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# Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death)

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**Cell death by apoptosis occurs in a wide range of physiological events including repertoire selection of lymphocytes and during immune responses *in vivo*. A hallmark of apoptosis is the internucleosomal DNA degradation for which a $Ca^{2+}$,$Mg^{2+}$-dependent endonuclease has been postulated. This nuclease activity was extracted from both rat thymocyte and lymph node cell nuclei. When incubated with nuclei harbouring only limited amounts of endogenous nuclease activity, the ladder pattern of DNA fragments characteristic of apoptosis was induced. This extractable nucleolytic activity was immunoprecipitated with antibodies specific for rat deoxyribonuclease I (DNase I) and was inhibited by actin in complex with gelsolin segment 1, strongly pointing to the presence of a DNase I-type enzyme in the nuclear extracts. COS cells transiently transfected with the cDNA of rat parotid DNase I expressed the enzyme, and their nuclei were able to degrade their DNA into oligosome-sized fragments. PCR analysis of mRNA isolated from thymus, lymph node cells and kidney yielded a product identical in size to that from rat parotid DNase I. Immunohistochemical staining with antibodies to rat DNase I confirmed the presence of DNase I antigen in thymocytes and lymph node cells. The tissue distribution of DNase I is thus extended to tissues with no digestive function and to cells which are known to be susceptible to apoptosis. We propose that during apoptosis, an endonuclease indistinguishable from DNase I gains access to the nucleus due to the breakdown of the ER and the nuclear membrane.**

*Key words:* apoptosis/deoxyribonuclease I/DNA/programmed cell death/thymocytes

## Introduction

Apoptosis, or programmed cell death (Duvall and Wyllie, 1986) is a fundamental feature of development by which unwanted cells are eliminated from tissues during embryogenesis (Hincliffe, 1981) (e.g. the disappearance of the interdigital cells during the formation of the digits from the solid limb paddle), in metamorphosis (the resorption of the tadpole tail), in endocrine-dependent tissue atrophy (prostate regression after castration) and following removal of essential growth factors (Duke and Cohen, 1986). Similar phenomena are observed in the immune system during the negative selection of autoreactive T-cell clones (Shi *et al.*, 1989; MacDonald and Lees, 1990) and attack by cytolytic T cells (Russell and Dobos, 1980; Duke *et al.*, 1983). Recent reports also demonstrated that mature CD4+ T-cells undergo apoptosis during an immune response *in vivo* (Kawabe and Ochi, 1991; MacDonald *et al.*, 1991), and following simultaneous stimulation by antibodies to both CD4 and the T-cell receptor (Newell *et al.*, 1990).

Immature thymocytes, many of which are deleted by negative selection, provide a model system in which apoptosis can be induced by glucocorticoids or antibodies to the T-cell receptor (Smith *et al.*, 1989). When these cells undergo apoptosis, their intracellular $Ca^{2+}$ concentration increases (McConkey *et al.*, 1989) and their chromatin DNA is degraded at internucleosomal sites by a neutral $Ca^{2+}$,$Mg^{2+}$-dependent endonuclease (Wyllie, 1980; Cohen and Duke, 1984; Arends *et al.*, 1990), giving rise to the 'ladder' pattern of oligonucleosomal-sized bands corresponding to multiples of 200 bp. The generation of this ladder pattern reflects the supramolecular organization of the nuclear DNA; the fragments are generated by a cleavage of chromatin in the linker region on either side of the nucleosome.

Besides its pH and metal ion dependence, little is known about the nature and origin of the endonuclease. A number of attempts have been made to isolate this endogenous endonuclease, but have been plagued by the apparent lability and scarcity of the enzyme. Two rat thymocyte nuclear proteins with prominent nuclease activity have been characterized, both of which were induced upon glucocorticoid treatments of the cells and thus represent excellent candidate proteins (Compton and Cidlowski, 1987). Doubt, however, was later cast on these results, after two groups independently identified these presumptive 'nucleases' as histones (Alnemri and Litwack, 1989; Baxter *et al.*, 1989).

In this report, experiments were designed to characterize the relevant endonuclease from rat thymocytes. We show that the nuclease activity extractable from isolated nuclei is functionally and antigenically indistinguishable from deoxyribonuclease I (DNase I).

## Results

### *Characterization of the endogenous endonuclease*

The neutral endonuclease activated during thymocyte apoptosis has been shown to be $Ca^{2+}$- and $Mg^{2+}$-dependent in the millimolar range, and inhibited in the presence of $Zn^{2+}$ (Wyllie, 1980; Cohen and Duke, 1984; Arends *et al.*, 1990). Isolated nuclei incubated with the appropriate combination of divalent cations yield a pattern of DNA fragments that is indistinguishable from those observed *in*

