Posttranscriptional Regulation of H1° and H3.3B Histone Genes in Differentiating Rat Cortical Neurons

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Accumulation of mRNAs encoding H1° and H3.3, two histone replacement variants, was studied in differentiating cortical neurons, cultured in a serum-free medium, with or without triiodothyronine (T3) supplementation. We found that the levels of both H1° and H3.3B mRNAs decrease in isolated neurons between the 2nd and 5th day of culture to the same extent as in vivo. At the same time, an active synthesis of the corresponding proteins was evidenced. The effects of transcription inhibition by actinomycin D and the results of nuclear run-on experiments suggest that H1° and H3.3 expression is regulated mainly at the posttranscriptional level. Concerning T3, only marginal effects were noticed, apart from up-regulation of both histone mRNAs at 2 days in culture. We propose one model for posttranscriptional regulation of the analyzed genes and discuss potential relationships to remodelling of chromatin.

KEY WORDS: Histone H1°; Histone H3.3; Neuronal terminal differentiation; posttranscriptional regulation; chromatin; thyroid hormones.

INTRODUCTION

The structural and functional integrity of eukaryotic chromatin is ensured by the controlled condensation of DNA by proteins. The first level of chromatin organization is the packaging of DNA into a nucleosome array; the proteins responsible for the nucleosomal structure (the "core" histones H2A, H2B, H3 and H4) are highly conserved in evolution. In spite of this, however, each class of histone proteins is composed of different non-allelic isotypes (27,49), some of which are expressed only in the S phase of the cell cycle, whereas others are expressed at low but constant level throughout the cell cycle (for reviews, see 34 and 42). Some of the cell cycle-independent histone variants accumulate, in particular, during cell differentiation; this is the case of the "replacement variant" H3.3 (26,32,38).

The variable length of linker DNA that lies between adjacent nucleosomal cores interacts with the H1 class of histones, accordingly referred to as "linker histones". These proteins are responsible for the folding of the nucleosomal array into higher-order chromatin structures (1,45) and seem to play an essential regulatory role in gene transcription (44). Linker histones may be grouped into different classes, depending on the timing and cell type-specificity of their expression (for review, see 31). At least five subtypes of H1 (H1a, b, c, d and e) are expressed in mammalian somatic cells (14) in addition to histone H1°, which is expressed when cells have been committed to terminal differentiation (2,23) or after growth inhibition (4,35). Recently it has been suggested that H1° gene expression is directly linked to the degree
of core histone acetylation (22). Concerning H1° function in chromatin structure, it has been suggested that it binds preferentially those genomic sequences that are active at the time of cell proliferation and inactive after the induction of differentiation (39,41). Moreover, H1° has been proposed to be enriched in peripheral chromatin (3).

Control of H1° expression seems to be a complex process which involves transcriptional as well as post-transcriptional regulation (24,30,40,43). In addition, hormone-dependent regulation has been evidenced in some cases (11,21,23).

Brain cortical neurons offer a unique opportunity to study cell cycle-independent histone gene expression. At the onset of terminal differentiation, neurons are definitively post-mitotic, but nevertheless undergo dramatic changes in the structural organization of chromatin (5,10,28,29,46). This latter event must depend on the synthesis and incorporation into chromatin of replication-independent histone variants. Accumulation of both H1° (9,17,36,37) and H3.3 histone variants (38) in differentiating neurons has been indeed demonstrated. Moreover, we have recently reported that, in developing rat brain, the relative abundance of H1° and H3.3 mRNAs start decreasing at a time when the levels of the corresponding proteins begin to accumulate (7).

In this study, using rat cortical neurons cultured in a synthetic medium as a model, we show that H1° and H3.3 expression is likely to be regulated mainly at the posttranscriptional level.

**EXPERIMENTAL PROCEDURE**

**Animals.** Sprague-Dawley rats (Stefano Morini, S. Polo D’Enza, Italy) were housed at constant temperature (21 ± 1 °C) and relative humidity (60 ± 5%) under a regular light/dark schedule (light, 7:00–19:00). Food and water were freely available. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (European Community Council Directive 86/609, OJ L 358 1, December 12, 1987; NIH Guide for the care and use of laboratory animals, NIH publication no. 85-23, 1985).

**Cell Cultures.** Neurons were purified from fetal rat cortical hemispheres at the 16th embryonal day (E16) and cultured, for different periods of time, in Maat medium, supplemented or not with 10−6 M triiodothyronine (T3), as previously described (12). To investigate the synthesis of nuclear proteins, cells were cultured for 24 h in l-lysine-free Maat medium, supplemented with H3lysine monohydrochloride (Amersham, UK; specific activity > 70 Ci/mmol) at a final concentration of 5 μCi/ml.

