

# Direct excision of 50 kb pair DNA fragments from megabase-sized fragments produced during apoptotic cleavage of genomic DNA

Piero Sestili, Flaminio Cattabeni, Orazio Cantoni\*

*Istituto di Farmacologia e Farmacognosia and Centro di Farmacologia Oncologica Sperimentale, Università di Urbino, Via S. Chiara 27, 61029 Urbino, Italy*

Received 16 September 1996

**Abstract** The DNA of U937 cells exposed to two different apoptotic stimuli, namely the cocktail  $H_2O_2$ /3-aminobenzamide (3AB) and etoposide, was analyzed using field inversion gel electrophoresis (FIGE) as well as programmable, autonomously controlled electrode electrophoresis (PACE). The results obtained indicate that FIGE is not appropriate for sizing apoptotic DNA fragments. PACE appears to be more accurate and reliable and the results obtained with this technique strongly suggest that the 50 kb DNA fragments are directly excised from Mb-sized DNA fragments without the intermediate cleavage of 200–300 kb products.

**Key words:** Apoptosis; Necrosis; High molecular weight DNA fragmentation; Pulsed field gel electrophoresis

## 1. Introduction

Apoptosis is a controlled form of cell death actively involved in the regulation of physiological processes, toxicity and disease [1,2]. At the biochemical level, the apoptotic process is characterized by a number of different events including an intense nucleolytic activity [1,2] which generally leads to the formation of oligonucleosomal DNA fragments [3,4]. For this reason, the appearance of a DNA ladder on conventional agarose gels has long been considered a biochemical hallmark of apoptosis, although it is important to point out that apoptosis can also occur in the absence of this characteristic event [5,6]. Internucleosomal fragmentation of genomic DNA in apoptotic cells is temporally and hierarchically preceded by the so-called high molecular weight (HMW) or 'large-scale' DNA cleavage [7–15]. This event leads to the formation of kb-paired DNA fragments which can only be detected by non-conventional gel electrophoresis, namely pulsed field gel electrophoresis (PFGE). These DNA fragments are estimated to range in size from 10 to 700 kb and, in particular, different investigators have identified discrete bands at 700, 580, 300, 200–250, 50 and 50–10 kb [8–14]. The formation of DNA fragments of 200–300 and  $\leq 50$  kb has been consistently reported in a number of different studies and is thus considered to be key evidence of apoptosis [2,8–14]. This observation also has an important mechanistic implication since it can be interpreted [2,10] as the consequence of the ordinate and sequential digestion of interphase chromosomes into rosettes (300 kb pairs) and loops (50 kb pairs).

It is important to point out, however, that no conclusive information has been presented in support of the notion that rosettes are directly excised during apoptosis and, to the best of our knowledge, only one report has provided convincing experimental evidence that the 50 kb fragments are in fact chromosomal loops [15]. Indeed, although the available technology allows an accurate determination of the size of DNA fragments in the kb up to the Mb size range [16–22], most of the studies reported above used FIGE [7–12,14], a strategy which has proved to be only partially adequate for sizing purposes because of its low resolving capacity [17,23]. As a consequence, it is still unclear whether the wide distribution of HMW DNA fragments reported in different cell lines in response to challenge with different stimuli reflects the heterogeneity of the apoptotic response or is more simply dependent on intrinsic limitations of the FIGE technology.

In this study we compared the pattern of apoptotic HMW DNA cleavage induced by two different apoptotic stimuli using FIGE and the powerful and flexible PACE electrophoresis [16–19] coupled with photostimulatable storage phosphor imaging [24]. Our results strongly suggest that, at least in the system utilized in the present study, apoptotic cell death is characterized by an early formation of Mb-sized DNA fragments followed by the direct excision of 50 kb fragments.

## 2. Materials and methods

### 2.1. Chemicals

Reagent grade chemicals and PFGE grade agarose were obtained from Sigma (St. Louis, MI, USA). *S. pombe*, *S. cerevisiae* and  $\lambda$  ladder pulse markers, chromosomal grade agarose, low melting agarose, TE and TBE buffers were obtained from BioRad (Richmond, CA, USA). Proteinase K was from Merck (Darmstadt, Germany). Cell culture media and reagents were obtained from Sera-Lab Ltd. (Crawley Down, UK). [methyl- $^{14}C$ ]thymidine was from Du Pont de Nemours (Bad Homburg, Germany).

