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Analysis of internucleosomal DNA fragmentation in apoptotic thymocytes by dynamic sieving capillary electrophoresis

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Abstract

The analysis, by slab gel electrophoresis, of internucleosomal DNA cleavage or laddering, characteristic of apoptosis in many cell systems, is labour intensive, difficult to automate and at best only semi-quantitative. In this report we show that CE, using dilute solutions of hydroxyethylcellulose as a replaceable sieving matrix, can be applied to the relatively rapid analysis of DNA laddering in whole digests of apoptotic rat thymocytes. Also, using the sensitivity of laser-induced fluorescence detection and the highly sensitive nucleic acid stain YO-PRO-1, the CE method reported here can use 1000–2000 fold fewer cells than needed for traditional slab gel methods.

1. Introduction

Traditionally, DNA fragments are analysed by slab gel electrophoresis using rigid, cross-linked polymers formed from agarose or polyacrylamide which serve the functions of sieving matrices to separate fragments by size and as anti-convective media to minimise the effects of Joule heating. The dissipation of Joule heat is achieved efficiently in CE by the use of very fine bore capillaries with a large surface area-to-volume ratio. Consequently, separations by CE can run at much higher field strengths allowing significantly shorter analysis times. CE has further advantages over traditional slab gel methods: automation of analysis; on-line detection; direct quantitative features, such as the ability to integrate peak areas, no staining or scanning densitometry; high mass sensitivity; analysis of minute amounts of material; and superior resolving capabilities. In contrast, slab gel electrophoresis is generally slow, labour-intensive, can suffer from poor reproducibility, uses large amounts of DNA, has limited quantitative capability and automation has proved difficult.

Although rigid cross-linked polymers, predominantly polyacrylamide, have been used in the CE format they suffer disadvantages such as problematic capillary packing procedures, expensive pre-packed capillaries, the use of chemically modified capillaries and a high probability of irreversible degradation of capillary performance. An alternative uses dilute entangled polymer solutions such as hydroxylated cellulose

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derivatives [1–3] as a sieving matrix, which can be replaced between runs. Sometimes termed dynamic sieving CE, this is a relatively new technique that has primarily been applied to the analysis of polymerase chain reaction products and restriction enzyme digests [4–6].

Apoptosis and necrosis are well recognised modes of cell death in eukaryotic cells. Whereas necrosis is a response to gross cytotoxic insult, apoptosis is a more ordered, metabolically controlled cellular demise which enables the elimination of cells without the release of intracellular components and pathological consequences such as excessive inflammation [7,8]. The primary morphological and biochemical features of apoptosis are chromatin condensation, plasma membrane blebbing, loss of cell volume and DNA fragmentation. Nuclear and cytoplasmic condensation are followed by nuclear fragmentation and the appearance of apoptotic bodies, arising from cell surface blebs, containing nuclear remnants and intact organelles [7,8]. Cells which are apoptotic can therefore be quantitated by identifying their overt morphology by light microscopy. It has also been shown that apoptotic cells can be distinguished by their increased fluorescent dye uptake, which can then be detected and quantified by flow cytometry [9–11]; these cells have been shown to be apoptotic by several criteria.

Endonuclease-mediated cleavage of DNA in the linker regions between nucleosomes during the later phases of apoptosis can result in an internucleosomal or 'ladder' fragmentation pattern following gel electrophoresis. Each 'rung' of the ladder represents a multiple of the basic nucleosome unit which contains 160–240 base pairs (bp), depending on the organism. The DNA ladder is regarded as a biochemical marker of apoptosis in many systems. Several approaches have been used to measure DNA fragmentation in apoptosis ranging from crude measures of the molecular size of DNA in cell populations or individual cells, to the specific study of internucleosomal cleavage in agarose gels using electrophoresis. One commonly used measure of DNA fragmentation involves the centrifugation of a cell lysate and the determination of DNA in the pellet (high-molecular-mass) and the supernatant (fragmented); their ratio being used as an index of fragmentation [12–14]. DNA strand breakage in individual apoptotic cells has been analysed by in situ nick translation, involving the incorporation of a nucleotide analogue by DNA polymerase I followed by detection of the analogue with a fluorescently labelled antibody [15,16]. Labelled cells are examined by microscopy or flow cytometry to quantitate apoptosis. Internucleosomal cleavage is most specifically identified by slab gel electrophoresis of DNA in whole cell digests or DNA extracted from cells. Semi-quantitative assessment of 'laddering' in ethidium bromide stained gels is achieved by scanning densitometry or visual assignment of relative band intensity [17–19].