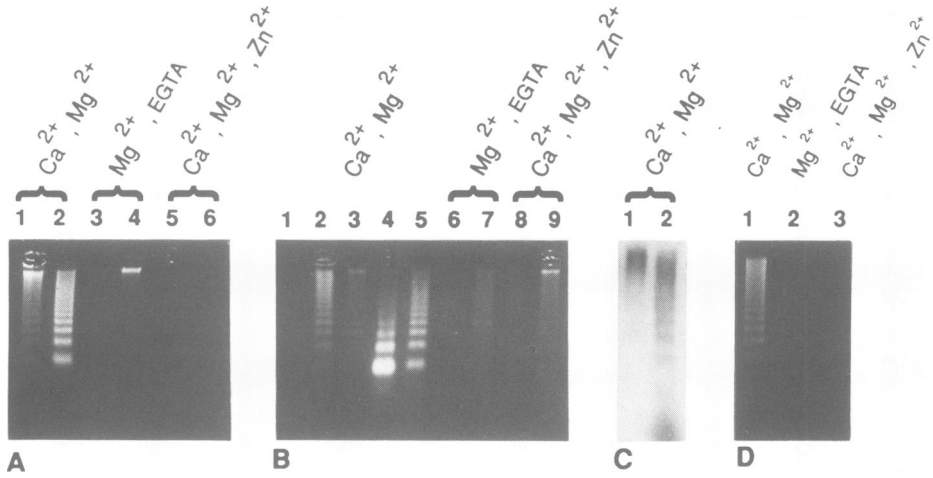


**Fig. 1.** (**A**) Nuclear DNA degradation in thymocyte (lanes 1, 3 and 5) and lymph node (lanes 2, 4 and 6) nuclei by the endogenous $Ca^{2+}$- and $Mg^{2+}$-dependent endonuclease in the presence of both $Ca^{2+}$ and $Mg^{2+}$ (lanes 1 and 2), $Mg^{2+}$ and EGTA (lanes 3 and 4) or $Zn^{2+}$ (lanes 5 and 6). (**B**) Nuclear extracts from thymocytes (lanes 2, 3, 6 and 8) and lymph node cells (lanes 4, 5, 7 and 9) degrade RG-17 nuclear DNA in the presence of both $Ca^{2+}$ and $Mg^{2+}$ (lanes 2–5), but not in the presence of either $Mg^{2+}$ and EGTA (lanes 6 and 7) or $Zn^{2+}$ (lanes 8 and 9); lanes 3 and 5 show the activity of 5-fold diluted extracts and lane 1 is the negative control in the presence of $Ca^{2+}$ and $Mg^{2+}$ and no added exogenous nuclease. (**C**) Thymocyte nuclear extracts produce a radioactive DNA ladder from [$^{125}$I]deoxyuridine labeled RG-17 in the presence of $Ca^{2+}$ and $Mg^{2+}$ (lane 2), whereas their level of endogenous nuclease is very low (lane 1). (**D**) DNA degrading activity of normal rat serum on RG-17 nuclei in the presence of either $Ca^{2+}$ and $Mg^{2+}$ (lanes 1), $Mg^{2+}$ and EGTA (lanes 2) or $Zn^{2+}$ (lanes 3). Since only fragmented DNA is loaded on the gels, the absence of DNA in a lane indicates that no (or very low levels of) DNA fragmentation has occurred (see Materials and methods).

*vivo* following induction of apoptosis. These nuclei therefore contain the endonuclease and represent a convenient cell free system for studying the endonuclease involved in apoptosis (Hewish and Burgoyne, 1973; Vanderbilt *et al.*, 1982).

We confirmed these findings using isolated nuclei of rat thymocytes and lymph node cells. Preliminary experiments showed that the presence of the endogenous endonuclease was independent of the extent of cell death; nuclei isolated from fresh thymocytes contained as much nuclease activity as those obtained from thymocytes treated with dexamethasone for 2 h, although the latter already displayed the typical morphology of apoptotic cells.

As shown in Figure 1A, nuclei exhibited the typical 'ladder' pattern when incubated in the presence of $Ca^{2+}$ and $Mg^{2+}$ for 2 h, whereas the addition of $Zn^{2+}$ or the removal of $Ca^{2+}$ by EGTA inhibited the endonuclease (results are shown only for untreated thymocytes). In our experiments, levels of endogenous endonuclease activity were consistently higher in lymph node nuclei than in thymocyte nuclei (Figure 1A).

In an attempt to characterize this endonuclease further, we took advantage of the very low endogenous $Ca^{2+}$,$Mg^{2+}$-dependent endonuclease activity found in the $CD4^+$ T-cell hybridoma RG-17 (MacDonald *et al.*, 1989) (Figure 1B, lane 1). Since nuclei represent a more physiological substrate than isolated DNA, we designed a transfer test, similar to that described by Compton (1991), in which nuclei from RG-17 cells were used as substrate for various endonuclease preparations. Endonuclease activity was extracted with 0.4 M NaCl from isolated nuclei of thymocytes and lymph node cells. When added to RG-17 nuclei, both extracts were able to cut DNA at internucleosomal sites in a dose-dependent manner, and displayed the same divalent cation dependence as the endogenous nuclease in thymocyte and lymph node cell nuclei. Further increase of the ionic strength during nuclear extraction did not result in any additional nuclease activity. Treatment of nuclei from RG-17 cells, prelabelled with [$^{125}$I]deoxyuridine, with thymocyte nuclear extracts yielded a radiolabelled apoptosis 'ladder', thus precluding the possibility that the DNA contained in the extracts could be responsible for the observed pattern (Figure 1C). Nuclear extracts of rat liver, spleen and pancreas also produced the DNA fragments (data not shown) with levels of endonuclease activity similar to that of the thymus. While testing possible sources of $Ca^{2+}$,$Mg^{2+}$-dependent endonuclease, we also observed that normal rat serum degraded RG-17 chromatin DNA into oligosome-sized fragments (Figure 1D).