### 2.2. Cell culture and treatments

U937 cells were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50  $\mu$ g/ml), at 37°C in T-75 tissue culture flasks gassed with an atmosphere of 95% air–5%  $CO_2$ . For experiments, cells were diluted in fresh complete medium to a final density of  $2.5 \times 10^5$ /ml.

### 2.3. Preparation of plugs for PFGE

Cells pre-labelled with [methyl- $^{14}C$ ]thymidine (0.05  $\mu$ Ci/ml) were embedded into agarose plugs as previously described by Sestili et al. [20], except that the final cell density in the plugs was adjusted to  $5.0 \times 10^6$ /ml.

### 2.4. DNA sizing by PACE electrophoresis

PACE electrophoresis was carried out using a BioRad DRIII variable angle system. The PACE assay used in the present study was a modification of the contour-clamped, homogeneous field electrophoretic assay that had been previously reported by Sestili et al. [20]. The present modification was developed in order to obtain the

\*Corresponding author. Fax: (39) (722) 327670.

**Abbreviations:** FIGE, field inversion gel electrophoresis; PACE, programmable, autonomously controlled electrode electrophoresis; HMW, high molecular weight; 3AB, 3-aminobenzamide; PFGE, pulsed field gel electrophoresis

simultaneous separation of three different sets of DNA markers (*S. pombe* and *S. cerevisiae* chromosomes, and  $\lambda$  ladder) ranging from 5.7 to <0.05 Mb in 42 h (Fig. 2B).

Briefly, these gels were cast using 1.0% w/v chromosomal grade agarose in 0.5×TBE buffer (composition of the 0.5×concentrated buffer: 44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM Na<sub>2</sub>EDTA [pH 8.3]) and run with a three block assay. During the first block, the switch time was 75 min for 5 h at 14°C, with a field strength of 1.2 V/cm and a reorientation angle of 106°. In the second block the temperature was decreased to 9°C, the switch time was 30 min for 30 h and the reorientation angle was 106° using a field strength of 2.2 V/cm. In the third block the switch time was linearly ramped from 10 to 90 s for 7 h and the field strength increased to 6 V/cm. The reorientation angle was changed to 120°.

#### 2.5. DNA sizing by FIGE electrophoresis

FIGE electrophoresis was carried out using a BioRad Pulsewave 760 Switcher connected to a BioRad 200/2.0 power supply. The gels were cast using 1.5% w/v PFGE grade agarose in 0.5×TBE. The switch time was linearly ramped from 1 to 15 s with a forward/reverse ratio of 3/1 over 6–8 h of run time. The current was kept constant at 50 mA and the temperature of the running buffer (0.5×TBE) was maintained at 8°C.

#### 2.6. Photostimulatable storage phosphor imaging

Phosphor imaging of desiccated gels was carried out according to the procedure described by Story et al. [24]. A BioRad GS 250 Phosphor Imager was used to pulse-scan (200  $\mu$ m resolution) and analyze the photostimulated screen. Data obtained were then elaborated and plotted using the resident BioRad Molecular Analyst software.

#### 2.7. Cytotoxicity assay

Cytotoxicity was determined using the trypan blue exclusion assay.

#### 2.8. DNA fragmentation analysis by the filter-binding assay

Secondary DNA fragmentation was quantified using the filter binding assay developed by Bertrand et al. [25] with minor modifications. Briefly, cells were labelled overnight with [*methyl*-<sup>14</sup>C]thymidine (0.05  $\mu$ Ci/ml) and incubated for a further 6 h in a medium containing unlabelled thymidine (1  $\mu$ g/ml). After treatment, cells were sedimented at 1000 rpm for 5 min at 4°C and the pellet was then resuspended in saline A containing 5 mM EDTA, pH 8.3. Cells ( $5 \times 10^6$ ) were loaded onto 2  $\mu$ m pore-size polyvinylchloride filters, washed with 10 ml of saline A containing 5 mM EDTA and lysed with a solution (5 ml) containing 0.2% sarkosyl-2 M NaCl-0.04 M EDTA (pH 10.1). Lysates were rinsed with 7 ml of 0.02 M EDTA (pH 10.1). The filters were then removed and the filter holders were washed twice with 3 ml of 0.4 N NaOH. Radioactivity was counted in the lysates, EDTA wash, filter and filter holder washes. DNA fragmentation was determined as the percentage of the <sup>14</sup>C-labelled DNA in the lysate plus the EDTA wash relative to total <sup>14</sup>C-labelled DNA.

