Prolactin activation of IRF-1 transcription involves changes in histone acetylation

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Received 8 December 2000; accepted 9 December 2000

First published online 20 December 2000

Edited by Ned Mantei

Abstract In response to prolactin (PRL) signaling, transcription of the interferon regulatory factor-1 gene (IRF-1) is rapidly induced during early G_1, declines in mid G_1, and rises again over the G_1/S transition phase of the cell cycle in Nb2 T cells. Using chromatin immunoprecipitation assays, we show that histone H4 acetylation increases over a 1.7 kb region of the IRF-1 promoter in early G_1 and again at the G_1/S transition in response to PRL stimulation. These results demonstrate a correlation between histone H4 hyperacetylation at the IRF-1 promoter and biphasic transcription of IRF-1 in response to PRL signaling in vivo.

Key words: Chromatin immunoprecipitation; H4 acetylation; Interferon regulatory factor-1; Transcription; Stat1; Prolactin

1. Introduction

The packaging of DNA into nucleosomes results in an overall repression of gene expression through mechanisms that may include masking transcription factor interactions with DNA or creating higher order chromatin structures across regions of DNA [1,2]. Recently, the characterization of proteins that can modify histones has elucidated how the transcriptional machinery can alter chromatin to modulate gene expression. Acetylation of histone tails is thought to disrupt higher order nucleosome structures as well as decreasing the affinity of histones for DNA, thereby creating a more 'open' chromatin architecture that promotes transcription [3–5]. The discovery that cointegrator or coactivator proteins which promote transcriptional activation also possess intrinsic histone acetyltransferase activity (HAT) supports the idea that histone acetylation and gene transcription are linked [1–3]. These coactivators, such as CBP/p300, SRCs, and PCAF, are present in large multi-protein complexes which are recruited to target promoters through interactions with sequence-specific transcription factors [6,7].

In contrast, proteins that mediate transcriptional repression are associated with histone deacetylase (HDAC) activity [4,8,9]. Reversal of histone acetylation can re-establish the 'closed' or more condensed chromatin state [1,4]. These HDACs can be found in large multi-protein complexes targeted to specific promoters through interactions with corepressors such as NCoR, SMRT and NuRD, or through sequence-specific transcription factors [8,10–12]. Thus the activities of both transcriptional coactivators and corepressors can be correlated with changes in histone acetylation. The recruitment of histone modifying enzymes to a target promoter by sequence-specific factors links changes in histone acetylation to signals that modulate gene expression.

Transcription of the interferon regulatory factor-1 gene (IRF-1) is induced in a biphasic manner over a prolactin (PRL)-induced cell cycle in Nb2 T cells [13]. In this model system, PRL stimulates greater than 90% of the Nb2 T cells in a synchronized manner to enter the cell cycle [14]. IRF-1 is an immediate–early gene, whose transcription dramatically increases from 15 min to 1 h following PRL stimulation. Transcription of IRF-1 is then downregulated by 4 h and upregulated a second time at 8–10 h after PRL stimulation, as the cells enter S phase. We asked if PRL stimulation of Nb2 T cells results in changes in histone acetylation at the IRF-1 promoter. Using chromatin immunoprecipitation (ChIP) assays, we show increases in acetylated histone H4 on the IRF-1 promoter during both G_1 and G_1/S transition phase, in parallel with the biphasic transcriptional activation of the IRF-1 gene. Further, the increased histone hyperacetylation is seen throughout the IRF-1 promoter up to -1.7 kb. These results indicate a role for histone acetylase activity in transcriptional regulation of the IRF-1 promoter in response to PRL signaling in vivo.

2. Materials and methods

2.1. Cell culture

Nb2 T cells were cultured as previously described [14]. Cells were made quiescent and synchronized by culturing in medium lacking newborn calf serum for 18–20 h and were stimulated to enter the cell cycle by the addition of 50 ng/ml oPRL (NIDDK-20). Under this culture condition, greater than 90% of the Nb2 T cells are synchronized in early G_1. Upon PRL stimulation, cells traverse G_1 over the first 8 h, G_1/S between 8–10 h, S phase from 10–14 h and complete a cell cycle by 24 h as previously described [14].

2.2. ChIP

Chromatin was prepared from Nb2 T cells (1×10^7/time point) using a ChIP kit according to the manufacturer's protocols (Upstate Biotechnology). Briefly, cells were treated with 1% formaldehyde to cross-link histones to DNA and sonicated to an average length of 600 bp (range 200–1000 bp; data not shown). Cell lysates were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, 167 mM NaCl, 1 mM AEBSF, and 1 μg/μl aprotinin) and precleared with ssDNA/Protein A beads.