In this report the development of dynamic sieving CE for the analysis of the characteristic internucleosomal DNA fragmentation pattern observed in immature rat thymocytes is presented. This CE application combines several advantageous characteristics of existing CE methodology and slab gel sample preparation for determination of internucleosomal DNA cleavage. These are, a replaceable sieving matrix at low pH in uncoated silica capillaries and the direct analysis of whole cell digests. In addition, the use of a sensitive fluorescent nucleic acid dye, YO-PRO-1, greatly increases the sensitivity of detection of DNA 'laddering' compared to that achieved by conventional slab gel electrophoresis.

2. Experimental

2.1. Chemicals

Two standard DNA digests were used during method development, *Hae*III digest of ΦX174 DNA (Sigma, Poole, UK), which contains eleven double stranded DNA (dsDNA) fragments in the range 72–1353 bp, and a 123 bp ladder containing dsDNA fragments from 123–4182 bp (Gibco, Paisley, UK). Digests were diluted to 4.9 μg/ml for *Hae*III digest and 0.75

μg/ml for the 123 bp ladder with distilled, deionised water and stored at −20°C in aliquots. Hydroxyethylcellulose (Aldrich, Poole, UK) was used as the sieving matrix. For DNA fragment detection, YO-PRO-1 diiodide {1-(4-[3-methyl-2,3,-dihydro-(benzo-1,2-oxazole)-2-methylidene]-quinolinium)-3-trimethylammonium propane diiodide} (Molecular Probes, Eugene, OR, USA) was used, this was supplied as a 1 m*M* solution in DMSO and further diluted with DMSO to a 50 μ*M* working stock and stored at 4°C. Proteinase K (from *Tritirachium album*) and ribonuclease A (RNase A, E.C. 3.1.27.5, Type IV from bovine pancreas) obtained from Sigma were used for thymocyte digestion. Prior to use the RNase working stock (400 Kunitz units/ml in 50 m*M* Tris, 0.5 m*M* EDTA, pH 8.0) was pre-treated to eliminate DNase activity. The pH of the RNase solution was first lowered to ca. 5.0 then heated in boiling water for 15 min, the solution was then cooled to room temperature and the pH re-adjusted to ca. 8.0. The pH adjustments were made with the minimum volume of 6 *M* hydrochloric acid or 6 *M* sodium hydroxide as necessary, to avoid excessive dilution. Foetal calf serum and RPMI-1640 used in thymocyte culture were from Gibco. All other chemicals were of reagent grade and purchased from Sigma.

2.2. Equipment

Capillary electrophoresis using laser-induced fluorescence (LIF) detection was performed with a P/ACE 2050 system and a laser module 488 housing a 3 mW 488 nm (air-cooled) argon ion laser (Beckman Instruments, High Wycombe, UK).

2.3. Thymocyte isolation and treatment

Thymocytes were isolated from male Fischer 344 rats (4–5 weeks old) as described previously [20]. The resulting cell suspension was diluted in RPMI-1640 containing 10% v/v foetal calf serum and 2 m*M* L-glutamine to give a cell suspension of $15–20 \cdot 10^6$ cells/ml. Incubations were carried out for 4 h at 37°C under an atmosphere of 95% air–5% CO_2, in the presence of 0.1 μ*M* dexamethasone or 10 μ*M* etoposide (VP-16), conditions known to induce differing levels of apoptosis in rat thymocytes [9,11].

