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Correlation between butyrate-induced histone hyperacetylation turn-over and *c-myc* expression

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Abstract

Transcriptionally active chromatin has an high level of histone acetylation, a post-transcriptional modification known to alter nucleosomal conformation increasing the accessibility of transcription factors to DNA. Recent studies have led new interest in histone acetylase and deacetylase because of their role as transcription factors.

Sodium butyrate, a known reversible inhibitor of histone deacetylase, modulates a large number of genes. This report is focused on the modulation of the *c-myc* oncogene expression by butyrate.

In HeLa cells, treated with butyrate and then exposed to butyrate-free medium, we established a correlation between the reactivation kinetic of *c-myc* expression and the increase in level of histone H4 acetylation. Both parameters, in cells exposed to butyrate-free medium, after showing a rebound effect, return to the control level. This trend was confirmed by quantitative analysis of the level of histone acetylation and of *c-myc* expression in the three distinct class of nucleosomal fragments with different transcriptional activity. In this chase process, we also detected a concomitant enrichment in *c-myc* sequences in the "active" chromatin fractions and decreased presence in the inactive nucleosomal fragment. Therefore we here demonstrate an excellent correlation between histone hyperacetylation and reactivation of a specific gene (*c-myc*).

Keywords: Butyrate; Histone deacetylase; *c-myc* chromatin structure; Gene expression

1. Introduction

The transcriptional regulation of a quite extensive number of cellular genes has been shown to be uniquely sensitive to the level of histone acetylation [1]. Recently it was shown that a number of transcriptional regulators have histone acetylase or deacetylase activities. Furthermore specific DNA binding transcription factors recruit histone acetylase and deacetylase to promoters activating or repressing transcription [2,3]. Treatment of cultured cell with sodium butyrate (NaB) is known to increase histone acetylation by inhibiting histone deacetylases and consequently causing an altered chromatin structure at nucleosomal level, in particular by leading to histone hyperacetylation in specific sites. These alterations determine the inhibition or enhancement of transcription and translation of quite a few genes [1,4]. In particular has been well established that the *c-myc* protoncogene expression is negatively affected by NaB [1,2,4,5]. Transcription of this gene, constitutively expressed by the cells, decreases significantly within 24 h of NaB treatment and remains suppressed until the NaB block is released. In particular, in many cell lines, *c-myc* is reversibly down regulated by exposure to millimolar amounts of butyrate.

At least a couple of mechanisms has been described to be involved in the NaB-induced *c-myc* gene reversible inhibition in cultured cells:

- down-regulation of *c-myc* is accomplished partially by a NaB-mediated acceleration in the degradation of mRNA and inhibition of the splicing of *c-myc* transcripts [6];
- NaB inhibits *c-myc* in all the steps of the cell-cycle suggesting that it acts at the transcriptional level and that its effect does not result from the arrest of the cells at the G1 phase [7].

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We previously described an affinity chromatography technique that allows the separation of transcriptionally active and inactive chromatin fragments, released by limited nuclease digestion, on an organomercurial-agarose column (Hg-agarose) [8]. This separation is based on the difference in accessibility in active and inactive chromatin of the sulphydryl groups of histone H3 and of specific non-histone proteins. A first class of chromatin fragments rich in maximally compacted, transcriptionally silent nucleosomes would not bind to the affinity column. A second class of fragments with nucleosomes of moderate gene transcriptional activity binds to the column because of a salt-labile association with thiol (SH) containing group of its non-histone nucleosomal proteins. This fraction is eluted increasing the ionic strength of the running buffer. A third class of fragments, rich in nucleosomes with an open structure and consequent maximum transcriptional activity, remains on the resin since the exposed histone H3 thiol groups bind strongly to the Hg-resin. These fragments can be displaced from the resin only by addition of an excess of competing thiol groups to the column running buffer. We here present a study, on active and inactive chromatin fractions, of the correlation between *c-myc* gene reactivation and sequence redistribution and level of histone acetylation when the NaB-induced silencing of the oncogene was reversed by exposing the cells to NaB-free medium.

2. Materials and methods

2.1. Cell culture

HeLa cells were cultured in suspension at 4–8 $\times$ 10^5 cells/ml in Joklik modified Eagle's medium, 10% fetal calf serum, (Biochrom KG, Berlin, DE). Cells were exposed for 15 h in the presence of 5 mM NaB, then washed and resuspended in butyrate-free medium for 0, 5, 60, 180, 240 min.