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0014-5793/01/$20.00
PII: S0014-5793(00)02385-1

For immunoprecipitation, half of the total lysate was incubated with anti-acetylated histone H4 antibodies (anti-acH4 Ab) (#06-866, Upstate Biotechnology) overnight at 4°C and the other half was analyzed as total chromatin input. The immunocomplexes were collected with ssDNA/Protein A beads, the beads were washed, the cross-links were reversed, and the DNA was recovered by phenol:chloroform extraction and ethanol precipitation.

PCR was used to detect regions of the IRF-1 promoter in complex with acetylated histone H4. All reactions were performed using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech) reconstituted in a final volume of 25 μl containing DNA, 0.5 μM of each primer, and a [γ-^{32}P]ATP (NEN)-labeled forward primer (300 000 cpm/reaction) as a tracer. PCR reactions were carried out for 30 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min 30 s. The primers for the IRF-1 promoter were: -0.6 kb region: forward 5′-GAAATTAAGAGTCCCTGCGTCC-3′ and reverse 5′-CTCCTAGCCTGCTGGTTCATGT-3′; −1.7 kb region, forward 5′-AGACGGACATTCCAGGACAC-3′ and reverse 5′-TCTGACACAGCCAAGGAAAA-3′; H3.3, forward 5′-GCAAGAGTGCGCCCTCTACTG-3′ and reverse 5′-GGCCTCACTTGCCTCCTGCAA-3′ [15] (Bio-Synthesis, Inc., Lewisville, TX, USA). All PCR products were detected by autoradiography and quantitated using a Storm phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA, USA). The results are expressed as the ratio of the immunoprecipitated DNA to the total chromatin input and plotted as a percent of the maximum ChIP to input ratio. Data presented are the average ± S.E.M. of $n = 3$ PCR reactions for each primer set and are representative of three or four independent PRL time course experiments. Graphs were generated and statistical analysis (S.E.M. and ANOVA) was performed using Origin 4.1 software (OriginLab Corporation, Northampton, MA, USA).

3. Results

To determine if changes in histone acetylation at the IRF-1 promoter are involved in IRF-1 transcriptional activation in response to PRL stimulation, ChIP assays were performed [16]. Briefly, formaldehyde cross-linked chromatin fragments were isolated from Nb2 T cells stimulated by PRL to enter the cell cycle. A 10 h time course covering G_1 (1 h) and G_1/S (8–10 h) transition phase of the cell cycle was examined [14]. Chromatin fragments were immunoprecipitated with anti-acH4 Ab and the precipitated DNA was analyzed by PCR for the presence of the IRF-1 promoter. Fig. 1 shows a diagram of the IRF-1 promoter and the location of the primers used for the ChIP assay. Previous nuclear run-on transcription studies have shown that the 0.2 kb promoter region mediates G_1 activation in response to PRL stimulation, while sequences spanning 0.2 kb and 1.7 kb, in cooperation with the proximal promoter, mediate G_1/S activation of the IRF-1 gene [13,17]. In quiescent Nb2 T cells, a basal level of acetylated histone H4 is detected in association with the distal region of the IRF-1 promoter (Fig. 2A). In response to PRL signaling, histone H4 acetylation in the 1.7 kb region of the IRF-1 promoter reproducibly increases by 1 h during G_1, but returns to near basal levels in mid G_1, between 2 and 4 h following PRL stimulation (Fig. 2B). An increase in histone H4 acetylation on the IRF-1 promoter is observed again 8–10 h following PRL stimulation as cells enter G_1/S phase of the cell cycle. This biphasic pattern of histone hyperacetylation at the distal 1.7 kb IRF-1 promoter region parallels the biphasic IRF-1 transcriptional response to PRL stimulation in vivo [13].

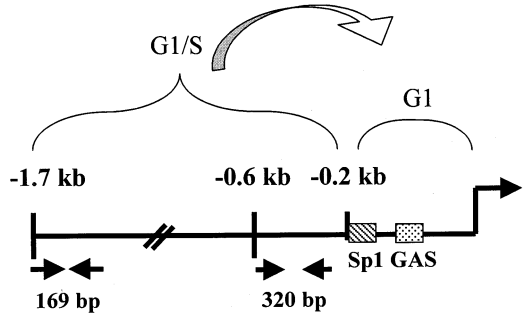

Fig. 1. Diagram of the IRF-1 promoter and location of PCR primers. The 0.2 kb region of the IRF-1 promoter is essential for G_1 transcription, while G_1/S transcription requires sequences extending to 1.7 kb in collaboration with the proximal 0.2 kb [17]. Primers used to detect the −0.6 kb and −1.7 kb regions of the IRF-1 promoter are indicated by arrows. The expected sizes of the PCR product are given below the arrows. The critical GAS element (−120 bp) and Sp1 element (−200 bp) are indicated.

