rabbit serum (Jackson ImmunoResearch Laboratories), and normal mouse IgG (Sigma). A ChIP assay was performed as described previously (23) with modifications. Briefly, cells on a 15-cm plate were treated with 1% formaldehyde for 10 min at room temperature. After washing, cells were lysed in 1 ml of the SDS lysis buffer and then sonicated in an XL2007 sonicator on wet ice for 25 s eight times using 15% of maximum power to shear the genomic DNA into 200–1000-bp fragments. After centrifugation, 100 μl of supernatants were diluted with 900 μl of the ChIP dilution buffer (23). Chromatin was precleared with 80 μl of protein A/G agarose-25% slurry (Santa Cruz Biotechnology) supplemented with salmon sperm DNA (200 μg/ml, Invitrogen) for 30 min at 4 °C. Precleared chromatin was incubated with an appropriate antibody overnight at 4 °C with rotation. For precipitation with monoclonal mouse antibody, anti-mouse IgM antibody was added during the last 1 h of incubation. Then 60 μl of protein A/G-25% slurry with salmon sperm DNA were added, and samples were rotated for 1 h at 4 °C to collect immune precipitates. Precipitates were then washed twice with each of the following buffers in order: low salt wash buffer, high salt wash buffer, LiCl wash buffer, and TE buffer (23). After elution of the chromatin complexes, the cross-link was reversed, and RNA and proteins were digested with RNase and proteinase K, respectively. DNA was then recovered by phenol/chloroform extraction followed by ethanol precipitation and resuspended in 100 μl of 5 mTris, pH 8. Five μl of each sample were used for quantification of the specific region of genomic DNA (40–100 bp) by duplicate real-time PCR amplifications. Input DNA (1%) was used for normalization.

**Reverse Transcription and Quantitative PCR**—Reverse transcription (RT) was performed as described previously (23) except that an oligo(dT)\(_{12–18}\) primer (Amersham Biosciences) was used. Quantitative PCR (qPCR) was performed using the SYBR Green PCR master kit (Applied Biosystems, Foster City, CA) and the ABI Prism 7000 sequence detection system (Applied Biosystems). Transcript levels were normalized by Gapdh mRNA and expressed as relative to those in unstimulated cells. Primer sequences used for qPCR are available upon request.

**Immunoblot Analysis**—For preparation of acid extracts, cell pellets were resuspended in lysis buffer (10 mM HEPES, pH 7.9,
1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol) with protease inhibitor mixture (Complete Mini, EDTA-free; Roche Applied Science), and then hydrochloric acid was added to a final concentration of 0.2 N. After incubation for 30 min at 4 °C with agitation, lysates were centrifuged, and supernatants were dialyzed against 0.1N acetic acid for 1 h and against H₂O three times for 1 h, 3 h, and overnight, respectively. Fifteen μg of extracts were separated on NuPAGE gels (Invitrogen) and were rendered near confluent, treated with IFNβ, and subjected to ChIP assay. Near confluent cells were tested because we wished to minimize replication-dependent H3 deposition. We first tested the Ifit1 gene, a representative of IFN-inducible genes (27, 28). As expected, Ifit1 mRNA expression rose within 1 h after IFN treatment and reached 1000-fold higher levels at 3 h irrespective of the expression of H3.3-YFP or H.3.1-YFP, comparable with the induction in parental NIH3T3 cells. Ifit1