### 3. Results

In these experiments, the cells were treated for 3, 4.5 or 6 h

with 1 mM H<sub>2</sub>O<sub>2</sub> in the absence or presence of a 1 mM concentration of the poly(ADP-ribose)polymerase inhibitor 3AB, conditions under which the oxidant induces necrosis or apoptosis, respectively [26].

Indeed, a large proportion of the U937 cells treated with H<sub>2</sub>O<sub>2</sub>/3AB displayed the typical morphological signs of apoptosis (i.e. extensive blebbing, not shown) and, while retaining the ability to exclude trypan blue (Table 1), also showed a time-dependent increase in DNA fragmentation (Table 1), resulting in a DNA ladder when analyzed by conventional gel electrophoresis (not shown).

Fig. 1 shows the photographs of ethidium bromide-stained gels obtained by running DNA samples from the same experiments with two different PFGEs (panel A FIGE gel; panel B PACE gel). FIGE analysis revealed that a large proportion of the DNA of U937 cells exposed to H<sub>2</sub>O<sub>2</sub>/3AB migrated out of the well, accumulating over two distinct bands at  $\leq 50$  kb and 200–300 kb (Fig. 1A, lanes 3, 5 and 7). The amount of DNA electrophoresed out of the well increased as a function of the treatment time and progressively accumulated in the 50 kb region while decreasing elsewhere. This information can be better appreciated by the size distribution profiles reported in panels 3A, 5A and 7A in which the DNA electrophoresed was sized with a photostimulatable storage phosphor imaging system.

The results obtained by analysing the same agarose blocks with the high-resolution three-block PACE technology are remarkably different since no distinct band at 200–300 kb was detected (Fig. 1B). A considerable amount of DNA, however, was found to accumulate in the very high molecular weight region (>2.2 Mb) after only 3 h of treatment with H<sub>2</sub>O<sub>2</sub>/3AB (Fig. 1B, lane 3 and panel 3B), a time at which apoptosis was not yet morphologically detectable (not shown). After 4.5 and 6 h of treatment, most of the DNA was found at  $\leq 50$  kb (Fig. 1B, lanes 5 and 7 and panels 5B and 7B).

The cells receiving only H<sub>2</sub>O<sub>2</sub> lost their viability in a time-dependent fashion and after 6 h less than 50% of these cells were still capable of excluding trypan blue (Table 1). Furthermore, the characteristic morphological (not shown) and biochemical (Table 1) features of these cells were consistent with the notion that, under these experimental conditions, H<sub>2</sub>O<sub>2</sub> induces necrosis. FIGE analysis of the DNA from these cells revealed the formation of a band at 200–300 kb (Fig. 1A, lanes 2, 4 and 6) similar to that detected in apoptotic cells (Fig. 1A, lanes 2 and 5). In marked contrast, PACE analysis

Table 1  
Cytotoxicity and secondary DNA fragmentation induced by H<sub>2</sub>O<sub>2</sub> ± 3AB or etoposide in U937 cells

Treatment	Exposure time (h)	Dead cells (%) <sup>a</sup>	DNA fragmentation (%) <sup>b</sup>
Control	6	2.13 ± 0.18	2.38
H <sub>2</sub> O <sub>2</sub> (1 mM)	3	22.4 ± 1.4	2.84
	4.5	42.6 ± 3.2	2.51
	6	54.7 ± 4.7	3.16
H <sub>2</sub> O <sub>2</sub> (1 mM)+3AB (1 mM)	3	5.2 ± 0.15	13.64
	4.5	8.3 ± 0.67	19.61
	6	9.6 ± 0.89	35.28
Etoposide 20 $\mu$ M	1+5 h in fresh medium	4.56 ± 0.34	7.5
Etoposide 40 $\mu$ M	1+5 h in fresh medium	4.73 ± 0.42	14.8
Etoposide 60 $\mu$ M	1+5 h in fresh medium	6.06 ± 0.63	23.4

<sup>a</sup>Cytotoxicity was measured using the trypan blue exclusion assay. Data are expressed as the percent ratio of trypan blue-stained vs. total number of stained and unstained cells (means ± S.E.M. from at least three separate experiments, each performed in duplicate).

<sup>b</sup>DNA fragmentation was measured using the filter binding assay. Data are expressed as the percent ratio of the DNA eluted vs. total DNA of the sample. Results are from a representative experiment.