2.4. Agarose slab gel electrophoresis

Agarose gel electrophoresis to detect DNA laddering was performed essentially as described by Sorenson et al. [17].

2.5. Thymocyte digestion for CE analysis

Post exposure, thymocytes were harvested by pelleting at 200 *g* for 5 min, washed once with filter sterilised (0.22 μm Acrodisc filter unit; Gelman Sciences, Ann Arbor, MI, USA) phosphate buffered saline (PBS) and re-pelleted. The pellet was resuspended to give an appropriate cell concentration, usually $1 \cdot 10^6$/ml. The required volume of cell suspension was removed to a 500 μl Eppendorf tube and the cells pelleted at 1500 *g* for 5 min. The supernatant was removed and the thymocytes resuspended in 40 μl of digestion buffer. The digestion buffer contained 50 m*M* Tris, 0.5 m*M* EDTA, pH 8.0 (TE buffer; pH adjusted with 1 *M* hydrochloric acid) was filter-sterilised and stored at room temperature in aliquots. Immediately before use, Triton X-100 was added to the digestion buffer to a final concentration of 1% v/v. At this concentration Triton X-100 permeabilised 100% of the cells as determined by Trypan Blue inclusion. In addition to digestion buffer 5 μl of proteinase K (32 units/ml in TE buffer, pH 8.0) and 5 μl of DNase-free RNase A (400 Kunitz units/ml in TE buffer, pH 8.0) were added. The thymocyte digests were incubated for 2 h at 37°C and were either analysed immediately or stored at 4°C for up to 24 h prior to analysis, with no difference in resultant electropherograms.

2.6. Capillary electrophoresis

Hydroxyethylcellulose (HEC) was used as the sieving matrix at a final concentration of 0.5% w/v in carrier electrolyte (50 m*M* potassium dihydrogen phosphate, 0.5 m*M* EDTA, pH 5.0);

HEC was solubilised by mild heating with constant stirring. The HEC solution was filtered through a 0.45-μm filter before use and stored at 4°C for up to two weeks. Immediately before use YO-PRO-1 was added to the HEC solution to give a final concentration of 400 n*M*; HEC containing YO-PRO-1 was prepared daily.

An uncoated fused-silica capillary 57 cm × 75 μm (50 cm to detector) was used. Prior to each sample injection the capillary was flushed for 5 min at 276 kPa with the 0.5% HEC. When the resolution of standard digest peaks was diminished and/or unacceptable baseline noise apparent, the capillary was flushed at high pressure (276 kPa) with ultrapure water (5 min), 1 *M* sodium hydroxide (30 min at 50°C), ultrapure water (10 min) and 0.5% HEC (5 min). Standard restriction enzyme digests and cell digests were loaded on to the capillary hydrodynamically at 3.5 kPa for 90 s. Before injection, each sample was degassed very briefly (1 s) in a sonicator bath to minimise artifactual 'spike' formation in the electropherogram. Separations were performed at −175 V/cm and at 25°C.

3. Results

3.1. Analysis of internucleosomal DNA fragmentation in apoptotic thymocytes

A complete electropherogram for a digest of $1 \cdot 10^5$ thymocytes treated with 10 μM VP-16 is shown in Fig. 1. There are two regions to note: between 16 and 24 min the ladder fragmentation pattern was visible with the smallest fragment migrating at the start of this region; between 24 min and the end of the analysis, large DNA fragments migrated as a substantial unresolved peak. Confirmation of the base pair size of the various peaks was achieved by co-injection of thymocyte digest with *Hae*III digest of ΦX174 DNA (Fig. 2). The results indicate that the broad ladder fragmentation peaks correspond to a distribution of DNA fragments around multiples of 180–200 bp, that is nucleosomal in size. The large DNA fragments are expected to partly consist of higher multiples of nucleosomal frag-

