CONSERVATION OF NON-HISTONE CHROMATIN PROTEINS DURING GROWTH IN HeLa CELLS.

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SUMMARY: The turnover kinetics of non-histone chromatin (NHC) proteins were measured relative to that of histones and DNA in HeLa cells growing in suspension culture. Turnover was examined in logarithmic cultures by short (30 minute) and long (one generation, 17 hour) labeled amino acid pulses followed by prolonged growth in unlabeled medium. Likewise, synchronized cells were labeled in S or G1 and then grown several generations in unlabeled medium. In all experiments the NHC proteins of HeLa cells were conserved. Decline in specific activity during prolonged chase is entirely accounted for by growth dilution.

The control of expression of genetic information in eukaryotes is probably controlled, at least in part, at the transcriptional level by DNA-binding proteins (1,2). Since the limited primary sequence variation of the five histones alone is unlikely to provide sufficient variability for this task (3,4,5), the balance of the non-DNA chromatin mass, i.e., the non-histone chromatin (NHC) proteins, has been invoked as potential genetic control elements.

Although there exists a paucity of information on this question, one property ascribed to NHC proteins compatible with regulatory functions is a high rate of turnover (6,7,8,9). Defining the role of rapidly turning over NHC protein may be of crucial importance to understanding genomic function, and thus, the decay properties of NHC proteins in HeLa cells maintained in exponential growth were studied here. By use of several variations of pulse-chase experiments designed to detect certain classes of labile NHC proteins, evidence was, instead, obtained to indicate that the bulk of the NHC proteins are conserved in HeLa cells during exponential growth.

MATERIALS AND METHODS

Cell culture, synchrony and labeling techniques

HeLa S-3 cells were maintained in spinner culture at a density of 2-4 x 10^5

cells/ml by daily dilutions with fresh medium containing 5% horse serum (Grand Island Biological Co.) as described (10).

In order to produce a synchronous S-phase culture, cells were incubated in medium containing freshly prepared 1 mM hydroxyurea for 12 hours (11). Upon washing twice and resuspension in fresh medium, 65% of the cells progressed through S. By reapplication of hydroxyurea for an additional 12 hours, after the 9 hour S-phase, the entire population is synchronized in S-phase upon the second release from hydroxyurea inhibition. During the second S-phase, cells were incubated with isotope as indicated in the text.

Cells synchronized in G_1 were produced by the pressurized nitrous oxide technique of Rao (12), after first being subjected to double S-phase synchrony with hydroxyurea. At the sixth hour following release of the second hydroxyurea block, which represents late S-phase, the cells were introduced into a stainless steel pressure tank (Millipore Corp.), which was flushed with 5% CO_2-air. N_2O was introduced to a final pressure of 80 psi. After 8 hours incubation with stirring, the culture had a mitotic index of 0.8. The pressure was then released and cells were allowed to grow at normal atmospheric pressure. The mitotic index fell to 0.05-0.07 within 2 hours, and the population was thus considered to be primarily composed of G_1 cells. Incubation with isotope for pulse-labeling was as described in the text. During incubation with isotope, 1 mM hydroxyurea was added to prevent any cells from entering S-phase.

Chromatin preparations were made from 1% Triton-washed nuclei by washing the nuclei in descending Tris·Cl,pH 7.6, concentrations to a final concentration of 1 mM (13). Histones were extracted from purified chromatin with 0.4 N H_2SO_4 twice and the residue was dissolved in 0.2 N NaOH. The NHC protein-DNA solutions were brought to 10% in trichloroacetic acid and heated at 90°C for 15 minutes. The precipitate was collected by centrifugation at 17,000 x g for 10 minutes, suspended in 95% ethanol and centrifuged again. The pellet was used for both protein (14), and radioactivity determinations.

