CELL CYCLE-DEPENDENT ALTERATIONS OF THE TWO TYPES OF RIBONUCLEASES H IN L5178y CELLS

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1. Introduction

Since the discovery of eukaryotic ribonuclease H (RNase H; EC 3.1.4.34) [1] the physiological role of this enzyme has remained conjectural. The existence of at least two distinct RNase H activities (RNase H I and RNase H II) [2,3] accounts for the, at least partially, conflicting reports about their physiological significance. Three main hypotheses have been propounded.

(1) Participation in DNA replication by hydrolyzing short RNA·DNA hybrid primers [3–8];
(2) Role in the regulation of RNA biosynthesis [9,10];
(3) Implication in conformation changes of chromosomal structure during the cell cycle [11].

Here we show that the extractable activities of RNase H I and II alter characteristically their activities during the different phases in the cell cycle of synchronized L5178y cells. Under the prerequisite that the enzyme activity is rate-limiting for a particular biochemical event in the cell the results presented in this paper might indicate that the RNase H II is involved in DNA synthesis, while RNase H I might have a function during the conformational changes in the chromatin.

2. Materials and methods

2.1. *Compounds*

The following materials were obtained, [*methyl*-^{3}H]thymidine (spec. act. 19 Ci/mmol) from the Radiochemical Centre (Amersham); Sephadex G-100 and dextran blue from Deutsche Pharmacia (Freiburg); *N*-ethylmaleimide from Sigma (St Louis, MO).

The poly(dT)·poly([^{3}H]rA) hybrid was prepared enzymatically [12] as in [8]. The specific activity was 4000 cpm/pmol poly([^{3}H]rA).

2.2. *Cell culture*

L5178y mouse lymphoma cells were grown in Fischer's medium for leukemic cells, supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, NY) in spinner culture [13]. Cell concentration and volume distribution were determined with a Model B Coulter Counter with a size-distribution plotter (Coulter Electronics, Hialeah, FL). The cells were synchronized at 2.5×10^5 cells/ml by the double thymidine block method [14,15]; after release of the block >90% of the cells were at the beginning of the S-phase as measured by [^{3}H]thymidine incorporation [15].

2.3. *RNase H assay*

The standard assay mixture (150 μl) contained 30 mM Tris–HCl (pH 7.8), 5 mM 2-mercaptoethanol, 10 μg bovine serum albumin, 10 mM $MgCl_2$, 100 mM KCl, 200 pmol ^{3}H-labeled (rA) nucleotide in the poly(dT)·poly([^{3}H]rA) hybrid form and 30 μl of enzyme fraction. The reaction was carried out for 30 min (the reaction kinetics were linear during the incubation time); 50 μl were placed on GF/C discs and processed as described [16]. In some experiments the specific activity was normalized to 10^7 cells. One unit is defined as the amount of enzyme necessary to solubilize 1 nmol poly([^{3}H]rA)/30 min under standard conditions.

2.4. *Separation of RNase H I and RNase H II*

$3–6 \times 10^7$ cells were suspended in 1 ml of the TMK-buffer (30 mM Tris–HCl (pH 7.8); 5 mM

2-mercaptoethanol, 10 mM $MgCl_2$, 100 mM KCl) and sonicated 5 times for 10 s with cooling intervals using a Branson S-75 Sonifier (microprobe, setting 3) at 0–2°C. Subsequently the homogenate was centrifuged (12 000 × g, 2°C, 2 min) and the supernatant was collected quantitatively. The total supernatant was applied on a Sepharose G-100 column (1 × 45 cm) in order to separate the RNase H activity into two fractions, RNase H I and RNase H II; fig.1. The first peak containing RNase H I is observed at a V_e/V_0 of 1.16 and a second distinct activity (RNase H II) appears at a V_e/V_0 of 1.72.

Protein was determined by Lowry's method [17].