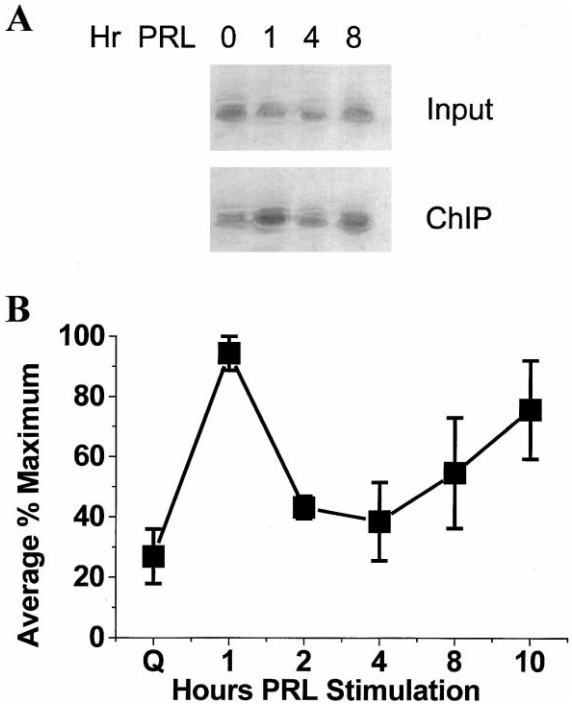

Fig. 2. Histone H4 acetylation at −1.7 kb of the IRF-1 promoter is biphasic in response to PRL signaling. ChIP analysis of the IRF-1 gene in Nb2 T cells across a PRL induction time course using anti-AcH4 Ab. PCR was used to detect the −1.7 kb region of the IRF-1 promoter. A: Representative PCR reactions from total chromatin (input) and immunoprecipitated (ChIP) samples (autoradiogram). B: Radioactive PCR products from an expanded PRL time course were quantitated, and the data are expressed as the mean ± S.E.M. of the ratio of ChIP to input material from $n = 3$ PCR reactions. Statistical significance of the data was analyzed by ANOVA ($P = 0.018$). The data are representative of three or four independent PRL time course experiments.

We next determined if the more central −0.6 kb region of the IRF-1 promoter also exhibits biphasic changes in histone H4 acetylation in response to PRL signaling. Interestingly, the −0.6 kb region of the IRF-1 promoter shows a reproducible increase in histone H4 acetylation at 1 h which persists at 2 h through early G_1 (Fig. 3). Subsequently, histone H4 acetylation at the IRF-1 promoter returns to basal levels by 4 h and increases again after 8–10 h of PRL stimulation. Although the biphasic histone acetylation patterns at both the −0.6 kb and −1.7 kb promoter regions are similar, differences do exist. Histone hyperacetylation after 2 h PRL at the −1.7 kb IRF-1 promoter region has already returned to near basal

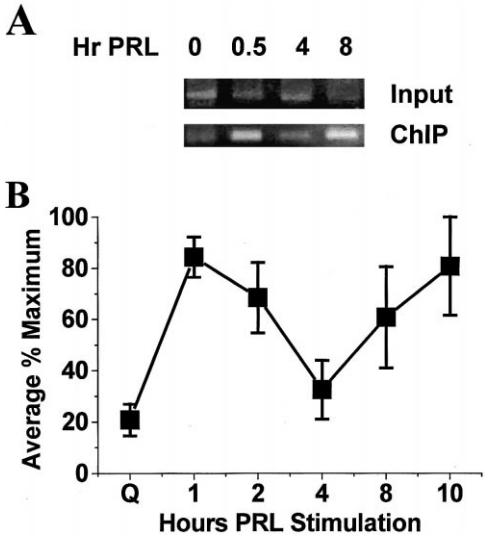

Fig. 3. Histone H4 acetylation at −0.6 kb of the IRF-1 promoter is biphasic in response to PRL signaling. The same ChIP samples as in Fig. 2 were assayed for the −0.6 kb region of the IRF-1 promoter using PCR. A: Representative PCR reactions from total chromatin (input) and immunoprecipitated (ChIP) samples (ethidium-bromide-stained gel). B: Radioactive PCR products from an expanded PRL time course were quantitated, and the data are expressed as the mean ± S.E.M. of the ratio of ChIP to input material from $n = 3$ PCR reactions. Statistical significance of the data was analyzed by ANOVA ($P = 0.032$). The data are representative of three or four independent PRL time course experiments.

levels (Fig. 2) while that for the −0.6 kb IRF-1 promoter region is still as highly elevated as the 1 h PRL time point (Fig. 3). This observation, coupled with the fact that chromatin is sheared to an average length of 600 bp (data not shown), suggests that distinct chromatin remodeling events are being detected at the −1.7 kb versus the −0.6 kb IRF-1 promoter in response to PRL stimulation.