Similarly, the acid hydrolysate was used for DNA determination by the diphen-

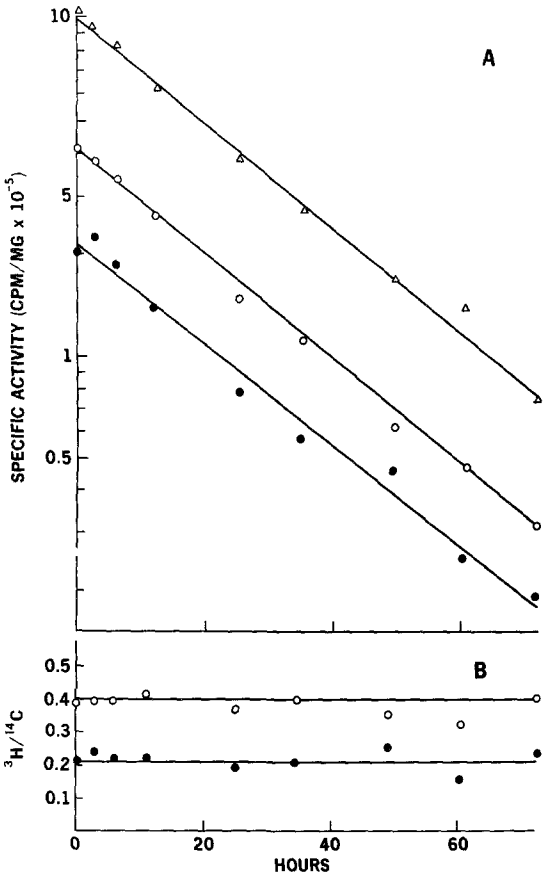

Figure 1. Turnover of chromatin proteins following an incubation for one generation with [^{3}H] lysine. At time zero the cells were washed and resuspended in non-radioactive medium as specified in the text. Panel A, (○) histones; (●) NHC proteins; (△) DNA. Panel B, (○) ^{3}H-histone: ^{14}C-DNA, (●) ^{3}H-NHC protein: ^{14}C-DNA.

ylamine reaction (15), and for radioactivity determination after drying aliquots on filters. Filter-dried samples were incubated with 0.2 ml NCS (Amersham/Searle)-water, 9:1, 12 hours prior to counting in 0.4% PPO in toluene.

RESULTS

It was expected, initially, that at least two populations of NHC proteins might exist, on the basis of turnover kinetics. These sets of proteins, relatively labile and stable, respectively, should be distinguishable by comparison of decay kinetics following short and long labeling periods. Long labeling periods selectively measure stable proteins which accumulate the most radioactivity in

this situation. Brief labeling periods detect unstable populations which, because they accumulate little radioactivity, have a greater proportion of total radioactivity in brief pulses as compared to long. Based on this premise, cells were labeled with ^{3}H-amino acids for varying periods, and then allowed to grow for prolonged periods to detect true degradation and replacement.

For the long labeling period, cells were incubated with 1.0 μCi ^{3}H-lysine and 0.02 μCi ^{14}C-thymidine/ml for 1 generation (17 hours). To terminate labeling cells were washed twice and resuspended in 500 ml fresh medium together with 2 liters carrier cells for sampling at the indicated times in Figure 1. In all experiments, the cells were fed by daily addition of an equal volume of fresh medium. The kinetics of the specific activity decline of NHC proteins are presented together with those of DNA and histones for comparison. All three populations decay at a constant logarithmic rate with a half-life of 20 hours (Figure 1A), a value indicating complete conservation. Decay in specific activity is entirely accounted for by growth dilution. In panel B, the data are expressed as the ratios of counts in protein to that in DNA. Any decrease in the ^{3}H:^{14}C ratio indicates loss of protein radioactivity. The curves have zero slope which again, indicates conservation.

A short pulse, prolonged-chase experiment was then performed in the same manner. Cells prelabeled 16 hours with 0.02 μCi ^{14}C-thymidine/ml were concentrated to 2 x 10^{6}/ml and incubated with 10 μCi ^{3}H-lysine/ml in lysine-free medium for 30 minutes. The cells were then washed twice and resuspended in 500 ml complete medium together with 3 liters of carrier cells for sampling at the times indicated in Figure 2. In panel A, the kinetics of specific activity decay of NHC proteins parallel those of DNA and histones with a half-life of 17 hours, indicating conservation as in Figure 1. The data, when represented as ^{3}H:^{14}C in Panel B also yield lines of zero slope.

The limits of error are empirically estimated in Figure 2A. The dashed line 1 is constructed to represent a loss of 10% radioactivity per generation and the dashed line 2 represents a loss of 20% per generation. From this, the limit of

