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Selective cleavage of 28S rRNA variable regions V3 and V13 in myeloid leukemia cell apoptosis

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Vigorous apoptosis is induced 3–4 hours after activation of cAMP-dependent protein kinase I in the rat myeloid leukemia cell line IPC-81 [J. Cell. Physiol. 146 (1991) 73–80]. We will report a novel feature of apoptosis in these cells: a selective and temporarily ordered cleavage within the two largest 28S ribosomal RNA variable regions (V3 and V13). The cleavage of 28S rRNA coincided with internucleosomal DNA fragmentation and cessation of cellular protein synthesis. The implication of 28S variable regions as targets in apoptosis is a clue to the function of these so far apparently superfluous parts of eukaryotic ribosomes.

Myeloid leukemia; Apoptosis; 28S ribosomal RNA

1. INTRODUCTION

Apoptotic cell death requires active participation from the affected cell, which typically shows shrinkage, surface blebbing, chromatin condensation and DNA fragmentation [2–5]. The molecular mechanisms involved in this physiological form of cell death are largely unknown [5]. In the rat myeloid leukemia cell line IPC-81 apoptosis can be triggered by activation of the cAMP-dependent protein kinase [1]. Cell death can be hindered if inhibitors of protein synthesis are given within three hours after cAMP-challenge [1]. When doing experiments aimed at finding genes that were induced in this three hour period, we fortuitously observed that the 28S rRNA became degraded in parallel with other indices of apoptosis, like DNA fragmentation and typical morphological changes. This novel feature of apoptosis may give a function to ribosomal variable regions and provides a new angle in the search for molecular mechanisms initiating cell death.

2. MATERIALS AND METHODS

2.1. *Cells and experimental set-up*

The rat myeloid leukemia cell line IPC-81 was maintained in RPMI with 8% horse serum [6]. Addition of 0.2 mM 8-(4-chlorophenylthio)-cAMP (or 8-CPT-cAMP) to the medium caused apoptotic cell death after a lag-phase of about three hours. The percentage of apoptotic cells at various time points after cAMP-challenge was measured by cell counting under bright-field microscopy. Sometimes cells were processed for electron microscopy, as described [7].

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2.2. *Preparation and analysis of RNA and DNA*

RNA and DNA were prepared, electrophoresed and blotted as previously described [8,9]. For preparation of RNA, the cell pellets were immediately lysed in 25 mM sodium citrate buffer pH 7 containing 4 M guanidinium thiocyanate, 0.5% sodium lauroylsarcosine (w/v) and 1% 2-mercaptoethanol (v/v), phenol extracted and isopropanol-precipitated. All hybridisations of Northern blots were done with 5′-^{32}P-labelled oligonucleotides complementary to various parts of rat 28S rRNA [9] (for details, see Fig. 2).

2.3. *Pulse-chase protein labelling*

The cells were washed in methionine-free DMEM and then grown for 30 min. in methionine-free DMEM with 1% horse serum and 0.2 mM 8-CPT-cAMP. At the indicated time 65 μCi/ml [^{35}S]methionine was added. After 15 min labelling, the cells were chased with DMEM containing 7% horse serum and 1 mM methionine. 15 min thereafter, the cells were lysed and prepared for SDS-PAGE as described [9]. All the media used had been pregassed and prewarmed.

3. RESULTS AND DISCUSSION

Within 3–6 hours after a strong cAMP-challenge more than 90% of the IPC-81 cells undergo classical apoptosis [1] or type 1 cell death as defined by Clarke [4] (Figs. 1 and 4). When scrutinising Northern blots from IPC-81 cells it was noted that the large ribosomal subunit RNA (LSU-rRNA or 28S rRNA) became degraded (Fig. 1). This degradation was in parallel with other indices of apoptosis, like DNA fragmentation and typical morphological changes (Figs. 1 and 4). The sizes of the different fragments were consistent with a double cleavage about 0.7 and 1.2 kb from the 5′ end and a single cleavage about 1.5 kb from the 3′ end of the 28S rRNA (Fig. 3), i.e. at locations corresponding to the two major, V3 and V13, of the 18 recognised variable regions of the LSU-rRNA [10–12]. Probing with oligonucleotides complementary to the regions flanking