The striking similarity between the endonuclease activated during apoptosis, all tested nuclear extracts, as well as normal serum, suggested that the nuclease contained in the serum and nuclear extracts was similar to the one involved in apoptosis. Serum and plasma are known to contain neutral endonuclease activity due to the presence of several isoforms of deoxyribonuclease I (DNase I) (Love and Hewitt, 1979; Chitrabamrung *et al.*, 1981; Miyauchi *et al.*, 1986; Peitsch *et al.*, 1992) secreted by different tissues (Kishi *et al.*, 1990). Divalent cations, particularly $Ca^{2+}$, for which two binding sites have been identified in the DNase I structure (Oefner and Suck, 1986), are essential co-factors for the enzymatic activity of DNase I and for its structural integrity. The presence of $Ca^{2+}$ effectively prevents proteolytic degradation (Price *et al.*, 1969a) and protects the essential disulfide bridge of DNase I from reduction (Price *et al.*, 1969b). Optimal enzymatic activity of DNase I is achieved in the presence of millimolar concentrations of both $Ca^{2+}$ and $Mg^{2+}$. Previous studies demonstrated the presence of rat DNase I in various tissues, including the parotid gland, small intestine, kidney, heart, prostate gland and lymph nodes, but in neither thymus, spleen, pancreas nor liver (Lacks, 1981). Since the zymogram method (see Lacks, 1981 for experimental procedures) used in these studies is at least 10-fold less sensitive than our RG-17 nuclei assay (data not shown), we did not exclude the presence and thus a possible role of DNase I in thymocytes and lymph node nuclear DNA degradation.

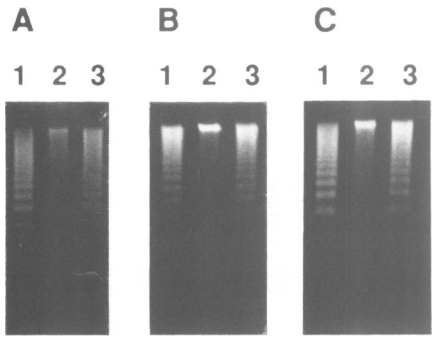


**Fig. 2.** (**A**) Normal rat serum, (**B**) thymocyte nuclear extract and (**C**) lymph node cells nuclear extract were immunoprecipitated with either non-immune rabbit antibodies (lanes 1), rabbit anti-rat parotid DNase I antibodies (lanes 2) or rabbit anti-bovine parotid DNase I antibodies (lanes 3).

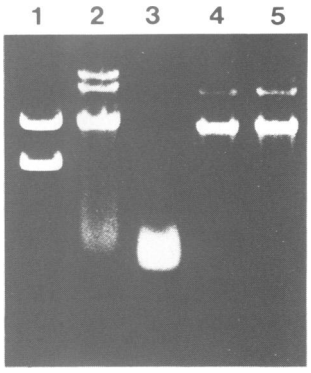


**Fig. 3.** Inhibition of digestion of plasmid DNA by nuclear thymocyte extract in the presence of the actin–gelsolin segment 1 complex. Plasmid DNA was treated with rat thymocyte nuclear extract in the absence (lanes 2 and 3) and in the presence (lanes 4 and 5) of the inhibitory actin–gelsolin segment 1 complex. Mixtures were incubated for 1 (even lane numbers) or 2 h (odd lane numbers) before agarose gel analysis. Lane 1 shows plasmid DNA incubated under identical conditions without added nuclear extract. One microgram was loaded per lane.

### *Immunodepletion of nuclease activity by anti-DNase I antibodies*

Thus, we immunoprecipitated our extracts and normal rat serum with antibodies to rat parotid DNase I raised in rabbits (Kreuder *et al.*, 1984); their specificity was assessed beforehand and no cross-reaction was found with other serum or nuclear proteins. By this treatment the nuclease activity was almost completely removed from both extracts and from normal rat serum (Figure 2). As an additional specificity control, we used normal rabbit IgG, and rabbit IgG against bovine parotid DNase I which does not cross-react with the orthologous rat enzyme (Kreuder *et al.*, 1984) in spite of >70% residue identity (Polzar and Mannherz, 1990). The endonuclease activities of all three samples were not affected by these control treatments (Figure 2).