2.2. Chromatin fractionation

Nuclei were prepared as previously reported [9]. Chromatin fragments were released from nuclei with Micrococcal nuclease (10 U/ml/10 mg DNA) (Sigma–Aldrich, Milan, Italy) limited digestion (digest). The digest was applied to a Affi-Gel 501 organomercurial agarose column (Bio-Rad Laboratories, Milan, Italy). Unbound chromatin was removed by extensive washing with the running buffer as previously described [8] (unbound: RO). The mercury bound fragments were fractionated in two subsequent steps. A fraction, bound through the salt labile association of the SH groups of non histone nucleosomal proteins was released making the buffer 0.5 M in NaCl (NaCl eluted: 0.5). The chromatin still bound to the column through the SH groups of the histone H3 was displaced by addition of 10 mM dithiothreitol (DTT) to elution buffer (DTT). Aliquots were taken from each fraction for DNA and histones analysis.

2.3. Histone extraction and analysis

Histones were extracted with 0.25N HCl and electrophoretically separated in their acetylated isoforms in acrylamide-Triton-urea gels as previously described [9]. Gels were stained and the five histone H4 isoforms quantified as a percentage of the total. The overall level of histone H4 acetylation is the sum of the percentage over the total H4 of each acetylated form multiplied by the number of acetyl groups [(%mono-acetylated $\times$ 1) + (%di-acetylated $\times$ 2) + (%tri-acetylated $\times$ 3) + (%tetra-acetylated $\times$ 4)].

2.4. Run-off

Newly transcribed *c-myc* mRNA was quantified for each time point. Cells were harvested, permeabilized with lysolecithin (Sigma–Aldrich) and incubated, as previously described, in the presence of [α-^{32}P]-UTP [10]. Transcription was allowed to proceed for 30 min at 37 °C. The probe for *c-myc*, a 9 kb *Eco*R1–*Hind*III fragment of the plasmid

Table 1
Level of acetylation of histone H4 in the various chromatin fractions

Time (min) NaB-free medium	Total nuclei	Digest	Unbound	NaCl eluted	DTT eluted
0	154 ± 3	240 ± 3	250 ± 3	254 ± 3	300 ± 3
5	146 ± 3	144 ± 3	224 ± 3	235 ± 3	293 ± 3
60	85 ± 3	106 ± 3	156 ± 3	157 ± 3	293 ± 3
180	118 ± 3	159 ± 3	117 ± 3	163 ± 3	291 ± 3
240	114 ± 3	171 ± 3	106 ± 3	165 ± 3	290 ± 3
NaB untreated cells	115 ± 3	165 ± 3	111 ± 3	163 ± 3	291 ± 3

We here present the variation in the level of the histone H4 acetylation in HeLa cells at all time points of the NaB chase process (first column). For each time point the level of histone H4 acetylation was determined for the different chromatin fractions (in each line). The overall level of histone H4 acetylation is the sum of the percentage over the total H4 of each acetylated form multiplied by the number of acetyl groups: [(%mono-acetylated $\times$ 1) + (%di-acetylated $\times$ 2) + (%tri-acetylated $\times$ 3) + (%tetra-acetylated $\times$ 4)]. The quantification of the non-, mono-, di-, tri-, tetra-acetylated histone H4 was achieved by analysis of the electrophoretically separated isoforms that were quantified, with S.D., from three separate experiments as percentage of the overall H4 total.