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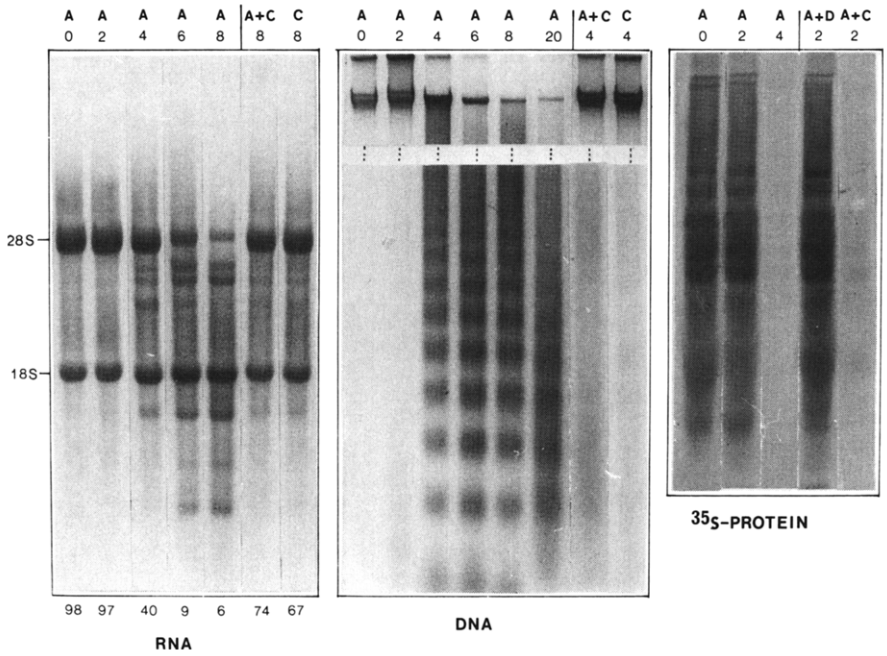

Fig. 1. Size distribution of leukemia cell RNA, DNA and protein synthesis were evaluated at indicated time (on top of each lane, in hours) of incubation with 0.2 mM 8-CPT-cAMP. Top legend: A, 0.2 mM 8-CPT-cAMP; C, 30 μg/ml cycloheximide; D, 10 μg/ml actinomycin D. Left panel: total cellular RNA (25 μg pr. lane) viewed in ethidium bromide-stained 1.5% denaturing agarose gel after electrophoresis (negative image). The number at the bottom of each lane represent the percentage of morphologically intact cells at each time point. Middle panel: total cellular DNA (10 μg/lane) viewed in ethidium bromide-stained 1.4% agarose gel after electrophoresis. Note that only the part of the gel with non-fragmented DNA (on top of each lane) and the part with DNA sizes from about 3 kb down to 0.2 kb are shown. Right panel: protein synthetic activity measured by pulse-chase labelling with [^{35}S]methionine, followed by electrophoretic separation of labelled proteins.

V3, V4 and V13, established that the double cut was in V3 and the single cut in V13 (Fig. 2). To the best of our knowledge, this is the first report of intracellular, selective cleavage of rRNA variable regions.

The time course of accumulation of 28S rRNA fragments indicated that the cut in V13 resulted in a stable 1.5 kb 3′ fragment and an unstable 3.3 kb 5′ fragment (Fig. 3). The latter fragment was subsequently cleaved in V3 and rapidly degraded (Figs. 1, 2). The pathway initiated by cut in V13 thus leads to rapid and extensive degradation. 28S rRNA initially cut at V3 was more stable towards further degradation (Figs. 1, 2). That some 28S rRNAs are preferentially cut at V3 and others at V13 may be due to pre-existing intraspecies LSU-rRNA variable region heterogeneity, already demonstrated [13–15]. No 1.2 kb fragment corresponding to the 5′ part of 28S rRNA cut at the 3′ extreme of V3 (V3b) was found (Fig. 2). This indicated that such a cut was immediately followed by a cut at V3a, or that it occurred after the V3a cut. The results shown in lane 4 and 5 in Fig. 2 limit the 3′ cut in V3 to an approx. 50 nucleotide segment in the very 3′ part of V3. Interestingly, the 3′ extreme of this 50 nucleotide segment comprises the only region which is conserved between eukaryotic variable regions (nucleotides 1213–1235) [10].