### *Inhibition of the nucleolytic activity of nuclear extracts by actin*

A highly diagnostic feature of DNase I is the inhibition of its DNA-degrading activity by monomeric (G-)actin (Lazarides and Lindberg, 1974; Mannherz *et al.*, 1975). We therefore assayed the DNA-degrading activity of nuclear extracts of rat thymocytes on plasmid DNA in the presence of actin. In contrast to bovine pancreatic DNase I, the rat DNase I (parotid) was shown to be unable to inhibit rabbit actin polymerization in the presence of high salt and unable to depolymerize F-actin (Kreuder *et al.*, 1984). Thus, actin was added to the assay system as a stoichiometric 1:1 complex with gelsolin segment 1. This complex is resistant to polymerization even in the presence of high salt and able to inhibit the DNA-degrading activity of bovine and rat DNase I (unpublished results). A typical result of four independent inhibition experiments is depicted in Figure 3. A clear inhibition of the degradation of circular plasmid DNA by nuclear extracts is observed in the presence of the actin–gelsolin segment 1 complex, suggesting the presence of DNase I in nuclear extracts. Gelsolin segment 1 alone did not inhibit this nuclease activity (data not shown).

### *Oligonucleosome-sized genome digestion induced by DNase I*

The capacity of DNase I to cut internucleosomal DNA in isolated nuclei was confirmed by analysing the effects of the recombinant protein. COS cells were transfected with a plasmid containing the full-length rat parotid DNase I cDNA (Polzar and Mannherz, 1990) under the control of the early SV40 promoter (Green *et al.*, 1988). Culture supernatant, as well as total extracts, of cells transfected with the insert in the correct orientation contained large amounts of DNase I after 24 h of expression, whereas cells transfected with the insert in the inverse orientation and untransfected cells expressed only limited amounts of nuclease activity (Figure 4). Similarly, nuclei isolated from COS cells that had been transfected with the DNase I insert in the correct orientation, degraded their DNA in the presence of both $Ca^{2+}$ and $Mg^{2+}$. In contrast, nuclei from untransfected cells or cells transfected with the insert in the inverse orientation showed little or no degradation (Figure 4) after 2 h. The addition of $Zn^{2+}$ or the removal of $Ca^{2+}$ inhibited this activity in every case (data not shown).

Since these experiments suggested that the endogenous nuclease was DNase I or DNase I-like, we next tested the digestion pattern generated by purified DNase I on RG-17 nuclei. We realized that most batches of DNase I obtained through commercial sources were not pure and contain multiple contaminating proteins, as assessed by silver staining (data not shown). Thus, highly purified rat DNase I obtained by sequential chromatography from the parotid gland was used (Kreuder *et al.*, 1984). Silver staining of overloaded SDS–polyacrylamide gels revealed the presence of only one band, migrating as a single band with an apparent molecular weight of 34 kDa (Kreuder *et al.*, 1984). Confirming previous observations (Vanderbilt *et al.*, 1982), the added purified DNase I was unable to mimic the apoptosis ladder, only resulting in a fuzzy pattern of digested nuclear DNA (Figure 5). The ladder pattern was, however, restored when the purified DNase I was first incubated with nuclear extracts from RG-17 cells and then added to the nuclei (Figure 5), suggesting that the restricted 'specificity' may be conferred on DNase I by (a) factor(s) present in nuclear extracts. A similar modification of the DNase I cleavage pattern has already been observed when purified DNase I was added to normal mouse serum (Peitsch *et al.*, 1992) instead of nuclear extracts. The nature of this factor is as yet unknown and is currently under investigation in our laboratories.

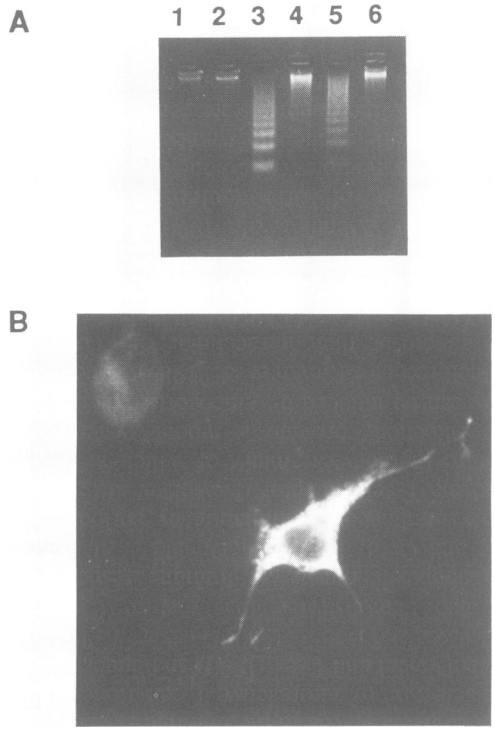


**Fig. 4**. Transfection of COS cells with the cDNA of DNase I. (**A**) Nuclease activity in the supernatants of untransfected (lane 1), transfected with DNase I in the inverse orientation (lane 2) or with DNase I in the correct orientation (lane 3) tested on nuclei of RG-17 cells. DNA degradation in nuclei isolated from untransfected COS cells (lane 4), and COS cells transfected with the DNase I insert in the correct (lane 5) or inverse (lane 6) orientation. (**B**) Immunofluorescence staining of COS cells transfected with the DNase I insert in the correct orientation. Nontransfected or weakly expressing cells are visible around the strongly positive cell in the centre of the micrograph.