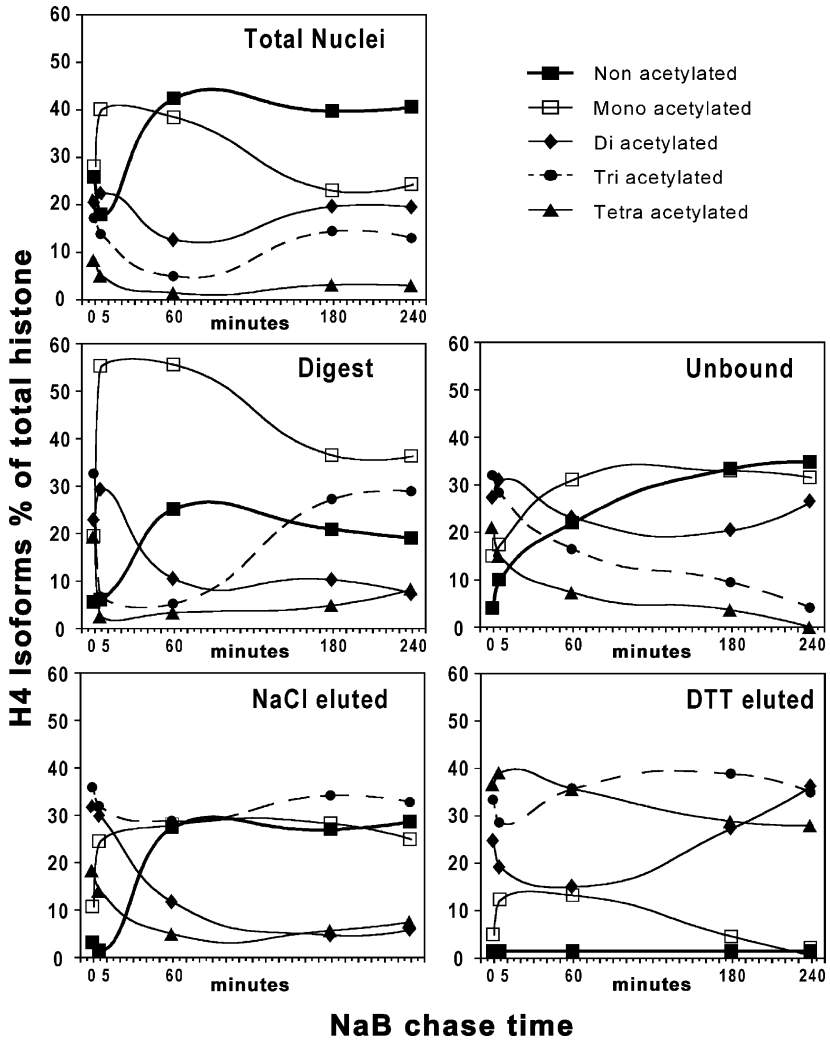

Fig. 1. Histone H4 level of acetylation. The correlation between the variations of the acetylated H4 isoforms in different chromatin fractions, at increasing time of HeLa cell exposure to NaB-free medium, is here represented. The abscissa represents the time of exposure (in min) of HeLa cells to NaB-free medium, at different chase time points. The ordinate shows the scale of each H4 isoform, at different level of acetylation, reported as percentage of the total histone H4.

pHSR-1 [11], was slot-blotted on Nytran filter (Schleicher & Schuell). Filters were hybridized with ^{32}P-mRNAs from each time point, washed and exposed to a Kodak film. The amount of newly transcribed *c-myc* mRNAs in each sample was detected and digitally analyzed as autoradiographic signal within the range of linear response of the film.

2.5. c-myc gene distribution in the chromatin fraction

DNA from total nuclei, crude total digest and the three chromatographically purified chromatin fractions of all the NaB chase time points and of untreated cells, was purified as described [10]. DNA fractions were then blotted on Nytran Plus filters and hybridized overnight at 60 °C in buffer containing a ^{32}P-dGTP random primed labeled *c-myc* probe (a 4314 bp ClaI/BglII restriction fragment of human *c-myc* sequence containing all the transcription promoters: P0, P1, P2 and P3; AC# X00364, base 1071–5385). Filters were washed and exposed to autoradiographic film. Filters are re-probed after stripping (by boiling in SDS 0.5%) with a probe for β-actin, a gene unaffected by NaB treatment (a 885 bp product of RT-PCR; AC# X00351) [12]. The distribution of *c-myc* in the chromatin fractions was expressed for each chase time point as the ratio between the *c-myc* gene and the matching β-actin gene.

3. Results

Exposure for increasing times of HeLa cells, that have been pre-treated for 15 h with 5 mM NaB, to butyrate-free medium resulted in a quite complex chain of molecular events. We analyzed, during this chase process, the correlation between the level of histone acetylation and of *c-myc*

oncogene expression. In particular we studied the trend of these parameters in total nuclei and in chromatin fragments released by nuclease limited digestion both in the original form and as chromatographic sub-fractions obtained by differential affinity on a Hg-resin.

In all our experiments the limited digestion (digest) fractions represented 13.5±1.5% of the total nuclear chromatin. The distribution of chromatin fragments with different transcriptional activity, purified by affinity chromatography, was quite similar in the untreated (unbound: 82.0 ± 3%; NaCl eluted: 8.8 ± 2%; DTT eluted: 9.2 ± 2%) and NaB treated cells (unbound: 80.0 ± 3%; NaCl eluted: 9.8 ± 2%; DTT eluted: 10.2 ± 2%).

We then studied the variation in the level of the histone H4 acetylation in the cells at all time points of the NaB chase process and for the different chromatin fractions. The quantification of the non-, mono-, di-, tri-, tetra-acetylated isoforms was achieved by analysis of their electrophoretic separation as from Section 2.

The overall level of acetylation of cells exposed for 15 h to NaB was much higher if compared to the one of untreated cells in all fractions but the DTT eluted (first and last line Table 1). When the NaB treated cells were exposed for increasing times to butyrate-free medium the level of acetylation went eventually back to the control level (180 min) for all the chromatin components (Table 1).

In total nuclei and digest at 5 min exposure to butyrate-free media brought a quite striking increase of the mono-acetylated form. At longer times of NaB chase the general trend was an increase of the non-acetylated H4 form and a decrease of all the other acetylated forms, eventually plateauing at control level in 180, 240 min (Fig. 1).