18S rRNA appeared intact after nearly all the 28S rRNA and the DNA had been fragmented (Fig. 1). This was verified using an 18S rRNA specific oligonucleotide probe (Fig. 2). Random RNase action appeared therefore not to be an important factor in the apoptotic process. Further evidence for this came from an experiment were IPC-81 cells underwent two cycles of freeze/thawing (liquid N_2/37°C water bath) followed by incubation at 37°C for up to two hours. Only a slow and general degradation was observed (no specific 28S rRNA fragments detected), affecting 18S and 28S rRNA to similar extents (data not shown).

The cAMP-induced apoptosis requires protein synthesis in the 2–3 hour period after cAMP-challenge [1]. Also the cAMP-induced 28S rRNA cleavage was blocked by an inhibitor of protein synthesis which also blocked DNA fragmentation (Fig. 1), underscoring that 28S rRNA fragmentation depended on similar triggering factors as did other apoptotic events. The protein synthetic activity was assessed every 15 minutes after cAMP-challenge by [^{35}S]methionine pulse-labelling. Only when the first signs of 28S rRNA degradation appeared (after 2.5–3 hours; Fig. 3), did the protein synthetic activity start to decline (not shown). Between 3–4 hours after cAMP-challenge protein synthesis decreased disproportionally faster than rRNA degradation. There was virtually no protein synthesis 4 hours after cAMP-challenge (Fig. 1), even though $^1/_3$ of the cells appeared morphologically non-apoptotic. This can be explained if inactivation of merely one or a few ribo-