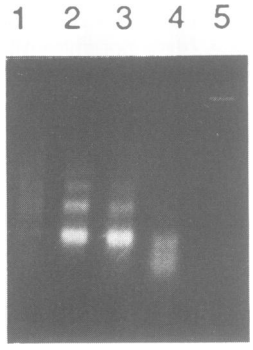


**Fig. 5**. Nuclear DNA degradation by purified rat parotid DNase I. 10 μl RG-17 nuclear extracts were incubated in the presence of 1, 2 and 5 μl rat parotid DNase I [1 μg/ml; purified according to Kreuder *et al.* (1984)] in the presence of 5 mM $Ca^{2+}$ and 5 mM $Mg^{2+}$ for 12 h at 37°C. RG-17 nuclei were then incubated in the presence of 1.0 μl rat parotid DNase I (lane 1), 2 μl of each of the above described mixtures (lanes 2–4) and RG-17 nuclear extract without DNase I (lane 5) as described in Materials and methods. All incubations were performed in the presence of 5 mM of both $Ca^{2+}$ and $Mg^{2+}$.

### *Expression of DNase I*

In order to distinguish between DNase I and a putative DNase I-like enzyme, we looked for the gene expression of genuine DNase I in cells that are known to digest their nuclear DNA upon triggering of apoptotic cell death. The

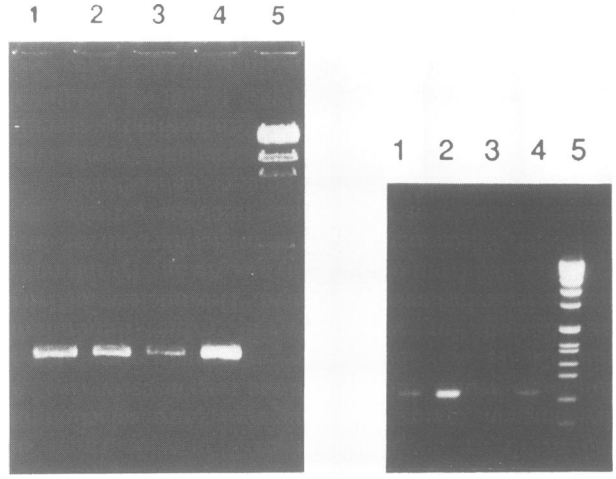


**Fig. 6**. PCR amplification of DNase I. (**A**) Rat cDNAs of thymus (lane 1), kidney (lane 2) and parotid (lane 3) cDNAs were subjected to PCR amplification using oligonucleotide primers derived from the known sequence for rat parotid DNase I as described in Materials and methods. Lanes 4 and 5 represent respectively a control amplification with the same primers on the cloned rat parotid DNase I cDNA and molecular weight markers (*Hin*dIII-digested lambda DNA). (**B**) Mouse cDNAs of thymus (lane 1), small resting lymphocytes (lane 3) and concanavalin-A blasts (lane 4) were amplified by PCR as described in Materials and methods. Lanes 2 and 5 represent respectively a control amplification with the same primers on the cloned rat parotid DNase I cDNA and molecular weight markers.

presence of DNase I mRNA in rat thymus and lymph node cells was analysed by PCR-mediated amplification of reversed transcribed mRNA, using specific primers corresponding to the $NH_2$-terminus (residues 1–7) and to the C-terminus (residues 252–260) of the mature protein. These two regions were chosen for their high degree of conservation among various species (rat, human, bovine and sheep; only two amino acid differences out of the 16 residues between rat and human). Two known DNase I-producing organs of the rat, the parotid gland and the kidney (Lacks, 1981), yielded an amplification product corresponding in size to the theoretical value calculated from the known nucleotide sequence (Polzar and Mannherz, 1990) (Figure 6A). Furthermore, the rat thymus displayed a clear amplification product of 700 bp which was also identical in size to the control product amplified from 10 ng of the cloned rat parotid DNase I cDNA (Figure 6A). Sequencing of the rat thymus amplification product revealed no difference from the published sequence of the rat parotid DNase I (Polzar and Mannherz, 1990). Since the presence of DNase I in thymocytes has not been described previously, we wanted to confirm its expression in the mouse organ. Using the same PCR primers, we achieved the amplification of the cDNA for DNase I from mouse thymus despite the species difference (Figure 6B). In addition, purified lymph node-derived small resting lymphocytes and concanavalin-A blasts derived therefrom were assayed, since for these lymphoid cells it has been reported that apoptotic cell death, including degradation of the genome, occurs *in vivo* and *in vitro* under various conditions (Newell *et al.*, 1990; Kawabe and Ochi, 1991; MacDonald *et al.*, 1991). Again, the DNase I PCR amplification product was generated, indicating that lymphocytes express DNase I message independently of their state of activation (Figure 6B). Using immunohistochemical staining methods with antibodies against rat DNase I, we

