DEOXYRIBONUCLEASE II ACTIVITY IN RELATION TO CELL CYCLE IN SYNCHRONIZED HeLa S3 CELLS

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Summary:

Studying the activity of DNase II in relation to cell cycle in synchronized HeLa S3 cells show a two to seven fold increase in DNase II activity at those times when DNA synthesis is taking place. The peaks of DNase II activity coincide with the peaks of DNA synthesis. The increased DNase II activity could be prevented by puromycin, suggesting that the enzyme activity increased at the S phase was caused by synthesis of new molecules rather than the activation of existing molecules. Acid phosphatase (as a marker for lysosomal enzymes) does not show an induction similar to that observed for DNase II in relation to cell cycle.

A correlation between DNase activity, cell division, and DNA synthesis has been reported by many researchers and was recently reviewed (1). A correlation between DNase II activity and the capacity of certain tissues to divide has also been demonstrated(2,3). Based on these and earlier findings it has been postulated that DNase II plays an important role in DNA metabolism in relation to cell division(4-6).

Two questions must be resolved in order to relate DNase activity to DNA synthesis. First, DNase II has been considered an exclusively lysosomal enzyme (7), but if it has a direct role in DNA synthesis it must be found in the nucleus. We have recently shown that besides being found in lysosomes it is also found in nuclei of calf thymus (8) and HeLa S3 cells (9). Our experiments ruled out the possibility that the presence of nuclear DNase II is caused by lysosomal contamination of the nuclear fraction(8) or by preferential binding of lysosomal or cytoplasmic DNase II to nuclear DNA during the purification of the nuclear fraction (9). Second, DNase II has been considered an acid DNase, optimally active at pH values between 4.5 and 5.5 in 0.15 - 0.2 M acetate buffer (10). But we have recently described conditions by which highly purified DNase II is active at neutral pH, given the appropriate conditions of a low ionic strength and divalent cations (11). This observation prompted us to suggest that the term "acid-DNase" for DNase II is inaccurate (11).

If DNase II is indeed involved in some aspects of DNA synthesis (e.g. in recombination events, degradation of DNA fragments which carry mistakes in their base sequence, making nicks in DNA for unwinding of the DNA during replication, etc.), its activity in the cell will probably have some correlation to the synthesis of DNA. This paper describes experiments with synchronized HeLa S3 cells that clearly show the existence of a correlation between DNase II activity and DNA synthesis.

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MATERIALS AND METHODS

Cell culture. HeLa S3 cells were grown as monolayers and maintained in equal parts of Eagle's MEM and M199 medium supplemented with 10% calf serum and antibiotics (200 iu/ml penicillin and 20 iu/ml dihydrostreptomycin). Cells were synchronized by the double thymidine blocking treatment(12) as slightly modified by Melnykovych et al.(13). In this procedure the cells were relaxed for 16 h between thymidine blocks in thymidine - free medium. Synchronized cultures were inoculated into T-30 tissue culture bottles at a concentration of 250,000 cells/ml medium (approximately 5×10^6 cells per bottle). Cells were harvested by trypsinization(0.25% versene-trypsin), washed 3 times with cold Earle's balanced salt solution (BSS) and sonicated for 30 sec (2 x 15 sec) in a MSE sonicator at an amplitude of 7 microns between peaks. Sonicates were centrifuged at 10,000 x g for 60 min at 4^oC and the supernatants were analysed for enzyme activity, DNA synthesis, and protein content(14).

DNA synthesis. DNA synthesis was determined by the incorporation of ^3H thymidine ([methyl ^3H]-thymidine TRA 120, 11.6 Ci/mM, Radioactive center, Copenhagen) into acid-insoluble material after 60-min pulse labeling with 1 μCi/ml. Cells were collected by trypsinization, washed 3 times with BSS, sonicated, and diluted with TCA to a final acid concentration of 7%. After 20 min in ice, the samples were collected on millipore filters, which were previously washed with cold 7% TCA containing 2 mM unlabeled thymidine to prevent nonspecific binding of free ^3H thymidine to the filters. Filters were washed with TCA and dried overnight (50^oC) and counted in 10 ml scintillation fluid in a Packard Tri-Carb spectrometer. Counting efficiency was 41% as determined by external standards.