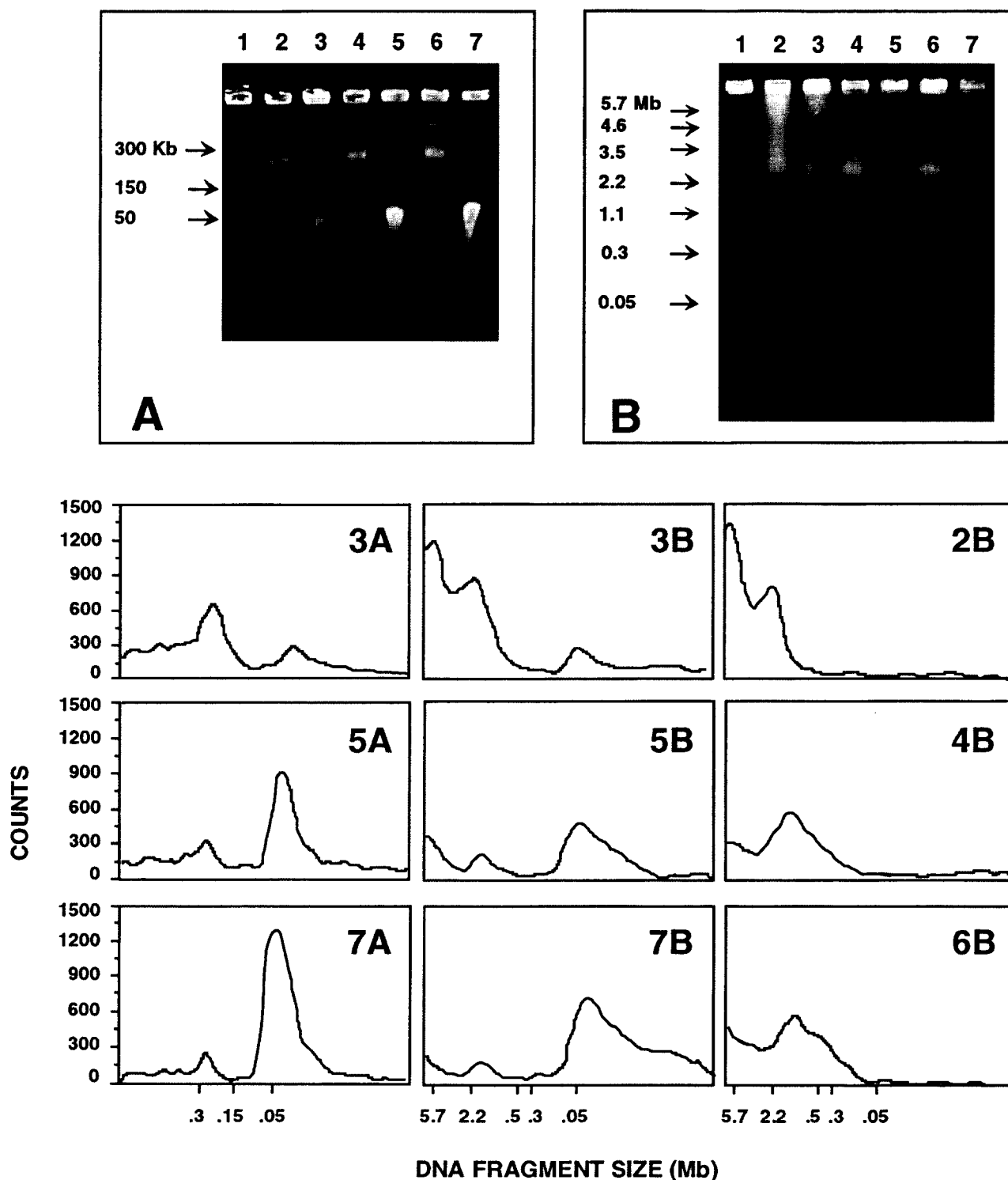


Fig. 1. PACE and FIGE analysis of the DNA from U937 cells treated for increasing lengths of time with 1 mM hydrogen peroxide in the absence or presence of 1 mM 3-aminobenzamide. Plugs from the same treatments were run according to both the FIGE (A) and PACE (B) assays. Treatments were: 1 mM  $H_2O_2$  for 3 (lane 2), 4.5 (lane 4) or 6 h (lane 6); 1 mM  $H_2O_2$  in the presence of 1 mM 3AB for 3 (lane 3), 4.5 (lane 5) or 6 h (lane 7). Lane 1 represents undamaged DNA from control U937 cells. The size distribution profiles obtained by digital phosphor imaging of the electrophoresed samples are labelled with the same lettering used in the lanes. Gels were scanned at 200  $\mu m$  resolution.

of the same samples clearly demonstrated a smeared distribution of the DNA from > 3 Mb to 300 kb fragments (Fig. 1B, lanes 2, 4 and 6).