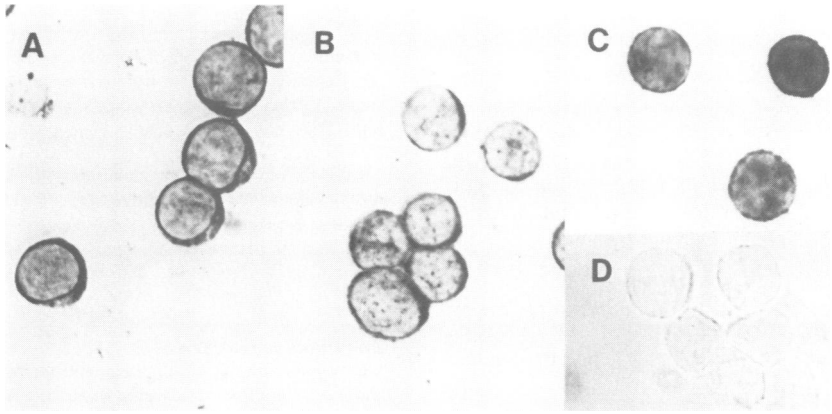


**Fig. 7.** Expression of DNase I in thymocytes and lymph node cells. The DNase I protein was detected with rabbit antibodies to rat parotid DNase I in rat lymph node cells (**A**) and thymocytes (**B**). As controls, rat lymph node cells stained with rabbit antibodies to rat Thy-1 (**C**) or no first antibody (**D**) are shown.

also detected the DNase I protein in rat lymph node cells (Figure 7A) and thymocytes (Figure 7B). The staining was specific and only low background was observed when the specific antibody was omitted (Figure 7D). When compared with the surface distribution of Thy-1 (Figure 7C), the grainy pattern of the signal found in untreated cells suggests that DNase I is located in the cytoplasm. Considering the presence of a signal peptide in the DNase I message (Polzar and Mannherz, 1990), it is most likely confined to an organelle involved in protein secretion.

## Discussion

An apoptosis-specific endonuclease displaying $Ca^{2+}$,$Mg^{2+}$-dependence has been repeatedly postulated. In an attempt to characterize this endonuclease at a molecular level, we used purified nuclei from thymocytes which are uniquely sensitive to the apoptotic cell death upon dexamethasone treatment. Our report now shows that the $Ca^{2+}$,$Mg^{2+}$-dependent endonuclease activity extractable from both thymocyte and lymph node cell nuclei is due to a nuclease which is functionally and antigenically identical to DNase I. The findings presented here are in harmony with a recent hypothesis of Ucker and colleagues (Ucker *et al.*, 1992), who described a DNase I-like activity in fibroblasts which was inhibitable by G-actin.

Our conclusion is based on the following observations. First, highly specific antibodies to rat DNase I deplete the nuclear extracts of the nuclease activity. In contrast, antibodies against bovine DNase I that do not cross-react with the mouse protein are ineffective, making it rather unlikely that the antiserum reacts nonspecifically with a different nuclear endonuclease or proteins associated with it. Second, the DNA-degrading activity of the thymocyte nuclear extract was found to be inhibited by the stoichiometric complex of actin and gelsolin segment 1. DNase I is so far the only endonuclease whose activity is known to be inhibited by G-actin. Third, the sizes of the DNA fragments generated by recombinant DNase I expressed in COS cells and nuclear extracts are indistinguishable, and have the same metal ion requirements. Moreover, isolated nuclei of COS cells having little endogenous endonuclease acquire the capability to degrade their DNA into multiples of 200 bp fragments upon transfection of the cells with the DNase I cDNA. Fourth, purified rat DNase I preincubated with nuclear extracts or serum (Peitsch *et al.*, 1992) generates DNA fragments mimicking the situation *in vivo*. In agreement with a report showing that DNase I occurs in a complexed and latent form in the cytosol of leukaemia cells (Blaszkiewucz-Malicka and Roth, 1983), these observations suggest that another factor(s) may combine with DNase I in serum and intracellularly, thereby modulating its activity allowing the 'apoptosis ladder' to be produced under physiological conditions. Finally, all cells and organs that we have tested so far for their capacity to degrade their nuclear DNA, express DNase I message and/or protein. Indeed, besides the known localization of DNase I in digestive tissues and other organs (Lacks, 1981), we show that thymocytes and lymph node cells, both known to be susceptible to apoptotic cell death, contain substantial amounts of DNase I as detected by immunohistochemistry and the more sensitive PCR. Although the present study was limited to a small selection of cells and tissues, it further underscores the broad distribution of DNase I also in organs with no digestive function.