Enzymic assays. DNase II activity was determined by measuring measuring the acid-soluble nucleotides released from the DNA by the enzyme. The reaction mixture contained 0.2 mg/ml calf thymus DNA(prepared as previously described (15)) in 3 ml of 0.175 M acetate buffer, pH 4.5, 1 mM EDTA, and enzyme. After incubation at 37^oC for a predetermined time, the tubes were chilled in ice and $HClO_4$ was added to a final concentration of 6%. Tubes were centrifuged for 15 min at 7,000 x g at 4^oC and the O.D at 260 nm of the supernatant was determined against an appropriate blank, in a Beckman DU spectrophotometer. When radioactive DNA was used as substrate, the final volume was 1 ml of the same buffer, containing 2,000 cpm HeLa S3 ^{14}C DNA (15,000 cpm/μg). The radioactivity of the acid-soluble nucleotides in the clear supernatant was determined in Bray's dioxane-based scintillation fluid . DNase I incubated in the DNase II reaction mixture did not degrade DNA at all, probably because of the presence of EDTA and the unfavorable pH.

Acid phosphatase activity was determined as previously described(8).

RESULTS AND DISCUSSIONS

Synchronized HeLa S3 were inoculated into tissue culture bottles. At various times

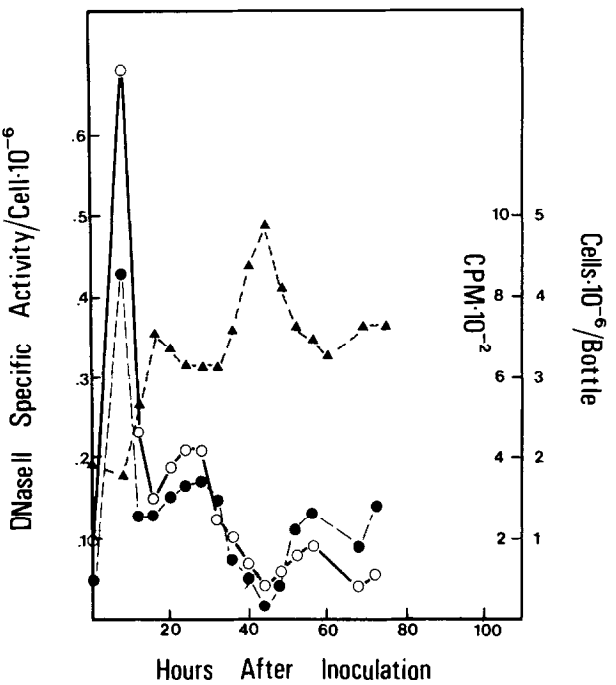

Fig. 1 DNase II activity in relation to DNA synthesis in synchronized HeLa S3 cells. Triplicate culture bottles were assayed every 4 h (each in triplicate) for 72 h after inoculation of 250,000 cells/ml medium. (○) DNase II specific-activity/cell, (●) acid insoluble cpm ^{3}H thymidine incorporated into DNA (counting efficiency of 38% as determined by external standards), (▲) number of cells/bottle.

triplicate cultures were pulse labeled with ^{3}H- thymidine, washed with BSS, collected by trypsinization, counted, and sonicated. The centrifuged sonicate was assayed for DNase II activity, protein content, and acid-insoluble radioactivity incorporated into the DNA (Fig. 1). Each point on the graph represents an average of 3 culture bottles assayed independently in triplicate. The variation between triplicate culture bottles was less than 15% and between triplicate assays of each bottle less than 5%. The peaks of increased DNase II specific activity per cell (DNase II units/mg protein) coincide with the peaks of DNA synthesis.

The amount of DNase II activity at the 8-h peak was more than 7 times greater than that at the time of inoculation. The initial inoculum in each culture bottle was high in order to have enough cells for the enzymatic assays. Therefore, cell number increased normally up to 40 h after inoculation, but then the cells became crowded and not only did not increase upon further incubation but declined in number, probably due to cell death. Only the first 40 h of incubation, which is essentially the time we interested in, should be regarded as typical. The sharp decline in DNase II specific activity at the end of the S phase cannot be explained by degradation of the enzyme alone. Preliminary studies (unpublished results) indicate that a DNase II-specific inhi-