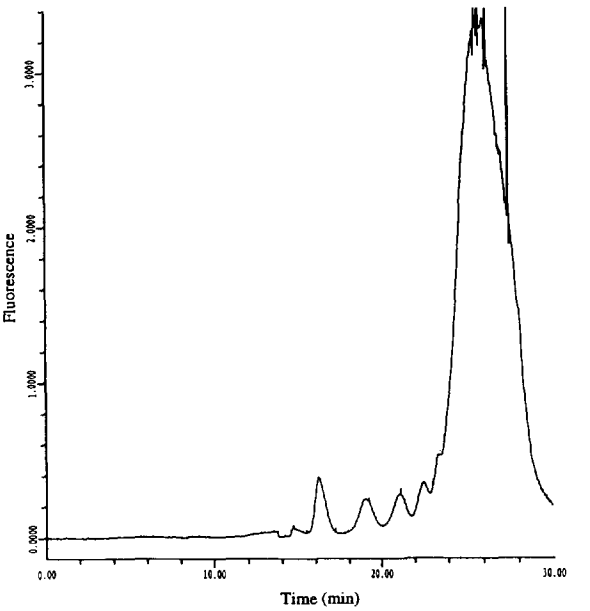

Fig. 1. A complete electropherogram for a digest of $1 \cdot 10^5$ VP-16-treated thymocytes analysed using the conditions noted in the Experimental section.

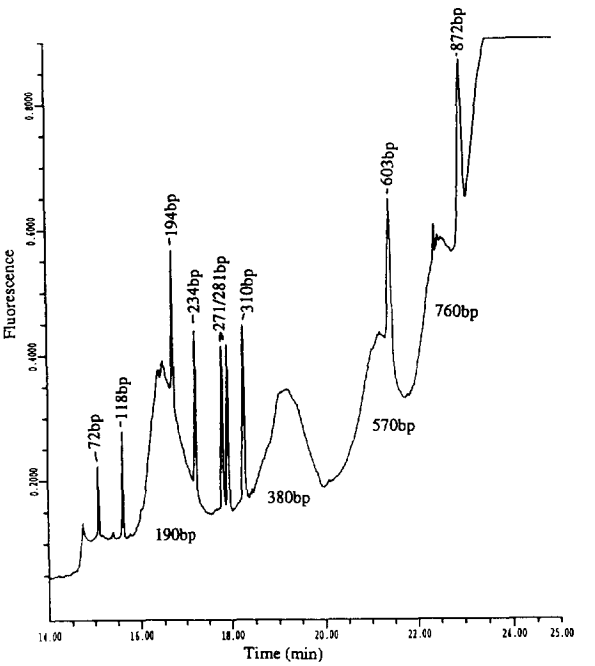

Fig. 2. Digest of $1 \cdot 10^5$ VP-16-treated thymocytes containing 98 ng/ml *Hae*III digest of ΦX174 DNA. Sizes of the ΦX174 DNA fragments and the nucleosomal fragments are indicated.

ments that remain unresolved by this CE method. A comparison of the ladder fragmentation region of electropherograms from all three types of cell exposure is shown in Fig. 3, along with slab gel data appended to Fig. 3a. A small degree of laddering was detected in control cells, consistent with faint laddering detected on the slab gel and the small percentage of apoptosis reported in the literature from flow cytometry studies [9–11]. VP-16 induced DNA laddering to a greater extent than dexamethasone, again consistent with the literature [11] and the slab gel electrophoresis data for the three thymocyte treatments showed a similar gradation of DNA laddering.

The electropherogram of a 123-bp dsDNA ladder showed up to 10 fragments with discrete, sharp peaks (Fig. 4) and a noticeable impairment of resolution above fragment 8 (984 bp). In contrast, the thymocyte nucleosomal peaks showed a Gaussian distribution, probably resulting from the inability of this CE method to resolve fragments differing in only a few base pairs and the biochemistry of the internucleosomal cleavage mechanism. The incubation of 20 μl of thymocyte digest with 2 μl of deoxyribonuclease I (E.C. 3.1.21.1, from bovine pancreas; 1012 Kunitz units/ml in deionised water) and 2 μl of phosphodiesterase I (E.C. 3.1.4.1., from *Crotalus atrox* venom; 1 unit/ml in deionised water) for 2 h at 37°C resulted in a marked attenuation of peak amplitude, further attesting to the identity of this material as nucleic acid, specifically DNA (data not shown).