**RESULTS**

**IFN Treatment Triggers H3.3-YFP Incorporation into IFN-stimulated Genes, Preferentially in the Distal Coding Region**—To study H3.3 deposition patterns following IFN-induced transcription, we first constructed NIH3T3 cells stably expressing H3.3-YFP or H3.1-YFP through retroviral transduction. GFP/YFP-tagged histone H3.3 has been used to study H3.3 replacement in Drosophila and human cells (8, 14, 15, 25). Levels of H3.3-YFP and H3.1-YFP expressed in NIH3T3 cells were similar and less than 5% of total histone H3 (supplemental Fig. S1 and see Fig. 4). To validate the use of H3-YFP constructs in assessing behavior of H3, we checked their localization during mitosis. H3.3-YFP localized to condensed mitotic chromosomes, as did H3.1-YFP (supplemental Fig. S2A). Further supporting proper association of H3.3-YFP with chromatin, fluorescence recovery after photobleaching showed that both H3.1-YFP and H3.3-YFP recovered very slowly after photobleaching as reported (26), indicating that they are part of cellular chromatin (supplemental Fig. S2B).

As shown in Fig. 1A, confocal microscopy analysis revealed a clear difference in the distribution patterns of H3.1-YFP and H3.3-YFP. Although H3.1-YFP was enriched at heterochromatic regions with intense DNA stain, H3.3-YFP was largely absent in the heterochromatic regions, showing a broad distribution pattern in the nucleus, indicative of a euchromatin-dominant localization pattern (Fig. 1A). A similar euchromatin-prevalent distribution was observed for H3.3 incorporation, NIH3T3 cells were rendered near confluent, treated with IFNβ, and subjected to ChIP assay. Near confluent cells were tested because we wished to minimize replication-dependent H3 deposition. We first tested the Ifit1 gene, a representative of IFN-inducible genes (27, 28). As expected, Ifit1 mRNA expression rose within 1 h after IFN treatment and reached 1000-fold higher levels at 3 h irrespective of the expression of H3.3-YFP or H.3.1-YFP, comparable with the induction in parental NIH3T3 cells. Ifit1

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**FIGURE 1. H3.3-YFP deposition at the Ifit1 gene after IFN stimulation.** A, distribution of H3.3-YFP and H3.1-YFP. NIH3T3 cells were transduced with H3-YFP vectors, and YFP signals were viewed by a confocal microscopy (×600). See relative levels and chromatin association of H3-YFP in supplemental Figs. S1 and S2. B, ChIP analysis of the Ifit1 locus in IFNβ-treated NIH3T3 cells. The diagram at the top depicts the exon-intron organization. The arrow indicates the transcribed region, and the gray boxes indicate exons. Positions of the primers used for ChIP were shown underneath. Cells were treated with IFNβ (100 units/ml) for 1, 3, 6, 12, 24, and 48 h. C, Ifit1 mRNA levels were measured by qRT-PCR. Transcript levels in IFN stimulation are expressed relative to those in unstimulated cells.