The outcome of the experiments performed with etoposide,

which had previously been shown to induce apoptosis in U937 cells [14], was basically identical to that obtained with  $H_2O_2$ /3AB. Table 1 shows that treatment for 6 h with 20–60  $\mu M$  etoposide does not affect the viability of U937 cells. Further

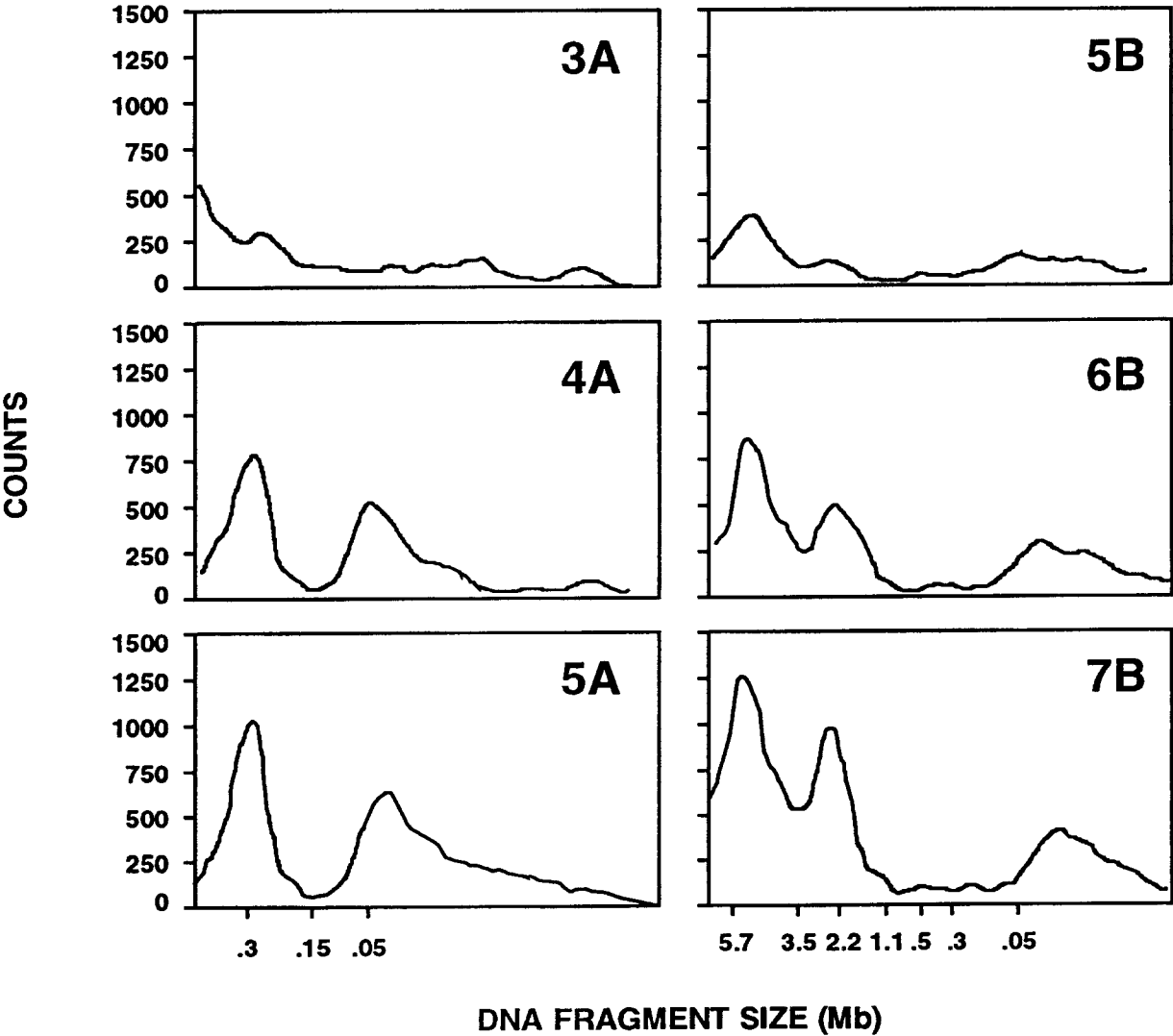
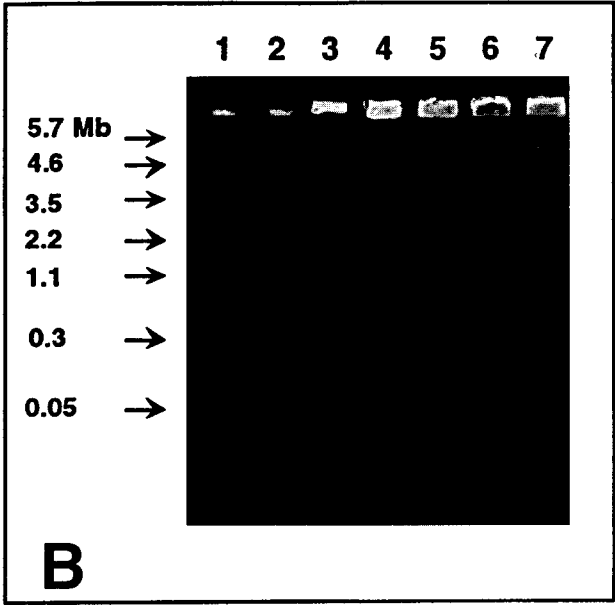
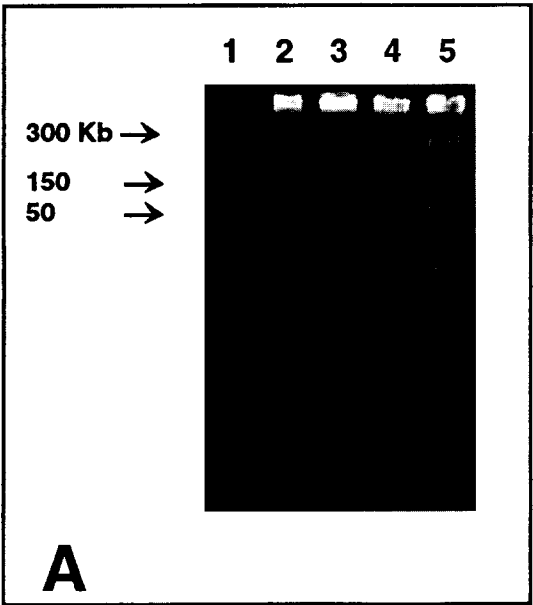