**Northern Analysis.** Total RNA was purified from neurons cultured for 2, 5, 10, and 15 days, according to Chomczynsky and Sacchi (13). RNA samples (20 μg) were separated by electrophoresis on 1.5% agarose-6% formaldehyde gels, transferred to nylon membranes (Hybond, Amersham) and hybridized to 32P-oligolabeled (19) fragments from either the pMH1° (EMBL acc.no. X70685; 8) or the pDH33 plasmids (EMBL acc.no. X73683; 7).

Hybridizations were carried out for 24/48 h at 42 °C in 50% formamide, 5 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 0.1 mM EDTA), 5 × Denhardt’s solution, 0.5% SDS, 0.2 mg/ml denatured herring sperm DNA. After hybridization, membranes were washed sequentially in: 2 × SSPE at room temperature for 30 min (with two changes of buffer), 0.5 × SSPE for 20 min at 55 °C and 0.2 × SSPE for 15 min at 55 °C. All washing buffers contained 0.5% SDS. Filters were finally exposed to Kodak X-Omat S films for 1–7 days at −70 °C with intensifying screens. For standardization, the same membranes were also probed with a 1.4 kb BamHI fragment derived from the human ribosomal gene cluster, which specifically hybridizes to the 28S rRNA (18).

The autoradiographs were scanned in a Beckman DU8 spectrophotometer and the planimetry of scans were used to calculate the relative abundance of histone mRNAs at different ages; values were normalized with respect to the total amount of rRNA in the lane.

**Isolation of Nuclei.** Neuronal nuclei were purified from rat brain cortices as described elsewhere (9). To purify nuclei from cell cultures, neurons were scraped from culture dishes, rinsed twice in cold phosphate-buffered saline and resuspended in nuclei buffer (NB: 0.32 M sucrose; 50 mM sodium phosphate buffer, pH 6.5; 50 mM KCl; 0.15 mM spermine; 0.15 mM spermidine; 2 mM EDTA; 0.15 mM EGTA and 1.0 mM phenylmethylsulfonyl fluoride, PMSF). After homogenization with several strokes of a tight-fitting pestle in a Dounce homogenizer, the homogenate was centrifuged at 1,000 g for 10 min and washed twice in reticulocyte saline buffer (RSB: 10 mM NaCl; 10 mM Tris-HCl, pH 7.4; 1.5 mM MgCl2).

**Purification and Analysis of 3H-Labeled Nuclear Basic Proteins.** Acid-soluble nuclear proteins were purified from nuclei as previously described (11) and resuspended in distilled water. Protein concentration was determined according to Lowry et al. (33), using calf thymus histones (Sigma, St. Louis, MO) as a standard. Incorporation of [3H]-lysine was measured by counting small aliquots in a liquid scintillation system.

Electrophoretic analysis of nuclear basic proteins on acetic acid/urea/Triton X-100/polyacrylamide slab gels (AUT-PAGE) was carried out as described elsewhere (7). Tritium-labeled proteins were fluorographed by treating stained gels with Amplify® (Amersham UK) for 30 min under shaking, after which the gels were dried and exposed to Kodak X-Omat S film at −70 °C for appropriate intervals.

**Nuclear Run-On.** Run-on experiments were performed by [32P]-UTP pulse labeling of partially purified nuclei, as already described (15). Briefly, neuronal nuclei from both brain cortices and cultures were resuspended in ice-cold transcription buffer (TB: 50 mM Tris-HCl, pH 7.9; 100 mM KCl; 12.5% glycerol; 18 mM β-matcaptoethanol; 6 mM MgCl2; 1 mM MnCl2; 0.1 mM EDTA; 2.0 mM ATP; 1.0 mM CTP; 1.0 mM GTP; 150 units/ml RNase inhibitor from human placenta; 2.0 mM 32P-UTP) and incubated for 25 min at 29 °C (or, alternatively, for 45 min at 26 °C); after that, DNase I and CaCl2 were added to final concentrations of 100 μg/ml and 1.0 mM respectively, and the incubation was prolonged for 5 min, at 37 °C. Total RNA was extracted from the mixture as described above. Purified labeled RNA was used as probe to reveal insert DNA blotted onto nylon membranes (0.5–1.0 μg DNA/spot).

**Actinomycin D Treatment.** To investigate the effect of transcription inhibition on the levels of H1° and H3.3 histone mRNAs, neurons were cultured for 2 or 5 days in Maat medium, supplemented or not with T3. After this, the medium was carefully removed, without disturbing cell aggregates, and replaced with fresh medium containing 5