The volume loaded into the capillary with the 90 s hydrodynamic load was calculated to be 134 nl. The volume of sample loaded was determined as follows: ca. 30 μM fluorescein sodium salt in cell digest was loaded into the capillary, against 0.5% w/v HEC in carrier electrolyte, at the sample loading pressure of 3.5 kPa. The time taken to reach the detector was 24.6 ± 0.5 min ($n = 3$) and the capillary volume calculated to be 2.2 μl. Therefore the capillary fill rate was 89.4 nl/s, and thus for a 90 s loading time 134.1 nl of sample was loaded. A mammalian diploid cell contains approximately 10 pg of DNA, therefore the routinely used digests of $1 \cdot 10^5$ cells/50 μl contain 20 ng DNA/μl, thus 2.7 ng of DNA is loaded per 90 s injection. Since control thymocytes are ca. 10% apoptotic after 4 h incubation, as assessed by flow cytometry, then the resultant nucleosomal fragments detected arise from ca. 270 pg of DNA. The limit of detection for nucleosomal fragments in terms of the cell number digested is dependent on the extent of apoptosis and the degree of internucleosomal cleavage occurring in the cells under study. For thymocytes treated with VP-16 for 4 h (45–50% apoptotic by flow cytometry), nucleosomal fragments were detectable by this CE method in a digest of $1 \cdot 10^3$ cells (in 25 μl digestion mixture); at least 1000-fold fewer cells than used for conventional slab gel electrophoresis (Fig. 5). This quantity of cells represents 54 pg DNA/injection. The limit of detection for *Hae*III digest of ΦX174 DNA, based on the smallest (72 bp) fragment was 70 ng/ml which corresponds to 9 pg of DNA digest injected on to the capillary with a 90-s hydrodynamic loading.

3.2. CE method development for thymocyte digests

The CE method was established using *Hae*III digest of ΦX174 DNA as the initial analyte, but method development was primarily oriented towards optimising conditions for the analysis of DNA fragments in cell digests. The effect of several variables on the migration times, total analysis time and resolution of the nucleosomal fragments was determined. The influence of parameters such as temperature, polymer concentration and field strength on the resolution of dsDNA fragments have been relatively well studied [21,22]; the final electrophoresis conditions were selected to keep analysis times below 30 min and achieve suitable resolution to allow the recognition of ladder fragmentation patterns.

Varying the concentration of YO-PRO-1 from 100 to 400 nM resulted in migration times for the nucleosomal peaks increasing by approximately 30 s with each concentration increase (Fig. 6a, b