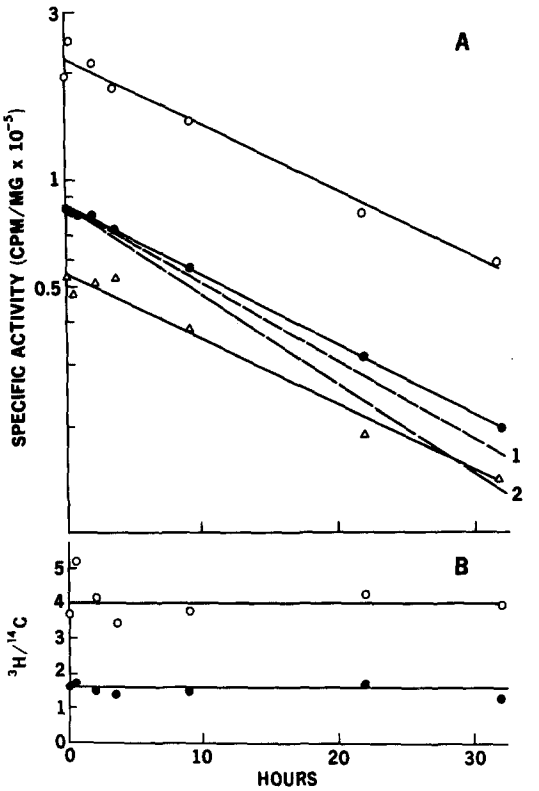

Figure 2. Turnover of chromatin proteins following incubation for 30 minutes with [^{3}H] lysine. At time zero the cells were washed and resuspended in non-radioactive medium for the indicated periods. Panel A (○) histones; (●) NHC-proteins; (Δ) DNA. Panel B, (○) ^{3}H-histone: ^{14}C-DNA; (●) ^{3}H-NHC protein: ^{14}C-DNA.

detection of decay is estimated at 10% per generation.

To delineate whether cell cycle specific populations of unstable NHC proteins exist, cells were synchronized and labeled either in G1 or S phase, which collectively account for 85% of the HeLa cell cycle. To minimize possible deleterious effects of multiple synchronizations, cells were released from synchrony after labeling and allowed to grow randomly for four generations during which samples were removed.

Cells were synchronized into S-phase by a double hydroxyurea block (11). Following release from the second round of hydroxyurea inhibition, cells were concentrated five-fold and incubated with 0.5 μCi ^{3}H-lysine and 0.03 μCi ^{14}C-thymidine per ml in 80% lysine-free medium for 7.5 hours. Cells were then washed twice and

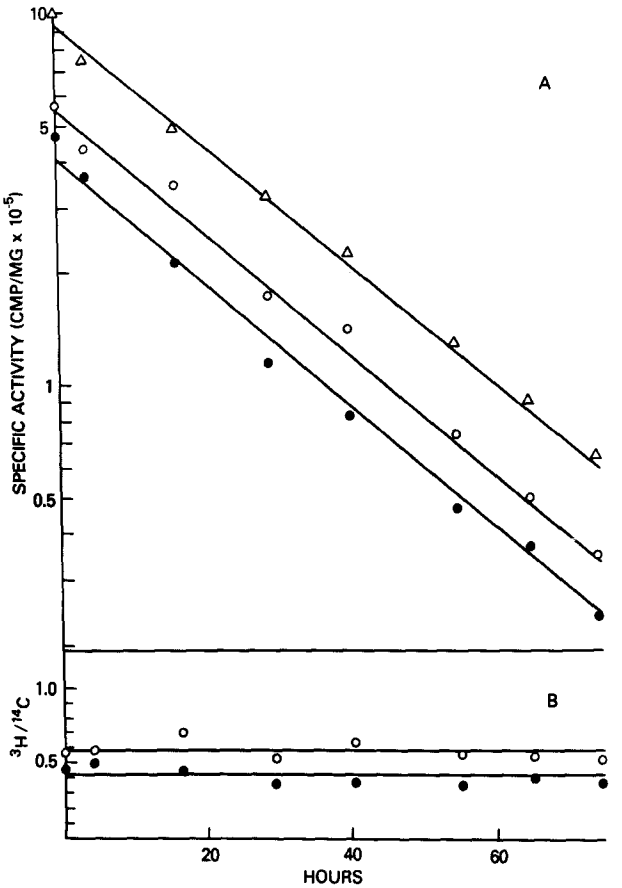

Figure 3. Turnover of S-phase chromatin proteins following incubation with [^{3}H] lysine. At time zero the cells were washed and resuspended in non-radioactive medium for the indicated periods. Panel A, (o) histones; (●) NHC proteins; (Δ) DNA. Panel B, (o) ^{3}H-histone: ^{14}C-DNA; (●) ^{3}H-NHC protein: ^{14}C-DNA.

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resuspended for sampling in 500 ml fresh medium together with 2 liters of carrier cells Turnover kinetics of S-phase NHC proteins are given in Figure 3. S-phase NHC proteins decay in specific activity at the same rate as DNA and histones. The ^{3}H:^{14}C ratios, Panel B, yield a horizontal line, again indicating total conservation.