3. Results

3.1. *Characterization of RNase H I and RNase H II*

The two RNase H species I and II are distinguished by their molecular weight. While RNase H I appears at a V_e/V_0 of 1.16, corresponding to mol. wt 95 000 [18], the species II elutes at a V_e/V_0 of 1.72 (mol. wt 32 000). The two enzymes were tested in assays containing instead of poly(dT)·poly([^{3}H]rA) as substrate, the polymers poly([^{3}H]rA), poly(U)·poly([^{3}H]rA) or poly([^{3}H]dT). These experiments were performed under conditions essentially as in [8]; none of these substrates were degraded to any measurable extent

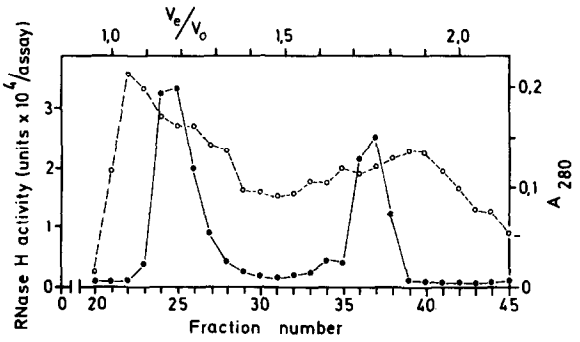

Fig.1. Separation of RNase H I from RNase H II activity on Sephadex G-100. The preparation of the crude extract obtained from 3 × 10^7 cells (3 h after the release from the thymidine block) is in section 2. Extract (950 μl) was applied on a Sephadex G-100 column (1 × 45 cm) equilibrated with TMK-buffer, containing 50 mM sucrose. Elution was performed with the same buffer. Fractions of 0.6 ml were collected and 30 μl aliquots were assayed for RNase H activity. The A_{280} (○– –○) and the RNase H activity/assay (●——●) are given. Abscissa (upper scale): V_e/V_0 value [18]; V_0 was determined with dextran blue.

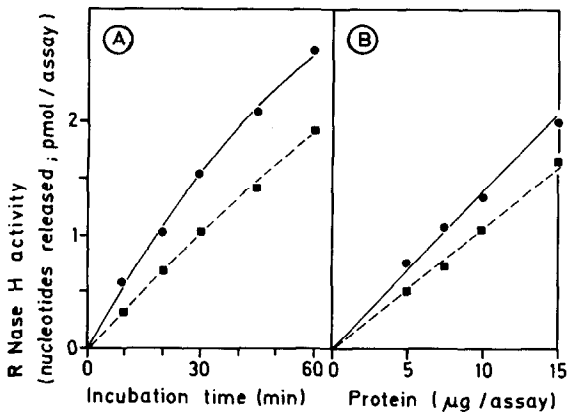

Fig.2. RNase H I (●——●) and RNase H II activity (■– –■) as a function of time (A) and of enzyme concentration (B). In the time kinetics experiments 0.0015 units of RNase H I and 0.001 units of RNase H II were used in the standard assay. In the experiments using various amounts of enzyme solution in the standard assay, RNase H I was spec. act. 0.65 units/mg protein (230 μg protein/ml) and RNase H II 0.56 units/mg protein (180 μg protein/ml). The RNase H I and II samples were obtained by Sephadex G-100 chromatography.

(data not shown). In the standard assay, containing 0.0015 units of RNase H I or 0.001 units of RNase H II (obtained after separation on Sephadex G-100), the RNase H I activity is not affected in the presence of 1 mM *N*-ethylmaleimide, while RNase H II activity is reduced by 95%.

The activity of both RNase H I and RNase H II shows a constant rate up to an incubation period of 30 min (fig.2A); beyond this period the rate declines. The rate of poly(A) hydrolysis is proportional to the enzyme concentration in the standard assay below 15 μg protein/assay, corresponding to 0.002 units of RNase H I and 0.0016 units of RNase H II (fig.2B).

3.2. *Alteration during cell cycle*

Each of the enzymes, both RNase H I and RNase H II, shows a characteristic variation in its activity during the cell cycle (fig.3). The two enzymes show a dramatic increase in their activity at the beginning of the S-phase. The maximum of RNase H II activity is only observed during S-phase; during G_2-phase the activity drops and remains at a low level during M- and G_1-phase. In contrast, the RNase H I activity shows high values not only during S-phase but also during G_2-, M- and the beginning of G_1-phase; a pronounced decrease of RNase H I level is observed at the end of G_1-phase.