The biphasic pattern of histone H4 acetylation at the −0.6 kb and −1.7 kb regions of the IRF-1 promoter is specific to the IRF-1 gene, as the level of histone H4 acetylation at the invariant histone H3.3 gene [15] remains essentially unchanged throughout the PRL induction time course (Fig. 4A). These results are consistent with the constant transcription of the histone H3.3 gene in PRL-stimulated Nb2 T cells [15], and further substantiate the specificity of the biphasic histone H4 acetylation pattern at the IRF-1 promoter in response to PRL stimulation in vivo. In addition, a no antibody (Fig. 4B) and an unrelated antibody (data not shown) control demonstrate that chromatin does not non-specifically bind to the ssDNA/Protein A beads during immunoprecipitation, thus demonstrating the specificity of the ChIP assay for acetylated histones.

4. Discussion

Chromatin modification has been shown to play an important role in transcriptional regulation. In this paper we show that changes in histone H4 acetylation at the IRF-1 promoter parallel the biphasic pattern of PRL-induced transcription of the IRF-1 gene in vivo. Using the ChIP assay, we show that histone H4 acetylation at the IRF-1 promoter increases in response to PRL signaling concomitant with the initial rise of transcription as cells enter G_1. Histone H4 acetylation subsequently decreases with kinetics similar to the downregulation of IRF-1 transcription in mid G_1. Histone H4 acetylation levels at the IRF-1 promoter increase again at the G_1/S transition, coincident with the second rise in IRF-1 gene transcription. This biphasic change in histone H4 acetylation is observed as far as 1.7 kb upstream of the transcriptional start site of the IRF-1 gene. These results show a strong correlation between the pattern of histone acetylation and biphasic transcription of the IRF-1 gene in vivo, implicating histone modification and changes in chromatin structure in transcriptional regulation of the IRF-1 gene.

Maximal induction of IRF-1 transcription in response to PRL signaling requires multiple elements within the 1.7 kb promoter. The minimal PRL-responsive elements are located in the proximal 200 bp of the IRF-1 promoter, including a critical GAS element at −120 bp [13,17,18]. PRL-inducible Stat1 interaction with the GAS element is required for transcriptional activation of the IRF-1 gene [18,19]. The transactivation potential of Stat1 at the 1.7 kb native IRF-1 promoter is further enhanced by interaction with the coactivator CBP/p300 [20]. The Stat1/CBP/p300 interactions may be one mechanism by which HAT activity is recruited to the IRF-1 promoter. Additionally, an Sp1 site at −200 bp of the IRF-1 promoter is required for maximal G_1 transcription of the IRF-1 gene (M.B. McAlexander and L.-y. Yu-Lee, in preparation). Sp1 can further enhance Stat1-mediated transcription-