In order to examine G1 NHC proteins, cells were reversibly arrested in mitosis by application of nitrous oxide under 5.4 atmospheres of pressure (12). To decrease the amount of time the cells were pressurized, and thus minimize damage, they were first synchronized twice with hydroxyurea. In late S phase, 6 hours after hydroxyurea

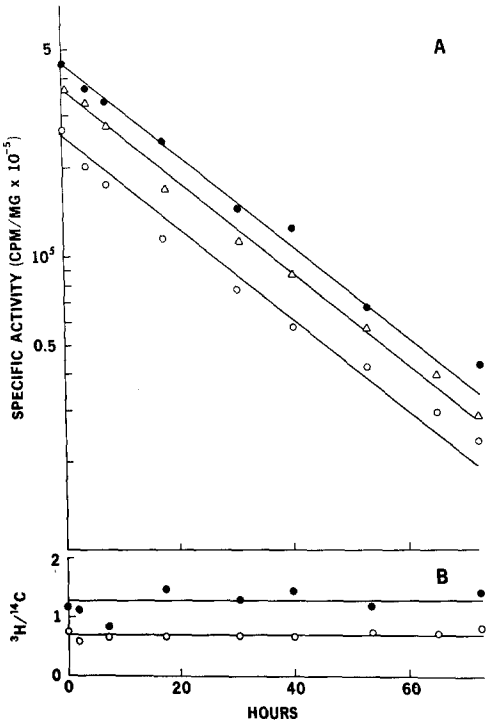

Figure 4. Turnover of G1 NHC proteins following incubation with [^{3}H] lysine. At time zero the cells were washed and resuspended in non-radioactive medium as specified in the text. Panel A, (○) acid-soluble protein; (●) NHC proteins; (△) DNA. Panel B, (○) ^{3}H-acid-soluble protein: ^{14}C-DNA; (●) ^{3}H-NHC protein: ^{14}C-DNA.

removal, cells were pressurized and maintained until over 80% of the population was arrested in mitosis. Pressure was relieved and cells were incubated with 2 μCi/ml ^{3}H-lysine and 1 mM hydroxyurea (to prevent entry into S) for 6.5 hours. Thymidine labeling was performed in the prior S phase at 0.02 μCi/ml. Labeling was terminated by washing twice and resuspending in complete medium. The labeled G1 cells (800 ml) were added to 2200 ml of carrier cells and sampling was performed at the times indicated in Figure 4. The results (Figure 4, Panel A), again show a specific activity decline in parallel with DNA, with a half-life of 19 hours. The specific activity ratios, Panel B, also indicate no detectable turnover. Acid-soluble G1 proteins with a specific activity 20% that of histones also decay in parallel with DNA.

DISCUSSION

Several variations of pulse-chase experiments were performed to detect certain

classes of labile NHC proteins. The turnover of NHC proteins of HeLa cells was examined relative to histones and DNA which are metabolically stable (16), with certain exceptions (17-19). NHC proteins from logarithmically growing cells were examined by decay of specific activity following short (30 minute) and long (one generation) labeled lysine pulses followed by prolonged incubation in unlabeled medium. Similarly, decay of NHC proteins labeled during S or G1 phases of the cell cycle was measured during the following four generations. Comparison of results with the decay of DNA labeled with ^{14}C-thymidine, provides additional support to the results. All four sets of experiments were also performed utilizing labeled leucine rather than lysine. Results were identical in all cases: no turnover could be detected that was not accounted for by growth dilution.

These results may not be in disagreement with reports on NHC protein turnover. Borun and Stein (8) reported decay in NHC protein specific activities which varied with the cell cycle stage during which the labeled pulse was administered. The two minute pulses utilized in that study would necessarily select for the most labile proteins present in a population. Such results do not, however, necessarily represent the turnover characteristics of the predominant protein population. In addition, specific activities were measured only for 120 minute periods and it is not clear whether those observations reflect temporal association-dissociation behavior, or true degradation and replacement.

The NHC proteins of Chinese hamster fibroblasts, when labeled in a non-dividing confluent monolayer, are reported to exhibit turnover upon renewed growth stimulation (9). HeLa cells on the other hand are not capable of growth arrest. It would be of considerable interest if such different populations of NHC proteins exist between growth-regulated and non-regulated cells, or between different metabolic states of a given cell. While these two reports (8,9) suggest that certain NHC proteins may turn over, and while the data reported here do not eliminate turnover of NHC protein subpopulations, this study demonstrates that the bulk of the NHC protein is metabolically as stable as histone and DNA.

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