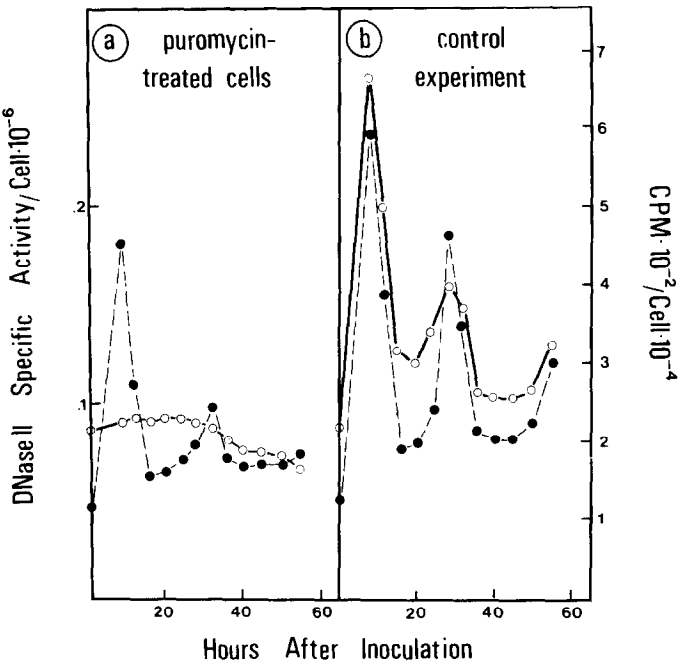

Fig. 2 Influence of puromycin on the induction of DNase II activity in synchronized HeLa S3 Cells. One μg/ml puromycine was added 1 h after inoculation. Every 4 h triplicate cultures were pulse labeled with ^{3}H thymidine and assayed in triplicate for acid-insoluble ^{3}H thymidine incorporation into DNA (●) and for DNase II specific activity (○) in both puromycin-treated cultures (a) and control untreated cultures (b).

bitor is being synthesized at this period and that the decline is a result of inhibition of existing DNase II molecules. This phenomena is probably part of the control mechanism by which the levels of DNase II (and probably other enzymes as well) activity in the cell is controlled by means of enzyme synthesis, enzyme degradation and enzyme inhibition.

Since approximately 90% of the DNase II activity in the cell is lysosomal and the rest nuclear (8,9), we were interested in determining whether the activity of a different lysosomal enzyme, such as acid phosphatase, would also show changes in specific activity in relation to cell cycle in the synchronized HeLa cells. We found that, contrary to DNase II, the specific activity of acid phosphatase remained constant for 96 h. This experiment suggested that the increase in DNase II activity was not caused by nonspecific increase of lysosomal enzymes during the synthesis of DNA at the S phase. The increase in DNase II activity could be explained either as a result of activation (or release from inhibition) of existing DNase II molecules or as a result of the synthesis of new molecules of DNase II. In order to differentiate between these two alternatives, we performed another experiment similar to the one shown in Fig. 1 in which we added puromycin (5 μg/ml) to the medium 1 h after inoculation. DNase II

activity was then assayed using HeLa S3 ^{14}C-labeled DNA as substrate. As can be seen in Fig. 2, there was no increase in DNase II in the puromycin treated cells, suggesting that the increased activity observed during the S phase is caused by synthesis of new DNase II molecules or a DNase II effector rather than the activation of previously existing molecules. Furthermore, there was a reduction of approximately 25% in DNA synthesis in the first peak (8 h) and a reduction of almost 50% in the second peak (32 h) in the puromycin-treated cultures compared to control cultures. Although the first DNA synthesis peak was in both treated and control cultures at 8 h, the second peak of DNA synthesis, which was at 28 h after inoculation in control cultures, was delayed in the puromycin-treated cells to 32 h after inoculation. This indicates that DNA synthesis in treated cultures was performed under unfavorable conditions that affected both the rate of synthesis and the time it occurred. The concentration of 5 μg/ml puromycin in this experiment inhibited 70% of the protein synthesis as measured by incorporation of DL ^{3}H-leucine into the acid-insoluble pronase-sensitive fraction(data not shown). Such inhibition level is similar to the results of Muller et al (17).

The reduction of DNA synthesis is probably due to the fact that enzymes essential to DNA synthesis were not synthesized in the puromycin-treated cultures and that DNA synthesis continued using existing enzymes which were not sufficient to carry DNA synthesis at an optimal rate.

In conclusion, we have shown an induction in the synthesis of new DNase II enzyme during the S phase in synchronized HeLa S3 cells and suggest that this phenomena might reflect the biological duty of DNase II in the cell, particularly during DNA synthesis, a duty which is by no meen yet understood.

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