These findings raise the question as to the cellular localization of DNase I and its possible route of access to the nuclear DNA. It has been proposed that the $Ca^{2+}$- and $Mg^{2+}$-dependent endonuclease involved in apoptosis is located in the nucleus (Bowen and Bowen, 1990), since isolated nuclei harbour the activity. However, this contention would be incompatible with the proposed role of DNase I, since it has been predicted from its cDNA sequence that this endonuclease has a signal peptide (Polzar and Mannherz, 1990). Thus, DNase I should be translocated into the lumen of the rough endoplasmic reticulum (ER) during biosynthesis before its secretion, a sorting route which was confirmed by our COS transfection experiments in which the active molecule was harvested in the supernatant. However, the ER is continuous with the nuclear envelope, with only the inner nuclear membrane separating the nucleus from the ER content. One means of initiating apoptosis is the sustained increase in intracellular $Ca^{2+}$ levels (McConkey *et al.*, 1989) (a prerequisite for DNase I activity) which is known to have a profound effect on ER morphology, causing it to disperse and dissociate (Booth and Koch, 1989). In addition, lamin phosphorylation and solubilization, and breakdown of the nuclear envelope are early events in cell death (Ucker *et al.*, 1992). We propose that these processes would allow the rapid access of ER enzymes to the cytoplasm and the

nucleus, resulting in the apparent 'activation' of DNase I, and ultimately in the autolytic destruction of the cell and its DNA. Such a loss of enzyme compartmentalization has already been proposed to explain the apoptotic DNA degradation observed in antigen presenting cells induced by $CD4^+$ T cells in the presence of protein and RNA synthesis inhibitors (Grogg *et al.*, 1992). Providing further support for this hypothesis are histochemical studies that revealed the appearance of ER-derived acid phosphatases in the cytoplasm (Jones and Bowen, 1979; Bowen and Bowen, 1990), consistent with the progressive disintegration and disruption of this organelle during apoptosis. In this way, the released DNase I (mol. wt 34 kDa) may reach the nucleus either by passive diffusion through the nuclear pores (Paine *et al.*, 1975) or directly via the impaired nuclear membrane. Similarly, during isolation of nuclei *in vitro*, the content of the ER is released and the nuclear envelope is damaged; this could explain the observation that nuclease activity is associated with isolated nuclei.

DNA fragmentation, however, does not represent a constant feature of apoptosis, and thus is not an absolute requirement for cell death (Lockshin and Zakeri, 1991); transformed fibroblasts lacking any DNA nicking activity were found to be susceptible to cell death mediated by cytotoxic T-lymphocytes (Ucker *et al.*, 1992). DNA degradation may thus be a physiological epiphenomenon which would serve rather to limit potentially dangerous genetic information spreading from the dying cells.

Although the molecular details of the actin–DNase I interaction have recently been elucidated for bovine pancreatic DNase I (Kabsch *et al.*, 1990), its role *in vivo* is still enigmatic. In view of our results, these interactions might be of physiological relevance during mitosis and apoptosis. Whereas the free DNase I is kept inactive during the nuclear membrane breakdown in the normal cell cycle by G-actin and inclusion into microsomal vesicles, this control may be impaired during cell death. Besides its nucleolytic activity, liberated DNase I may also influence the supramolecular organization of the microfilament system. DNase I may thus participate in the morphological changes, such as membrane blebbing, observed during apoptosis.

## Materials and methods

### *Cells and nuclei*

Thymi and lymph nodes of 4–5 week old Lewis rats were freshly collected. RG-17 cells (MacDonald *et al.*, 1989) were harvested from exponentially growing cultures in DMEM medium containing 5% fetal calf serum. Nuclei were isolated from single cell suspensions of thymocytes or lymph node cells with 0.05% NP-40 according to Hewish and Burgoyne (1973). Nuclei of RG-17 cells and untreated COS cells were prepared according to the same procedure except that 0.3% NP-40 was used.

Small resting lymphocytes, obtained from freshly harvested murine lymph nodes, and concanavalin-A blasts (2 days, 5 μg/ml Con-A) were separated by Percoll gradient centrifugation.

Labelling of RG-17 cells was performed with 4 μCi [$^{125}$I]deoxyuridine (Amersham) for 5 h prior to cell harvest and isolation of nuclei.

Nuclear extracts were obtained by disrupting $5\times10^8$ nuclei in 1 ml of 20 mM Tris–acetate buffer (pH 7.5) containing 0.4 M NaCl on a vortex mixer; after a 30 min incubation on ice the extracts were centrifuged for 30 min at 205 000 *g* at 4°C.

### *DNA degradation in isolated nuclei*

All incubations were carried out at 37°C for 2 h with $2\times10^5$ nuclei in 25 μl of 15 mM HEPES buffer (pH 7.5) containing 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol and 10% sucrose in the presence of divalent cations at the following concentrations: either 5 mM of both $Ca^{2+}$ and $Mg^{2+}$ or 10 mM $Mg^{2+}$ in presence of 2.5 mM EGTA or 5 mM of each $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$. In assays involving nuclei of RG-17 the endonuclease sources were added as follows: (i) 1 μl of either normal rat serum or nuclear extracts, (ii) 0.5 μl of culture supernatants from transfected or diethylaminoethyl dextran-treated COS cells. Reactions were stopped with 10 μl ice cold 50 mM EDTA and DNA was extracted with 50 μl phenol/chloroform/isoamyl alcohol (50:49:1). In order to see only fragmented DNA on the agarose gels, we omitted the proteinase K digestion step used by other investigators (Compton, 1991). Samples were centrifuged at 12 000 *g* for 10 min and the aqueous phase was loaded onto a 1.8% agarose gel equilibrated in 40 mM Tris–acetate pH 8.0 containing 1 mM EDTA. After 1–2 h migration at 3 V/cm, gels were stained with ethidium bromide and photographs were taken on a UV transilluminator.