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To study the dynamics of H3.3 incorporation, NIH3T3 cells were rendered near confluent, treated with IFNβ, and subjected to ChIP assay. Near confluent cells were tested because we wished to minimize replication-dependent H3 deposition. We first tested the Ifit1 gene, a representative of IFN-inducible genes (27, 28). As expected, Ifit1 mRNA expression rose within 1 h after IFN treatment and reached 1000-fold higher levels at 3 h irrespective of the expression of H3.3-YFP or H.3.1-YFP, comparable with the induction in parental NIH3T3 cells. Ifit1
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transcript levels rapidly declined thereafter and returned almost to the basal level by 12 h in these cells (Fig. 1C). Cells transduced with H3.3-YFP responded to IFNβ to express transcripts for Ifit1 and other IFN-inducible genes in a manner very similar to that of parental NIH3T3 cells, confirming that IFN responsiveness is not appreciably altered by exogenous H3.3-YFP expression under these conditions (supplemental Fig. S3). ChIP assays were performed over three distinct region of the 8.75-kb-long Ifit1 gene, i.e. the promoter region (−120), the proximal and distal sites of the coding regions (+2.9 and +7 kb, respectively, see a map in Fig. 1B). Results of ChIP assays performed using anti-GFP antibody are shown in Fig. 1D. IFN treatment rapidly induced H3.3-YFP enrichment, most noticeably over the distal site of the coding region (red line, +7 k). Detailed time course analysis showed that increased H3.3-YFP incorporation took place immediately after IFN treatment and continued for the initial 12 h followed by a slower rise continuing up to 24 h (see the early time course in Fig. 1D, right panel). Although modest, H3.3-YFP was also enriched over the proximal coding region (blue, +2.9 k) after IFN treatment. However, H3.3-YFP incorporation did not increase over the promoter region; rather it seemed slightly reduced after IFN treatment (Fig. 1D, green). Similarly, H3.3-YFP deposition in an upstream promoter region (−1.5 k) was low and did not change after IFN treatment (data not shown). In contrast to the dramatic rise in H3.3-YFP deposition, the amount of H3.1-YFP incorporation remained similar before and after IFN treatment over both the upstream promoter and the coding regions. We noted that H3.1-YFP incorporation, like that of H3.3-YFP, showed a 2-fold reduction in the promoter region after IFN treatment. The reduction of both H3.3-YFP and H3.1-YFP following IFN stimulation likely reflects transcription-induced nucleosome depletion as is known for many genes both in human and in yeast (29, 30). To further substantiate increased H3.3 deposition at the distal coding region, we calculated the ratio of incorporated H3.3-YFP to that of H3.1-YFP over these regions and found that IFN treatment enriched H3.3-YFP over the coding regions but not at the promoter region (Fig. 1B, lower right panel). It is of note here that prior to IFN treatment, H3.3-YFP was, although modest, enriched at the promoter region relative to H3.1 (2:1), in line with reports indicating that some constitutively expressed and poised genes show H3.3 enrichment in the promoter regions (9, 16). Induced H3.3 deposition at the 3′-coding region after IFN treatment was confirmed when H3.3-YFP data were normalized with total H3 (supplemental Fig. S4). In these experiments, the amount of DNA precipitated with control Ig was consistently less than 0.01% of total input and did not change after IFN treatment (not shown). These data indicate that IFN treatment triggers H3.3 deposition in the Ifit1 gene and deposition continues long after completion of transcription, showing a striking preference for the 3′-coding region.

To assess whether this unexpected pattern of H3.3-YFP enrichment is a general feature for IFN-stimulated genes, ChIP assays were performed for three additional IFN-inducible genes, Oas1, Stat1, and Mx1, in the above NIH3T3 cells (Fig. 2, A–C). The length of the coding regions for these genes ranged from 11.2 to 41.5 k. As expected, the transcripts for these genes were robustly induced following IFN treatment, showing similar kinetics as those of Ifit1 induction (see Fig. 6). A remarkably similar pattern of H3.3-YFP enrichment was observed with these genes (Fig. 2, A–C). In all genes, H3.3-YFP was enriched after IFN treatment, most heavily at the 3′-distal sites of the coding regions. Mirroring the long lasting deposition kinetics seen for the Ifit1 gene, H3.3-YFP accumulation continued in these genes for 24 h followed by a plateau that lasted for an additional 24 h.

It was important to ascertain whether H3.3-YFP enrichment seen after IFN treatment occurred only in IFN-stimulated genes. To this end, ChIP analysis was performed for constitutively expressed genes not activated by IFN, namely the Gtf2b (encoding TFIIIB) and Tbp gene (encoding TATA-binding protein) (Fig. 2, D and E). In both cases, IFN treatment hardly affected H3.3-YFP deposition. Interestingly, however, the distal sites of the coding region showed the highest constitutive enrichment relative to the promoter and proximal coding regions in these genes. In addition to NIH3T3 cells, 3′-biased H3.3-YFP deposition was observed for the Gtf2b and Tbp genes in a hematopoietic cell line, Tot2 (not shown). These data suggest that the coding region-biased H3.3 enrichment may be a widespread feature of actively transcribed genes.