Fig. 2. PACE and FIGE analysis of the DNA from U937 cells treated with increasing concentrations of etoposide. PFGE analysis of high order DNA fragmentation in U937 cells treated with increasing concentrations of etoposide for 1 h and then postincubated for a further 5 h in complete, drug-free medium. Plugs from the same treatments were run according to both FIGE (A) and PACE (B) assays. Treatments were: 20 (lane 3, panel A and line 5, panel B), 40 (lane 4, panel A and lane 6, panel B) or 60  $\mu$ M etoposide (lane 5, panel A and lane 7, panel B). Lane 2 in panel A and lane 4 in panel B represent undamaged DNA from control U937 cells. Lane 1 (panels A and B) shows the mobility of the DNA MW standard  $\lambda$  ladder. Lanes 2 and 3 (panel B) show the mobilities of the *S. cerevisiae* and *S. pombe* chromosomes, respectively. The size distribution profiles obtained by digital phosphor imaging of the electrophoresed samples are labelled with the same lettering used in the lanes. Gels were scanned at 200  $\mu$ m resolution.

morphological and biochemical characterization demonstrated that a proportion of these cells was apoptotic in nature. Indeed, treatment with etoposide was found to promote the formation of blebs and DNA fragmentation that was determined by both conventional gel electrophoresis (DNA ladder, not shown) or by the filter binding assay (Table 1). Once again, FIGE sizing showed the existence of a dose-related accumulation of DNA fragments over two bands in the  $\leq 50$  and 200–300 kb regions (Fig. 2A, lanes 3, 4 and 5; panels 3A, 4A and 5A). On the contrary, PACE sizing of the same samples (Fig. 2B, lanes 5, 6 and 7; panels 5B, 6B and 7B), while confirming the existence of the  $\leq 50$  kb centered band, did not detect any discrete band at 200–300 kb (note that, as illustrated in panels 6B and 7B, the radioactivity associated with the region between 1.1 Mb and 100 kb is hardly over the baseline). In addition, PACE-electrophoresed samples revealed that large amounts of Mb-sized DNA fragments accumulate, in a dose-related fashion, at  $\geq 5$  Mb and 3.5–2.2 Mb.

