EVIDENCE FOR THE RESUMPTION OF DNA REPLICATION PRIOR TO HISTONE SYNTHESIS IN HeLa CELLS AFTER RELEASE FROM TREATMENT WITH HYDROXYUREA

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1. Introduction

Hydroxyurea, which brings about a rapid but reversible inhibition of DNA replication and histone synthesis in HeLa cells [1–7], has provided valuable information regarding the relationship between these metabolic events. Although time course and biochemical changes associated with inhibition have been extensively examined [1–7], little is known regarding the release from the metabolic block imposed by hydroxyurea. Because of the potential value of this inhibitor for further assessing the apparent functional coupling of DNA replication and histone synthesis, we have examined histone and DNA synthesis at various times after removal of hydroxyurea from HeLa cell cultures. These results indicate that the resumption of DNA synthesis precedes that of histone synthesis.

2. Materials and methods

2.1. *Cell culture and synchronization*

HeLa S_3 cells were grown in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum and were synchronized by a single 2 mM thymidine block [6].

2.2. *Hydroxyurea treatment*

Two hours after release from thymidine block (early S phase) the cells were split into 3 equal cultures. Two of the cultures were treated with hydroxyurea at 10 mM (one for 30 min, the other for 120 min). Hydroxyurea treatment was stopped by pelleting cells and resuspending into fresh medium. The third (control) culture was not treated with hydroxyurea. In some experiments cells of control cultures were pelleted and resuspended at the same time as the hydroxyurea-treated cells. Cells were removed at various times before and after treatment with hydroxyurea and both DNA and histone synthesis were measured.

2.3. *DNA synthesis*

HeLa S_3 cells (2 ml, 5×10^5 cells/ml) were incubated for 30 min at 37°C with 0.2 μCi [^{14}C]thymidine (57 mCi/mmol, Schwarz/Mann). The reaction was stopped by the addition of 4 ml ice-cold Earle's balanced salt solution and the cells were pelleted by centrifugation at $800 \times g$ for 5 min. Ice cold 10% trichloroacetic acid (5 ml) were added to each pellet and the samples were kept at 0°C for 5 min. The samples were filtered through 0.45 μM Millipore HA filters, washed with 10% trichloroacetic acid and dried. Filters were dissolved in 1 ml cellosolve; 10 ml cellosolve cocktail (2365 ml toluene, 788 ml cellosolve, 132 ml Liquifluor) were added and radioactivity was determined by liquid scintillation spectrometry.

2.4. *Histone synthesis*

At each time point 1×10^8 cells were harvested and resuspended to 1×10^7 cells/ml in Earle's balanced salt solution containing 2% fetal calf serum and 5 μCi/ml of [^{3}H]leucine (Schwarz-Mann, 61 Ci/mmol). Following 30 min incubation at 37°C, cells were

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harvested and chromatin was isolated as in [6]. Histones were extracted by washing chromatin in 0.4 N H_2SO_4 (twice for 30 min) and precipitated at −20°C from the combined acid extracts by the addition of 2 vol. ethanol. The histones were fractionated electrophoretically in acetic acid–urea polyacrylamide gels [8]. The gels were stained with amido black, scanned at 600 nm and then sliced transversely into 2 mm sections. The gel slices were placed in 5 ml vials, dried and dissolved at 80°C in 0.2 ml 30% H_2O_2. Of Triton–toluene scintillation fluid (42 ml Liquifluor–333 ml Triton X-100–625 ml toluene), 3 ml were added to each vial and radioactivity was determined by liquid scintillation spectrometry.

3. Results and discussion

The ability of cells to resume DNA replication and histone synthesis following release from treatment with hydroxyurea was examined. Exponentially growing HeLa S_3 cells were synchronized by 2 mM thymidine block [6]. Two hours after release from the thymidine block (early S phase) cells were treated with 10 mM hydroxyurea for either 30 min or 2 h. At various times during and after hydroxyurea treatment the synthesis of DNA and histones was assayed as in section 2.