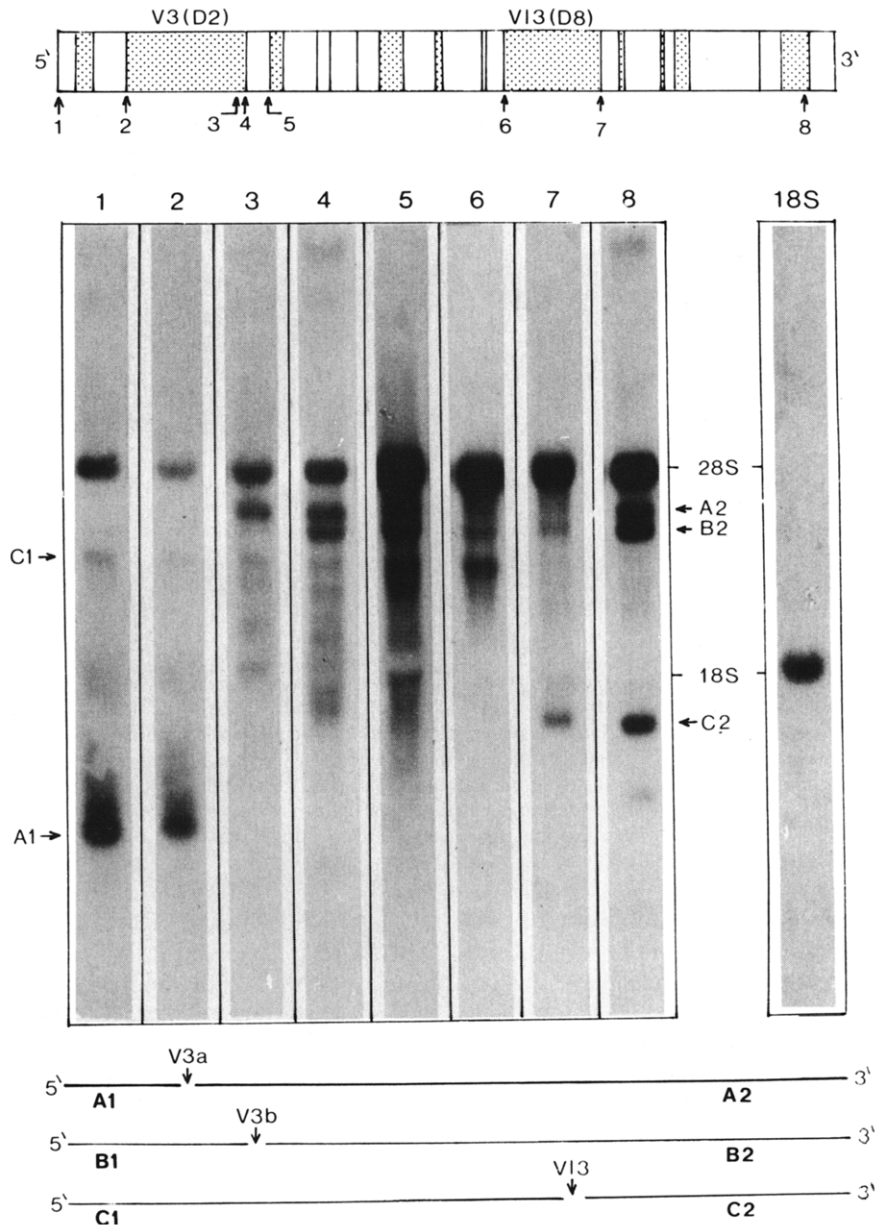

Fig. 2. Northern blots of fragmented 28S rRNA hybridised with oligonucleotides complementary to the ends of 28S rRNA (1 and 8) and the flanking regions of variable regions V3 and V13 (2 to 7), as indicated on the 28S rRNA linear map (top). 28S rRNA variable regions (V-regions; sometimes called D-regions) are shown in grey, conserved regions are white. The oligonucleotides were complementary to the following nucleotides of rat LSU-rRNA [13]: 1, 17–52 (5'28S); 2, 298–433 (5'V3); 3, 1166–1197 (3'V3a); 4, 1214–1249 (3'V3b); 5, 1265–1296 (5'V4); 6, 2700–2735 (5'V13); 7, 3337–3371 (3'V13); 8, 4608–4643 (3'28S, within V18). A probe complementary to nucleotide 1387–1418 (3'V4) gave the same hybridization pattern as in lane 5 (5'V4). The arrows labelled A1–C2 indicate the various primary fragments arising from the two cuts at V3 (V3a and V3b) and the one cut at V13, as illustrated on the bottom of the figure.

somes per polysome can stop protein synthesis, as shown [16]. Another possibility is that translationally active ribosomes were targeted preferentially. Although cessation of protein synthesis certainly causes cell death, other factors (selective protein synthesis in the early stage of rRNA affection ?) must have contributed to IPC-81 cell apoptosis, since cycloheximide, while efficiently blocking protein synthesis, induced apoptosis slower (within 8–16 hours) than did cAMP (Fig. 1; data not shown).

A mechanism for ribosome inactivation involving the variable regions is without precedence. Eukaryotic ribosomal toxins (the ricins, Shiga-toxin and α-sarcin) all attack the conserved α-sarcin loop known to be essential for protein synthesis [17,18]. Interestingly, these toxins can also cause cell death with the characteristics of apoptosis [19].

The function of ribosomal variable regions is so far unknown [10,12,20,21] , in fact they are thought to be dispensable for ribosome function [22,23]. They are