Recruitment of the Elongation Form of RNA Polymerase II and Trimethylation of H3K36 Correlate with Increased H3.3-YFP Deposition in the Coding Region. To gain insight into the coding region-biased, continuous H3.3-YFP deposition after IFN stimulation, we performed ChIP analysis over the Ifit1 gene for total RNA polymerase II (pol II) and pol II phosphorylated at serine 2 in the C-terminal domain, the elongation form of pol II (Fig. 3A, Pol II-2P) (31). pol II was not found on the Ifit1 gene prior to simulation but was rapidly recruited to the promoter region following IFN addition as noted for other IFN-inducible genes (32). pol II recruitment peaked at 3 and 6 h and declined thereafter, although it did not return to the basal level at least for 48 h. The phosphorylated, elongation form of pol II (pol II-2P) showed similar recruitment kinetics. Interestingly, however, the highest recruitment was seen at the 3′ region of the gene, suggesting that serine-2 phosphorylation in the pol II C-terminal domain increased as pol II moved through the gene during transcript elongation. pol II-2P recruitment returned to the basal level by 10 h, correlating with the kinetics of Ifit1 mRNA induction. It has been shown that the promoter regions of actively transcribed genes are enriched with trimethylation of histone H3 lysine 4 (H3K4me3) and acetylation of H3 lysine 9 and 14 (H3ac) (30, 33, 34). In agreement, we found that IFN treatment increased H3K4me3 modification and H3 diacetylation, mostly in the proximal site of the coding region, which remained for 48 h (Fig. 3B). These marks were, however, not increased in the 3′-coding region of the gene. Methylation of H3K9, although generally associated with repressed chromatin, has recently been shown to occur transiently in the transcribed region of activated genes, an event requiring transcriptional elongation (35). In addition, trimethylation of H3K36 has been shown to be enriched in the coding regions of transcriptionally active genes, which is also likely linked to elongation (36). We found that both H3K9me3 and H3K36me3 modifications were triggered by IFN treatment in the Ifit1 gene. Interestingly, H3K36me3 was seen predominantly over the 3′-coding region.
region, rather than the promoter region, and remained for 48 h (Fig. 3C). On the other hand, H3K9me3 was found on both the promoter and the coding regions and rapidly diminished after induction. These data indicate that recruitment of pol II-2P and trimethylation of H3K36, events linked to transcriptional elongation, take place in the 3'/H11032-coding region of the Ifit1 gene prior to or concurrent with H3.3-YFP deposition.

The Global H3K36me3 Mark on H3.3-YFP—It has been reported that histone modifications that mark a transcriptional active status, such as acetylation and H3K4 methylation, are enriched in H3.3 (6, 7, 9). Because the data above showed a correlation between H3.3-YFP enrichment and trimethylation of H3K36 on the Ifit1 gene, we sought to examine whether the H3K36me3 modification correlates with global H3.3 deposition. In Fig. 4, total histones extracted from NIH3T3 cells expressing H3.3-YFP or H3.1-YFP were immunoblotted with antibody for H3K36me3. The K36me3 mark on H3-YFP could be distinguished from that of endogenous H3.3 through a difference in molecular mass. Results showed that H3.3-YFP carried about four times higher amounts of K36me3 than H3.1-YFP. The H3.3-selective K36me3 was also observed with HeLa cells (Fig. 4). In agreement with previous reports, K4me3 and acetylation at Lys-9 and Lys-12 were found preferentially on H3.3-YFP, whereas K9me3 was on H3.1-YFP (6, 7, 9). These data indicate that trimethylation of H3K36 occurs globally on the H3.3 variant in these cells. We also examined whether the global correlation between H3.3 and H3K36me3 observed in untreated cells holds true after IFN stimulation. Levels of H3.3-YFP and H3.1-YFP were very similar between untreated and treated cells, and H3.3-YFP was similarly enriched with H3K36me3 in IFN-stimulated cells as in untreated cells (supplemental Fig. S5).