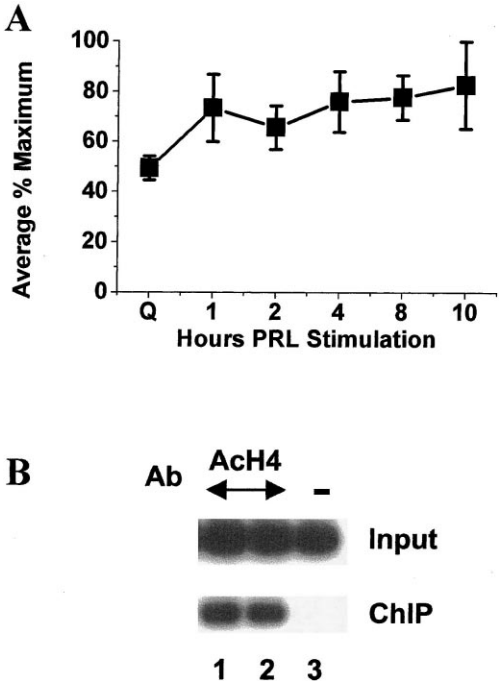

Fig. 4. Histone H4 acetylation does not change within the invariant histone H3.3 gene in response to PRL signaling. A: The same ChIP samples as in Fig. 2 were assayed for the invariant histone H3.3 gene using PCR. Radioactive PCR products from an expanded PRL time course were quantitated, and the data are expressed as the mean ± S.E.M. of the ratio of ChIP to input material from $n = 3$ PCR reactions. The data are representative of three or four independent PRL time course experiments. B: A representative PCR reaction of invariant histone H3.3 from total chromatin (input) and immunoprecipitated (ChIP) samples using anti-AcH4 antibody (lanes 1 and 2) or no antibody control (lane 3) as indicated (autoradiogram). Lanes 1 and 3, 8 h PRL; lane 2, 10 h PRL.

al activity by recruiting its own coactivator complex CRSP [21], the coactivator CBP [22], and components of the basal transcription machinery to regulate IRF-1 transcription. Our data suggest that during G_1, PRL-inducible Stat1 and constitutively bound Sp1 work together to recruit HAT activities which increase histone H4 acetylation throughout the 1.7 kb promoter to mediate IRF-1 transcription.

Which factors act to reverse H4 acetylation and thereby shut down IRF-1 transcription in mid G_1 are as yet undefined. The loss of Stat1 binding to the IRF-1 GAS at 4 h after PRL stimulation [13], due to either nuclear tyrosine phosphatase or proteasome activity [23,24], may ultimately lead to a loss of HAT activity at the IRF-1 promoter. It is also possible that PRL-induced Stat5 squelches limiting amounts of the coactivator CBP/p300 and thus antagonizes Stat1-mediated activation of the IRF-1 promoter [20]. Alternatively, Sp1 could recruit a deacetylase [11] to the IRF-1 promoter, reversing histone H4 acetylation thereby shutting down promoter activity. Experiments are underway to distinguish among these possibilities.

IRF-1 transcription at G_1/S phase of the cell cycle is also correlated with an increase in histone H4 acetylation on the IRF-1 promoter (Figs. 2 and 3). Previous studies have shown that G_1/S transcription requires IRF-1 promoter sequences beyond −0.2 kb extending to −1.7 kb [13]. Recent studies have shown that the −0.6 kb IRF-1 promoter region is sufficient to mediate the G_1/S response (M.B. McAlexander and L.-y. Yu-Lee, unpublished observations). Further, functional interactions between promoter distal elements and the proximal GAS element are required for PRL-induced IRF-1 gene transcription, as the region encompassing −0.2 to −1.7 kb is not PRL-responsive when taken out of context of the native 0.2 kb IRF-1 promoter [17]. A Stat-like factor binds the IRF-1 GAS during G_1/S transition in PRL-stimulated Nb2 T cells [19], but how this factor is involved in G_1/S transcription of the IRF-1 gene is currently unknown. Histone H4 acetylation at the IRF-1 promoter extends from −0.6 kb to −1.7 kb (Figs. 2 and 3), indicating that the entire 1.7 kb region of the IRF-1 promoter is targeted for PRL activation in both early G_1 and during G_1/S transition. Although studies at other promoters have shown a more localized region of hyperacetylation in conjunction with gene transcription [25,26], the acetylation of a broad region of the IRF-1 promoter agrees with data from the yeast *HO* gene promoter which is hyperacetylated over a 1 kb region in a temporal manner that correlates with *HO* transcription [27]. Interestingly, the temporal patterns of histone acetylation at the −0.6 kb and −1.7 kb IRF-1 promoter regions are similar but not identical. The 2 h time point shows a higher level of histone H4 acetylation in the −0.6 kb region (Fig. 3B) than in the −1.7 kb region (Fig. 2B), indicating possible temporal differences in chromatin remodeling enzyme activities in the distal promoter when compared to the more central region of the IRF-1 promoter. This may provide insight into mid-G_1 transcriptional down-regulation of IRF-1, which is currently under investigation.

Our studies demonstrate the involvement of HAT activity and chromatin remodeling at the IRF-1 promoter in PRL stimulation of IRF-1 gene transcription at two distinct phases of the cell cycle. Further ChIP studies employing antibodies against specific transcription factors [28,29] and acetylated histone H3 should elucidate how promoter-specific factors, together with chromatin remodeling and/or cell cycle regulated factors, coordinate the transcription of the IRF-1 gene in vivo.

Acknowledgements: We would like to thank Drs. Jiemin Wong and Sophia Tsai for helpful suggestions and critical comments. This work was supported by Heilig-Meyers Foundation Fellowship (M.B.M.) and NIH RO1 DK-44625 (L.-y.Y.-L.).

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