Thus, while FIGE mapping of apoptotic HMW DNA fragments generated by two different apoptotic stimuli would indicate the existence of two bands at 200–300 and  $\leq 50$  kb, PACE mapping of the same DNA samples indicated the presence of the  $\leq 50$  kb band alone. Furthermore, the FIGE assay detected a band at 200–300 kb in necrotic cells as well.

#### 4. Discussion

We separated the HMW DNA cleavage products of U937 cells triggered into apoptosis by  $H_2O_2$ /3AB or etoposide using PACE and FIGE coupled with a photostimulatable storage phosphor imaging system, a highly sensitive method for the direct detection of DNA fragments pre-labeled with weakly emitting isotopes in agarose gels [24].

The conflicting results obtained with these two techniques can only be explained in terms of non-linear migration of the DNA fragments under the lower-resolution electrophoretic condition, namely FIGE. In fact, while PACE has been firmly established as the first choice technique for the accurate sizing of DNA fragments up to the Mb range [16–19], FIGE is known to have some major disadvantages. In particular, as summarized by Elia et al. [17] and Carle [23], FIGE electrophoresis cannot resolve fragments larger than 700 kb, may produce sharp-appearing bands due to a compression effect and is prone to the so-called ‘band inversion’ (or ‘resonance’) effect. The band inversion effect, caused by a higher mobility of large vs shorter DNA fragments, could explain the appearance of bands at 200–300 kb (Figs. 1A and 2A) in cells undergoing either apoptosis or necrosis that was observed only under FIGE conditions (see Fig. 1A, lanes 2, 4 and 6).

It is important to note, however, that the conclusion that PACE is more reliable than FIGE also finds support in the experiments obtained in the present study. Indeed, as illustrated in Fig. 2, the PACE technology allows a good resolu-

tion of the DNA MW standard  $\lambda$  ladder (lane 1) and of the *S. cerevisiae* (lane 2) and *S. pombe* (lane 3) chromosomes. Thus, consistent with our previous results [20], the migration of these DNA markers is linear over a range of 0.05–5.7 Mb. In marked contrast, FIGE analysis of the same DNA MW standard demonstrated that while the *S. pombe* chromosomes do not migrate out of the well, the *S. cerevisiae* chromosomes form a very large band in the same region where  $\lambda$  ladder standards of 200–250 kb also migrate (not shown). In the PACE run, the *S. cerevisiae* standards migrate as distinct bands at 2.2, 1.6, 1.1, and 0.9 Mb (Fig. 2B). In analogy with these results, we also find the appearance of bands at 200–300 kb when the DNA of cells triggered into necrosis with  $H_2O_2$  is analyzed with FIGE (Fig. 1).

The results presented in this study are in apparent conflict with the commonly held theory (mainly based on data obtained with FIGE [7–12,14]) that apoptotic DNA fragmentation proceeds via the hierarchically ordered cleavage of chromosomes into fragments of 300 kb (possibly rosettes) and, subsequently, into fragments of 50 kb (possibly loops) [7–15]. Rather, our results strongly suggest that, at least in the apoptotic process triggered by either  $H_2O_2$ /3AB or etoposide in U937 cells, 50 kb fragments are directly excised from Mb-sized DNA fragments. The 50 kb fragments could then be further digested down to the nucleosomal level and, indeed, the formation of oligonucleosomal DNA fragments was also detected (not shown) and previously reported under conditions similar to those utilized in this study [26].

In conclusion, although the possibility that the appearance of 200–300 kb fragments is a very rapid and transient phenomenon cannot be completely ruled out, the data presented herein suggest that, at least in the system utilized in the present study, cleavage of genomic DNA in the course of apoptosis does not proceed by excision of rosettes and then loops, but rather that 50 kb fragments (possibly DNA loops) are directly cleaved from precursor fragments of  $\geq 2.2$  Mb in size. As a corollary, it would appear that a reliable marker of apoptosis, at the HMW DNA fragmentation level, is represented by the sole appearance of the  $\leq 50$  kb band. Moreover, in agreement with Bicknell and Cohen [14], we show that digestion of genomic DNA taking place in the course of necrotic death gives rise to DNA fragments which, using FIGE, migrate in the kb region and thus may be superimposed and/or confused with those produced during apoptosis.