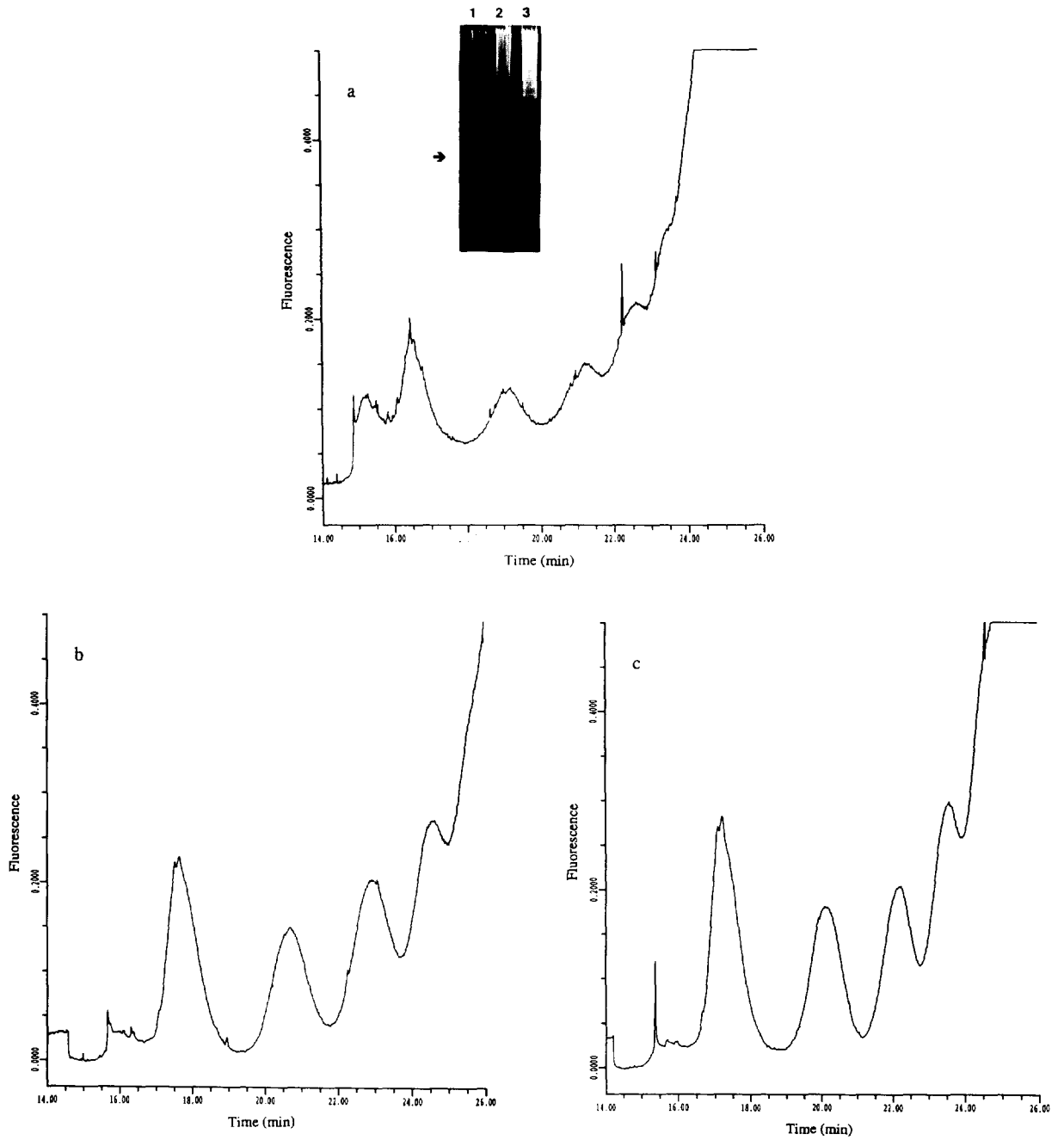

Fig. 3. DNA ladder regions for digests of $1 \cdot 10^5$ thymocytes. (a) Control; (b) dexamethasone; (c) VP-16. Appended to (a) is the agarose slab gel analysis for digests of $2 \cdot 10^6$ thymocytes. (1) Control; (2) dexamethasone; (3) VP-16. The arrow indicates the position of a 564 bp marker.