There was a >90% inhibition of both DNA and histone synthesis during hydroxyurea treatment (fig.1). When cells were treated for 30 min with hydroxyurea and then resuspended in fresh medium, DNA synthesis recovered to control levels within 10 min (fig.1A). In contrast, 10 min after resuspension of cells in fresh medium histone synthesis was only 30% of control level. Histone synthesis did not return to control levels until 60 min after removal of hydroxyurea (fig.1A). The level of inhibition of histone synthesis during hydroxyurea treatment and during the period following removal of the drug was the same for all histone species (H1, H2A, H2B, H3, H4) (fig.2). When HeLa cells were treated with 10 mM hydroxyurea for 2 h, then resuspended in fresh medium, DNA synthesis rapidly (within 10 min) returned to control levels (fig.1B). Although the inhibition of histone synthesis was completely reversible following 2 h hydroxyurea treatment, the initial rate at which histone synthesis resumed was higher for cells treated with hydroxyurea for 30 min than for cells treated for 120 min. Histones were synthesized at control

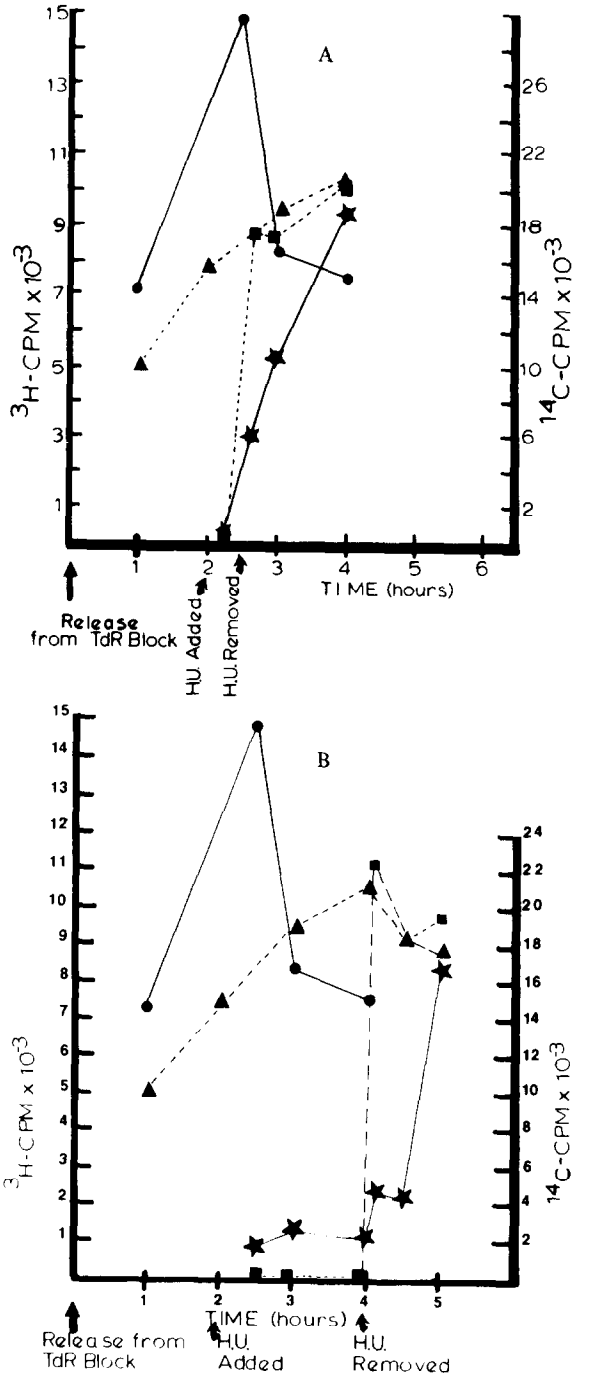

Fig.1. Effect of a 30 min (A) or 2 h (B) hydroxyurea treatment (10 mM) on histone and DNA synthesis. Incorporation of [^{14}C]thymidine into DNA and [^{3}H]leucine into histones was assayed during S phase in HeLa S_3 cells prior to, during and following hydroxyurea treatment. (•) Histone synthesis in S phase controls; (▲) DNA synthesis in untreated controls; (■) DNA synthesis in hydroxyurea-treated cells; (★) histone synthesis in hydroxyurea-treated cells.