### *Immunoprecipitations*

The rabbit antisera (non-immune, anti-rat parotid DNase I and anti-bovine parotid DNase I) were adsorbed on protein A–Sepharose (Pharmacia) and washed in 10 mM Tris–HCl (pH 7.5) containing 150 mM NaCl. 20 μl of either normal rat serum preadsorbed with protein A–Sepharose, or nuclear extracts were added to 20 μl packed Sepharose containing the rabbit immunoglobulins prepared as described above. After 60 min incubation at room temperature with gentle shaking, the Sepharose was centrifuged and the supernatants tested on RG-17 nuclei for their DNA-degrading activity.

### *Inhibition of plasmid DNA digestion*

Rat thymocyte nuclear extract (2 μl of 50 μg/ml) was preincubated in either the absence or presence of 2 μg of the stoichiometric 1:1 complex of actin and gelsolin segment 1 in 20 μl (final volume) 5 mM HEPES–OH pH 7.4, 0.1 mM $CaCl_2$ and 0.2 mM $NaN_3$ for 1 h at 32°C. To initiate the reaction, 1 μg of plasmid DNA (BlueScript vector) was added and the $CaCl_2$ and $MgCl_2$ concentrations were adjusted to 0.2 mM and 1.0 mM respectively. The reactions were stopped after 1 and 2 h by addition of 2 μl 0.1 mM EDTA and analysed by electrophoresis on a 1% agarose gel.

Actin was prepared as reported in Kreuder *et al.* (1984); the cloned gelsolin segment 1 has been described by Way *et al.* (1989). The actin–gelsolin segment 1 complex was formed by mixing both proteins at an equimolar ratio.

### *Histochemistry*

For immunoperoxidase stainings, freshly isolated thymocytes and lymph node cells were cytocentrifuged at 100 *g* for 10 min, allowed to dry, and kept at room temperature overnight. A three layer biotin–avidin–peroxidase technique was used (Guesdon *et al.*, 1979) in combination with rabbit antibodies to rat parotid DNase I (Kreuder *et al.*, 1984) and to rat Thy-1. The peroxidase activity was revealed with 3-amino-9-ethylcarbazol (Sambrook *et al.*, 1989)

Immunofluorescence staining was performed on COS cells grown and transfected on cover slips. The cells were fixed for 10 min in acetone at −15°C, washed in cold phosphate-buffered saline (PBS) and then incubated for 60 min with affinity purified rabbit antibodies to rat parotid DNase I (Guesdon *et al.*, 1979). After three washes with PBS a second incubation of 15 min was performed with a fluorescein-conjugated goat anti-rabbit IgG prior to fluoromicrography.

### *COS cell transfections*

COS cells were transfected with the pSG5 plasmid (Green *et al.*, 1988) containing the rat parotid DNase I insert (Polzar and Mannherz, 1990) using the diethylaminoethyl dextran method (Sambrook *et al.*, 1989). The DNase I insert was excised with *Eco*RI from the cloning vector (Polzar and Mannherz, 1990), purified on an agarose gel and ligated into the *Eco*RI site of pSG5. Untransfected cells were also treated with diethylaminoethyl dextran.

### *Polymerase chain reaction*

cDNAs of rat parotid gland, kidney and thymus, and of mouse thymus and lymph node-derived small resting lymphocytes and concanavalin-A blasts, obtained using kits available from Invitrogen (San Diego), were subjected to PCR using primers derived from the known rat parotid DNase I cDNA sequence (Polzar and Mannherz, 1990). The primers correspond to the beginning and the end of the mature protein: (i) 5′-ccgaattccgaccatg-CTGAGAATTGCAGCCTTCAACA (corresponding to the amino acid sequence $NH_3$-LRIAAFN) and (ii) 5′-cggaattccgtcaTCTGAGTGTCA-CCTCCACTGGGTAATG (HYPVEVTLR-260). One microlitre of each cDNA (10 ng) was used for 30 cycles of amplification with *Taq* polymerase (Perkin-Elmer) in the presence of 10 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 50 mM KCl and 0.2% gelatin. Annealing was allowed at 56°C (50°C for mouse cDNAs) for 1 min, elongation was performed at 72°C for 1 min and denaturation at 94°C for 1 min. 5 μl of the reaction mixture were loaded

on 1.2% agarose gels in TAE buffer. After 1–2 h migration at 3 V/cm, gels were stained with ethidium bromide and photographs were taken on a UV transilluminator.

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