The IFN-induced H3.3 Mark Is Reduced Following Cell Division—Of particular interest to us was the long term continuity of the H3.3 mark and its relationship with cell division. To date, the effect of cell growth on the stability of H3.3 deposition has not been fully investigated. To this end, near confluent NIH3T3 cells were first stimulated with IFN for 24 h. Cells were then separated into two groups. In the first group, cells were allowed to stand in fresh medium without splitting for an additional 2 days (Fig. 5A). In the second group, cells were divided into four plates, allowing them to proceed with extensive cell division. As expected, cell numbers in the first group remained

![Figure 2.](image-url)
unchanged, whereas cell numbers in the second group increased by ~3.5-fold. Similarly, as expected, IFN treatment caused induction of Ifit1, Oas1, and Stat1 transcripts at 4 h followed by a fall 24 h later (Fig. 5B). In Fig. 5B, the right panel depicts H3.3-YFP deposition on the 3′-coding region of the Ifit1 gene in the two groups of cells. H3.3-YFP levels remained essentially unchanged in non-dividing cells. In contrast, levels of H3.3-YFP dropped to less than half in rapidly dividing cells (Fig. 5B). We also observed that the H3.3-YFP mark was stably retained in non-dividing cell populations for up to 5 days, whereas this

**FIGURE 5. Reduction of H3.3 deposition following cell division.** A, confluent NIH3T3 cells expressing H3.3-YFP were treated with IFNβ for 24 h. Cells were allowed to stand or were split into four plates and cultured for two additional days. Total cell yields on days 0, 1, and 3 are shown. B, transcript levels at the indicated times (left panel) and H3.3-YFP deposition in the 3′-coding region of indicated genes (right panel). See data for H3K36me in supplemental Fig. S5.
mark was further diminished in rapidly dividing cells (not shown). These results indicate that cell proliferation negatively affects the stability of H3.3 deposition, presumably due to the contribution of replication-coupled H3.1/H3.2 deposition. We also found, in separate experiments, that the retention of the H3K36me3 mark was much greater in confluent cells as compared with rapidly dividing cells, suggesting an H3K36me3 mark on and H3.3-YFP (supplemental Fig. S6).

**H3.3 Knockdown Inhibits IFN-induced Transcription**—To address the biological significance of H3.3 enrichment in IFN-dependent transcription, we knocked down the expression of H3.3 in NIH3T3 cells by shRNA. In the mouse, two H3.3 genes, *H3f3a,b* and *H3f3b*, encode the identical H3.3 protein sequences. We designed an RNA interference that corresponds to a common region of the genes and introduced it into NIH3T3 cells through a retroviral vector. This strategy achieved a consistent, ~75% reduction in endogenous H3.3 transcript expression, without affecting H3.1 transcript levels (Fig. 6A). However, the lack of available antibody for H3.3 prevented us from assessing the amount of H3.3 proteins in knockdown cells. The H3.3 knockdown cells did not show a noticeable difference in morphology and grew at a rate comparable with cells expressing control shRNA for at least 2 weeks. Cells expressing H3.3 shRNA or control shRNA were stimulated with IFNβ, and transcript induction was tested by qRT-PCR at 1, 3, 6, 12, and 24 h. As seen in Fig. 6B, induction of *Ifit1*, *Stat1*, *Oas1*, and *Mx1* transcripts was reduced in H3.3 knockdown cells as compared with control shRNA cells for all genes. Although the inhibition was partial, peak transcript levels were consistently reduced by up to 40% in H3.3 knockdown cells. In line with these data, additional IFN-responsive genes, *Isg15* and *Trim21*, also showed a similar, partial reduction in expression in H3.3 knockdown cells (Fig. 6B). These results