*Acknowledgements:* This work was supported by a grant from CNR.

#### References

- [1] Corcoran, G.B., Fix, L., Jones, D.P., Treinen Moslen, T., Nicotera, P., Oberhammer, F.A. and Buttyan, R. (1994) Toxicol. Appl. Pharmacol. 128, 169–181.
- [2] Orrenius, S. (1995) J. Int. Med. 237, 529–536.

- [3] Arends, M.J., Morris, M.G. and Wyllie, A.H. (1990) *Am. J. Pathol.* 136, 593–608.
- [4] Cohen, J.J. and Duke, R.C. (1984) *J. Immunol.* 132, 38–42.
- [5] Cohen, G.M., Sun, X.M., Snowden, R.T., Dinsdale, D. and Skilleter, D.N. (1992) *Biochem. J.* 286, 331–334.
- [6] Oberhammer, F.A., Fritsch, G., Schmied, M., Pavelka, M., Printz, D., Purchio, A.F., Lassmann, H. and Schulte-Hermann, R. (1993) *J. Cell Sci.* 104, 317–326.
- [7] Walker, P.R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J.F. and Sikorska, M. (1991) *Cancer Res.* 51, 1078–1085.
- [8] Brown, D.G., Sun, X.M. and Cohen, G.M. (1993) *J. Biol. Chem.* 268, 3037–3039.
- [9] Oberhammer, F.A., Wilson, J.W., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R. and Sikorska, M. (1993) *EMBO J.* 12, 3679–3684.
- [10] Walker, P.R., Kokileva, L., LeBlanc, J. and Sikorska, M. (1993) *Res. Rep.* 15, 1032–1040.
- [11] Sun, X.M. and Cohen, G.M. (1994) *J. Biol. Chem.* 269, 14857–14860.
- [12] Walker, P.R., Weaver, V.M., Lach, B., LeBlanc, J. and Sikorska, M. (1994) *Exp. Cell. Res.* 213, 100–106.
- [13] Zhivotowsky, B., Cedervall, B., Jiang, S., Nicotera, P. and Orrenius, S. (1994) *Biochem. Biophys. Res. Commun.* 202, 120–127.
- [14] Bicknell, G.R. and Cohen, G.M. (1995) *Biochem. Biophys. Res. Commun.* 207, 40–47.
- [15] Lagarkova, M.A., Iarovaia, O.V. and Razin, S.V. (1995) *J. Biol. Chem.* 270, 20239–20241.
- [16] Chu, G. and Gunderson, K. (1991) *Anal. Biochem.* 194, 439–446.
- [17] Elia, M.C., Deluca, J.G. and Bradley, M.O. (1991) *Pharmacol. Ther.* 51, 291–327.
- [18] Gunderson, K. and Chu, G. (1991) *Mol. Cell. Biol.* 11, 3348–3354.
- [19] Elia, M.C. and Nichols, W.W. (1993) *Int. J. Radiat. Biol.* 63, 7–11.
- [20] Sestili, P., Cattabeni, F. and Cantoni, O. (1995) *Carcinogenesis* 16, 703–706.
- [21] Sestili, P., Cattabeni, F. and Cantoni, O. (1995) *Biochem. Pharm.* 50, 1823–1830.
- [22] Sestili, P., Brandi, G., Brambilla, L., Cattabeni, F. and Cantoni, O. (1996) *J. Pharmacol. Exp. Ther.* 277, 1719–1725.
- [23] Carle, F.G. (1992) in: *Pulsed Field Gel Electrophoresis* (Burmeister, M. and Ulanovsky, L., Eds.), Vol. 12. pp. 1–18, Humana Press, Totowa, NJ.
- [24] Story, M.D., Mendoza, E.A., Meyn, R.E. and Tofilon, P.J. (1994) *Int. J. Radiat. Biol.* 65, 523–538.
- [25] Bertrand, R.M., Sarang, M., Jenkin, J., Kerrigan, D. and Pommer, Y. (1991) *Cancer Res.* 51, 6280, 6285.
- [26] Palomba, L., Brambilla, L., Cattabeni, F. and Cantoni, O. (1999) *FEBS Lett.* 390, 91–94.