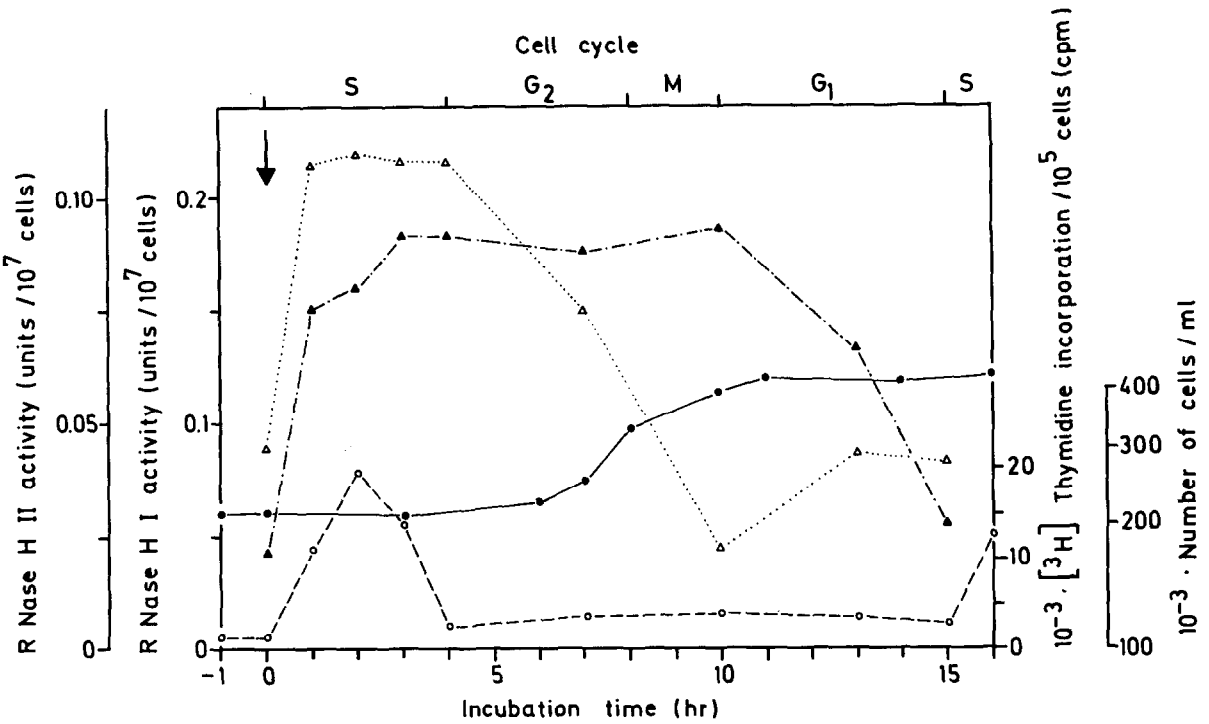

Fig.3. Levels of RNase H I and RNase H II in L5178y cells at different cell cycle stages. The cells were synchronized by two thymidine blocks; the last hour of the second thymidine block is marked in the graph (−1). At time zero (arrow) the cells were transferred into fresh medium and incubated further for up to 16 h. The cell number (•——•) and the thymidine incorporation rate (○— —○) were determined as in section 2. On the upper abscissa the approximate duration of the cell cycle phases are given. Cellular extracts were prepared from 3–6 × 10^7 cells. At the time indicated the cells were harvested and RNase H I activity was separated from RNase H II activity as described in fig.1. (▲–.–▲) RNase H I; (△.....△) RNase H II.

4. Discussion

The aim of this paper was to determine the alteration of RNase H I and RNase H II levels during the cell cycle. For that purpose a separation method (gel filtration) was chosen, which allowed a high yield during the fractionation procedure. The two enzyme species isolated from L5178y cells showed the same characteristics as the ones known from other mammalian cells [3,6,11]: absolute dependence on a DNA·RNA hybrid as substrate, insensitivity of RNase H I to *N*-ethylmaleimide, sensitivity of RNase H I to -SH blockers and the difference of the molecular weight between the RNase H I (75 000–110 000, depending on the determination method used [2,3]) and RNase H II (30 000–40 000 [2,4]).

The two RNase H species show characteristic alterations in their activity. While RNase H II activity shows a high level only during S-phase (5-fold higher than during M-phase), RNase H I activity increases at the beginning of S-phase, remains constant till the beginning of G_1-phase and decreases during G_1. From this finding we favor the involvement of RNase H II in the removal of RNA primer during DNA replication; this assumption is also supported by data in [3]. Concerning the fluctuation of RNase H I activity during cell cycle it is too early to formulate even an hypothesis, although the idea about its role during changes in chromatin structure appears to be attractive.

Acknowledgements

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