**FIGURE 6.** Inhibition of IFN-stimulated gene expression in H3.3 knockdown (KD) cells. A, NIH3T3 cells were transduced with a vector for H3.3 shRNA or control (Ctrl) shRNA for 4 days, and levels of *H3f3a,b* (H3.3) or *Hist1h3a* (H3.1) transcripts were quantified by qRT-PCR. Values represent the average of three determinations ± S.D. B, the above cells were treated with IFNβ for 24 h, and the levels of indicated IFN-responsive transcripts were measured at the indicated times by qRT-PCR. C, levels of *Tbp* and *Gtf2b* transcripts are measured in the above cells. Note that the y axis is enlarged to emphasize the slight reduction in transcript expression in H3.3 knockdown cells.
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indicate that H3.3 plays a role in IFN-dependent transcription. The partial inhibition may be attributed to the persistent presence of residual H3.3 proteins. We noted that expression of Tbp and Gtf2b transcripts was also reduced in IFN-stimulated H3.3 knockdown cells relative to control cells, albeit to a lesser extent than IFN-stimulated genes (Fig. 6C). The slight, but measurable down-regulation of Tbp and Gtf2b in H3.3 knockdown cells may be consistent with the deposition of H3.3 in these genes seen in Fig. 2, D and E.

DISCUSSION

IFN treatment triggered rapid deposition of H3.3-YFP in multiple IFN-stimulated genes, showing prolonged accumulation on their 3′-coding regions. Indicative of a functional link between H3.3 deposition and transcription, cells with H3.3 shRNA were less efficient in expressing IFN-responsive genes than control cells.

Dynamics of H3.3-YFP Incorporation—Spatial and temporal analysis of H3.3-YFP incorporation demonstrated that H3.3-YFP enrichment begins with transcriptional induction and shows a clear spatial selectivity. Moreover, H3.3 enrichment continued to increase for several days in all IFN-inducible genes examined, even after transcription had completely ceased. H3.3-YFP deposition was overwhelmingly biased toward the 3′-coding regions and coincided with the coding region-predominant recruitment of phosphorylated pol II and enrichment of trimethylated H3K9, the events associated with transcriptional elongation (35, 37). Our results are in line with induced H3.3 enrichment on Drosophila HSP70 genes reported by Schwartz and Ahmad (14). In that report, H3.3 enrichment was found to be dependent on an elongation-related event because the enrichment was not observed with an artificial promoter-only array. This raises the possibility that IFN-induced H3.3 deposition may also be dependent on elongation. However, given that recruitment of phosphorylated pol II and the H3K9me3 mark declined rapidly, coincident with the cessation of transcription, whereas H3.3-YFP incorporation continued, H3.3 deposition may not have a direct causal relationship with the event of transcription or elongation. It is possible that there are multiple ways in which H3.3 deposition is regulated and that in the initial stage, H3.3 incorporation may be mechanistically linked to elongation, but in a later stage, H3.3 incorporation may depend on another, presently undefined mechanism. We noted that IFN treatment increased trimethylation of H3K36, prominently on the 3′-coding region of IFN-stimulated genes. Genetics of H3K36me3 suggested that this modification may precede IFN-induced H3.3-YFP deposition, pointing to the possibility that the H3K36me3 modification contributes to sustained enrichment of H3.3 in the 3′-coding region. In support of a mechanistic link between this modification and H3.3 incorporation, K36me3 was found globally associated with H3.3-YFP in NIH3T3 and HeLa cells before and after IFN stimulation. We found the coding region-biased H3.3 enrichment not only in IFN-induced genes but in the constitutively expressed genes, such as Gtf2b and Tbp. It has been shown that K36me3 is present mainly in the coding regions of active genes (36, 38, 39). It is thus possible that deposition of H3.3 occurs in the transcribed regions of many genes.

In our study, modest H3.3 enrichment was observed in the promoter regions of IFN-inducible genes prior to activation. However, the promoter regions showed little increase in H3.3 deposition after IFN stimulation. The apparent absence of activation-induced H3.3 enrichment in the promoters may be in part due to transcription-coupled nucleosomal depletion shown to occur in the promoter region of many genes (29, 30). The mechanism of a constitutive H3.3 mark in the promoters is unclear but may be relevant to the assembly of transcriptional machinery prior to initiation.