Each chromatin fraction followed a different trend during the NaB chase process (Fig. 1).

The RO unbound fraction (compact, inactive) showed a quite sharp parallel increase of non- and mono-acetylated forms and matching parallel decrease of the tri- and tetra-acetylated ones. These isoforms plateaued at longer times of exposure. The amount of the di-acetylated form remained fairly constant during the NaB chase process. The 0.5 chromatin fraction (moderate open structure and transcriptional activity) once exposed to NaB-free medium had an early sharp rise of the non- and mono- and a slow steady decrease of di-acetylated form. The tri- and tetra-acetylated forms were constant during the NaB chase. Steady state was reached at 180 min (Fig. 1).

The DTT eluted fraction (open and active) had the highest level of histone H4 acetylation (Table 1) that was basically maintained throughout exposure to butyrate-free medium (Fig. 1).

We then analyzed the *c-myc* gene transcriptional activity during the NaB chase process. As detected by the run-off transcription assay *c-myc* transcription was highly inhibited by NaB exposure of the cells. Once these cells were exposed to NaB-free medium *c-myc* transcription increased steadily reaching up to 145% of the control at 180 min (Fig. 2). This

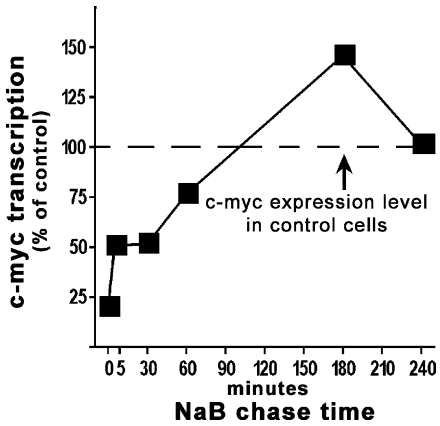

Fig. 2. *c-myc* expression. *c-myc* transcription has been monitored by run-off transcription assay in HeLa cells pre-treated with 5 mM NaB for 15 h at increasing time (min) of exposure to NaB-free medium as indicated (■) in the abscissa. *c-myc* expression is reported in the ordinate as percentage of the mRNA of the oncogene in the untreated cells (control: broken line at 100%).

rebound effect is concomitant to the one observed in level of histone acetylation. Histone H4 acetylation and *c-myc* mRNA returned to control level at 240 min. These data derived from a series of three experiments with a S.D. consistently below 10% (Fig. 2).

We also determined the distribution of the *c-myc* gene sequences in the different chromatin fractions (Fig. 3). We found that right after the NaB treatment *c-myc* gene sequence was mainly in the inactive chromatin. At increasing time of

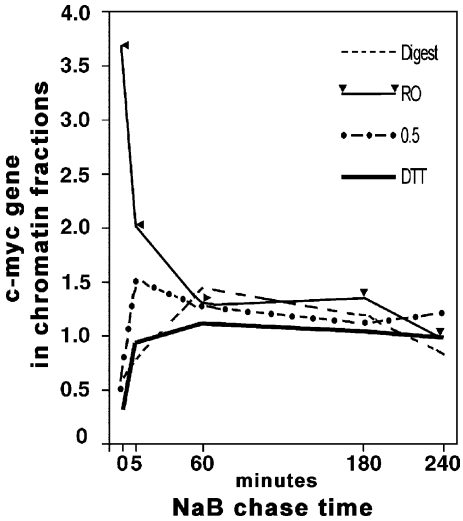

Fig. 3. *c-myc* gene sequence distribution in chromatin. *c-myc* distribution in each type of chromatin fractions at every chase time point is expressed as the ratio between the *c-myc* gene and the reference β-actin gene. In the abscissa is represented the time of exposure (in min) of HeLa cells to NaB-free medium. In the ordinate is expressed the distribution of the *c-myc* gene in the different chromatin fractions for each chase time point as ratio between the *c-myc* gene and the matching β-actin gene as control. First *c-myc* was quantified in each DNA preparation by a ^{32}P-GTP random primed labeled *c-myc* probe and the same sample was subsequently re-probed for β-actin, a gene unaffected by NaB treatment.

exposure of the cells to butyrate-free medium the presence of the *c-myc* gene sequences steadily decreased in the inactive and increased in the transcriptionally active chromatin fractions.

4. Conclusions

The kinetics of recovery of *c-myc* expression/activity, by exposure to butyrate-free medium (Figs. 2 and 3), are in good correlation with the decreasing trend in the level of histone H4 acetylation (Table 1, Fig. 1).

c-myc expression dowregulation by NaB treatment has been a well established fact for many years [1,4]. We here demonstrate that chromatin structure at the *c-myc* gene level is indeed reversibly altered to and from a compressed "non transcribing" conformation as a consequence of NaB treatment.

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