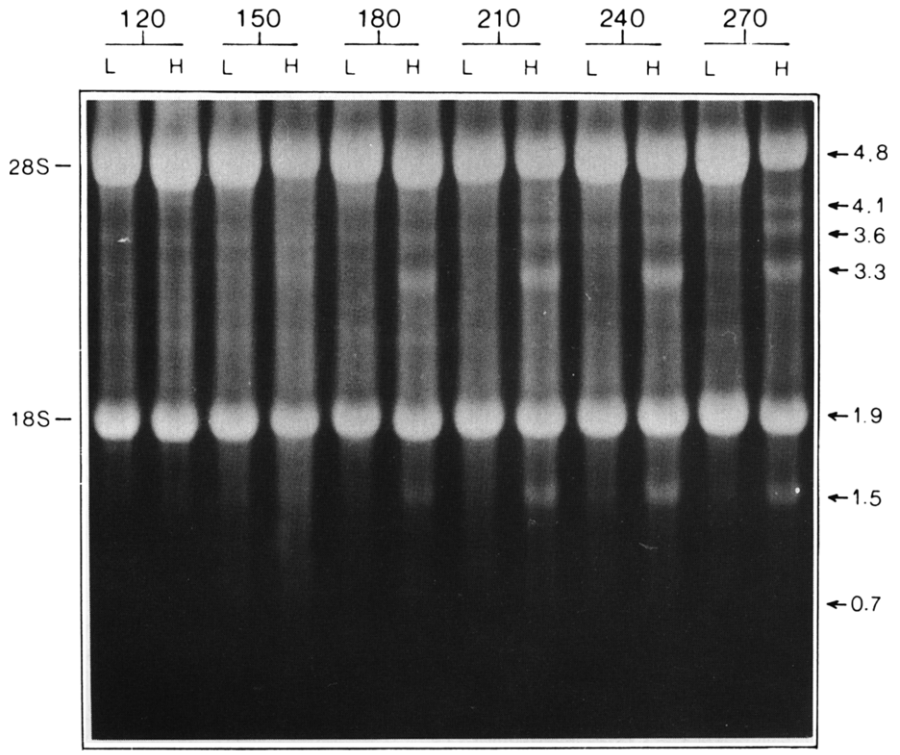

Fig. 3. Photograph of agarose-electrophoresed total cellular RNA (25 μg/lane) from IPC-81 cells at indicated time points (on top, in hours) of incubation with 8-(4-chlorophenylthio)-cAMP. L = low dose 8-CPT-cAMP (5 μM). H = high dose 8-CPT-cAMP (0.2 mM). The low dose 8-CPT-cAMP does not induce apoptosis but can activate cAMP-inducible genes (data not shown). Note that fragmentation resulting from V13 cleavage (3.3 and 1.5 kb) appear earlier than those produced by the V3 cuts (4.1, 3.6 and 0.7 kb).

characteristic of eukaryotic rRNA, and there is correlation between the size of the variable regions and the complexity of the organism [12,21]. The variable regions V3 and V13 account for more than 70% of the total enlargement observed from prokaryotic to eukaryotic LSU-rRNAs [12]. Noteworthy, the V3 and V13 regions have evolved in a tightly concerted fashion during evolution [24]. The sequence variability is due to a 'compensatory slippage' mechanism which causes variation in the length of the major loops within a conserved secondary structure [12,21,25]. In some cases variable regions are excised during rRNA processing without religation of the rRNA, but excision of V3 or V13 has not been reported [12].

It is tempting to speculate on the function of the variable regions V3 and V13 in light of the present findings. If these regions are early targets for apoptotic regulation, a functional role in development can be expected. Variable region heterogeneity might provide differentiated cellular sensitivity towards apoptotic induction. Developmental variegation involving variable regions, possibly controlled by genetic homing regulated by developmentally controlled transposases/gene-specific endonucleases [26,27], is an attractive model for stage-specific regulation of apoptotic sensitivity. Stage-specific ribosome populations have been observed in *Plasmodium* [28]. The tight connection between selective

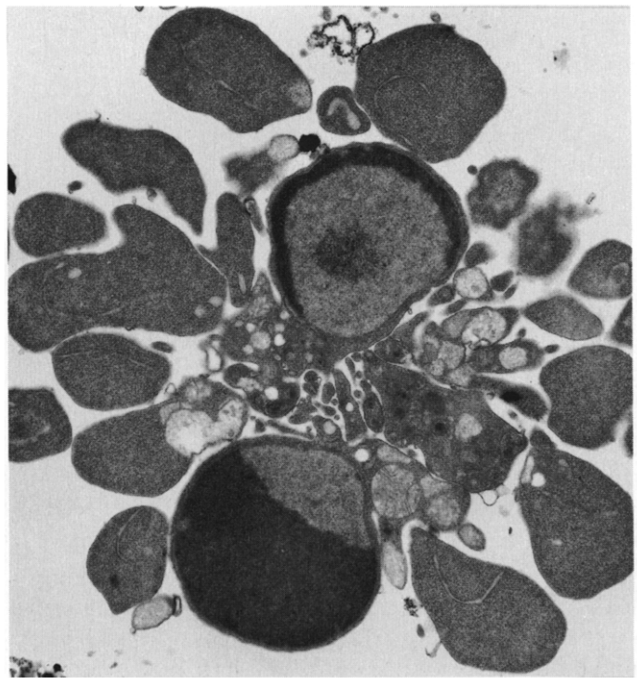

Fig. 4. Section of an IPC-81 cell in apoptosis (after 4 h of incubation with 0.2 mM 8-(4-chlorophenylthio)-cAMP) (9000×). Note the chromatin condensation, nuclear fragments and pronounced cellular disintegration. Mitochondria appeared intact, even at larger magnification (not shown).

cleavage of LSU-rRNA and the apoptotic process, as disclosed in the present study, gives a new angle for understanding the rDNA evolutionary paradox [29].

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