It is worth noting that previous studies on the spatial distribution of H3.3 reported varied deposition patterns. A genome-wide analysis of Drosophila cells showed that H3.3 is clearly enriched throughout active genes with prevalence on the 5′ promoter regions (9, 40). The promoter-preferred H3.3 enrichment was also reported for an Ig light chain gene in B lymphocytes (16). However, Jin and Felsenfeld (18) did not find a simple, unified pattern of H3.3 distribution in the chicken folate and globin loci. Further, varied H3.3 distribution was reported for cell cycle-regulated genes as well (17). These reports indicate that H3.3 deposition is not necessarily synonymous with ongoing transcription, which may be consistent with the observations that H3.3 is enriched in some poised promoters (18, 40). It may be reasonable to envisage that H3.3 deposition is regulated by multiple mechanisms that create varied patterns of distribution. Although H3.3 may be predominantly associated with gene expression, the overlying, replication-coupled histone deposition may modulate the global pattern of H3.3 distribution. In addition, H3.3 may be turned over differentially depending on the region of the genome. In this regard, H3.3 deposited on the ribosomal DNA is shown to be rapidly replaced (14).

Biological Role of H3.3 Deposition—IFN-induced transcription was significantly reduced in H3.3 knockdown cells following IFN stimulation, providing evidence that H3.3 deposition contributes to IFN-stimulated transcription. Constitutively expressed Tbp and Gtf2b in which H3.3-YFP was deposited also showed somewhat reduced expression in H3.3 knockdown cells, supporting the view that the H3.3 variant has a role in active transcription. Our results are in line with the report that overexpression of H3.3 enhances expression of several genes (18). In keeping with the functional significance of H3.3 deposition in gene expression, Placek et al. (41) recently reported that down-regulation of H3.3 deposition by HIRA shRNA reduces expression of herpesvirus genes during infection. At present, however, the mechanisms by which H3.3 deposition regulates IFN-stimulated transcription are not clear. H3.3 may have a structural property that facilitates transcription factor access to DNA. Hake and Allis (3) pointed out that H3.1 has a cysteine at amino acid 96, which may be involved in the compartmentation of chromatin, and that in H3.3, this amino acid is replaced by aspartic acid, making chromatin potentially more favorable to transcription. By setting the chromatin environment conducive to transcription, H3.3 may affect specific steps of transcription, such as assembly of transcription complexes and promotion of transcription initiation. The preexisting H3.3 in the promoters may play such a role. On the other hand, IFN-stimulated H3.3 deposition in the coding regions may modulate an
elongation step, given coincidental recruitment of phosphorylated pol II at serine 2 in the C-terminal domain, H3K9me and H3K36me3 are implicated in elongation (31, 35, 42).

The most striking observation made in this study was the prolonged H3.3 deposition that continued long after IFN-induced transcription ceased, which was most prominently seen in the distal region of multiple IFN-stimulated genes. What is the biological significance of this deposition? Given the prolonged duration of H3.3 uptake, it may be tempting to speculate that this H3.3 incorporation leaves a lasting, epigenetic mark on IFN-stimulated genes, capable of modulating transcription of these genes in the subsequent IFN stimulation.

Lastly, the H3.3 mark generated following IFN response was more stable in confluent, non-dividing cells than in rapidly dividing cells, suggesting that the H3.3 mark has a larger impact on terminally differentiated, post-mitotic cells as compared with proliferating cells. It may thus be anticipated that in terminally differentiated cells, H3.3 plays a major role in gene expression specifying differentiated properties. On the other hand, rapid reduction of H3.3 marks may also be important for proliferating cells because it may allow cells to erase previous gene expression patterns and to adjust to a new and shifting environment.

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