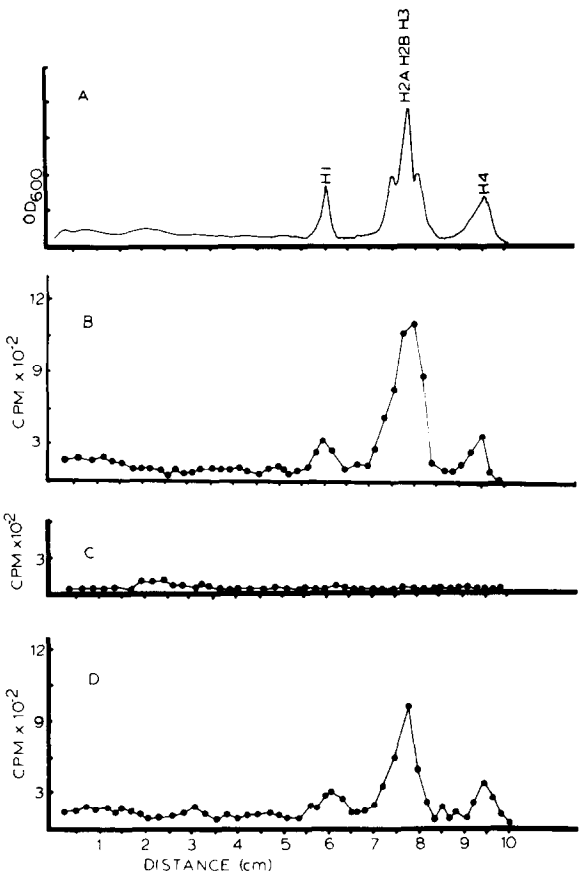

Fig.2. Electrophoretically fractionated histone polypeptides of S phase HeLa S_3 cells prior to, during and following release from treatment with 10 mM hydroxyurea. (A) A_{600} scan of a Coomassie blue-stained gel containing electrophoretically fractionated histones from S phase HeLa cells 2 h after release from thymidine block. (B) Incorporation of L-[^{3}H]-leucine into electrophoretically fractionated histones from S phase HeLa cells 2 h after release from thymidine block. (C) Incorporation of L-[^{3}H]leucine into electrophoretically fractionated histones from S phase HeLa S_3 cells 30 minutes after treatment with 10 mM hydroxyurea. (D) Incorporation of L-[^{3}H]leucine into electrophoretically fractionated histones from S phase HeLa S_3 cells treated with 10 mM hydroxyurea for 30 min followed by resuspension in normal growth medium for 60 min.

levels 60 min after release from the 120 min drug block.

We cannot completely dismiss the possibility that histone gene expression and DNA replication resume concomitantly following release from hydroxyurea treatment. The apparent delay in resumption of histone biosynthesis could be explained by the time required for transcription and processing of histone mRNAs, translation of polypeptides and association of the newly synthesized histone proteins with chromatin. However, the rapid association of histone mRNAs with polysomes and the rapid transfer of newly synthesized histones into chromatin are not compatible with such an explanation.

While it is well documented (though not uncontested [11]) that histone biosynthesis and DNA replication occur concomitantly during the S phase of the cell cycle of many continuously dividing cells and also following stimulation of non-dividing cells to proliferate [1–7,12], the nature of the functional inter-relationship between these 2 events remains unresolved. A further understanding of the levels at which the hydroxyurea block of histone gene expression and resumption thereof after release are controlled may provide insight into the nature of this relationship. Despite the caution which must be exercised when using inhibitors, our observation that during recovery of HeLa cells from hydroxyurea treatment there is a period of 30 min when DNA is synthesized at control levels in the apparent absence of significant amounts of histone synthesis may prove useful for elucidating the coupling of histone gene expression and DNA replication. However, an assessment of the representation of histone mRNA sequences in various intracellular compartments during hydroxyurea block as well as following release from hydroxyurea inhibition is necessary. Preliminary results [10] indicate that when DNA synthesis is inhibited histone mRNA sequences are lost from the polysomes, yet are still detectable in the nucleus and post-polysomal cytoplasmic fractions. Present studies are directed towards determining the representation of histone mRNA sequences following release from hydroxyurea treatment along with their structural and translational properties. While histone proteins were reported [11] to be synthesized throughout the cell cycle but become associated with DNA only during S phase in S 49 and CHO cells, in HeLa cells histone proteins are synthesized and histone mRNAs are present on polysomes only during S phase [12].

Acknowledgements

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