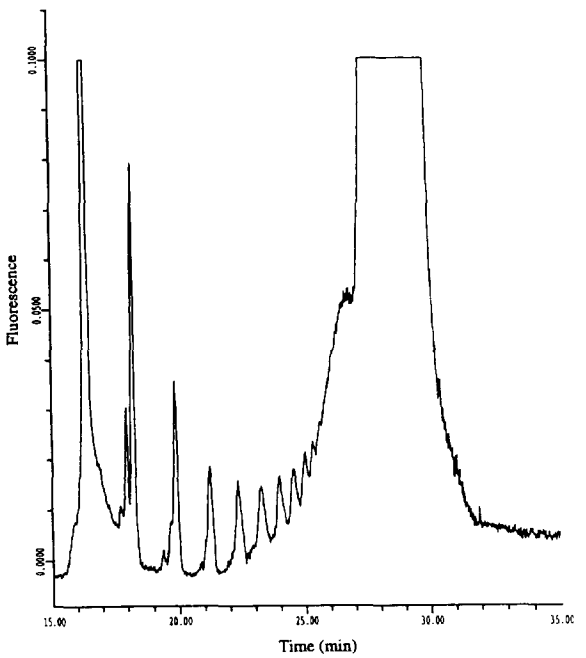

Fig. 4. 123 bp dsDNA ladder at 325 ng/ml analysed using the conditions noted in the Experimental section.

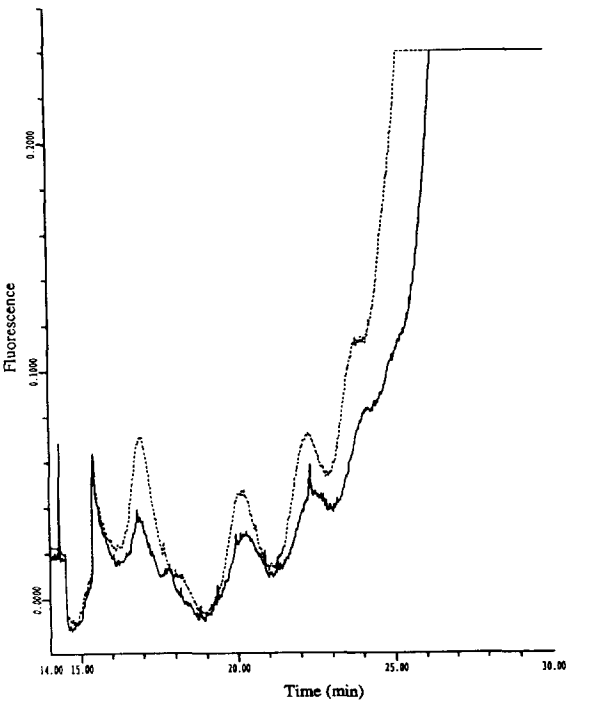

Fig. 5. Comparison of the nucleosomal fragmentation patterns from digests of $5 \cdot 10^3$ VP-16-treated thymocytes (· · ·) and $1 \cdot 10^3$ VP-16-treated thymocytes (—) in a 25-μl digestion volume.

and c). Increasing the YO-PRO-1 concentration from 400 to 800 n*M* resulted in only a marginal increase in migration time (Fig. 6c and d). This increase results from two influences of intercalation of YO-PRO-1 with the fragments: first, an increase in positive charge imparted by the dye; second, a decrease in the flexibility of the fragments which would then decrease fragment mobility through the sieving matrix. The latter effect is the basis for the enhancement of resolution between dsDNA fragments of similar length when adding an intercalating dye such as ethidium bromide [4]. Nucleosomal peak areas increased using 400 n*M* YO-PRO-1 compared to 100 n*M* and less so after increasing the concentration to 800 n*M*. Resolution between nucleosomal peaks was negligibly affected by increasing the YO-PRO-1 concentration from 100 to 800 n*M* (Fig. 6). Since the differences between 400 and 800 n*M* YO-PRO-1 on the analysis were relatively small, the dye was used at 400 n*M* to achieve suitable results and economise on reagent.

Initially, electrokinetic injection (− 35 kV/cm for 5 s) was used for the analysis of restriction enzyme digests, since this gave sharper peaks, better resolution and a less noisy baseline compared to hydrodynamic injection. Electrokinetic injection was therefore expected to be suitable for the thymocyte digests, since small nucleic acid fragments would be preferentially loaded. However, hydrodynamic injection was found to give better electropherograms for the cell digests since the resolution of nucleosomal fragments was still achieved with less noise and more defined peak shapes. Using capillary loading times of 30 s, 60 s and 90 s gave progressively increasing peak heights and spatial areas, however, resolution did not noticeably deteriorate at longer loading times (data not shown).

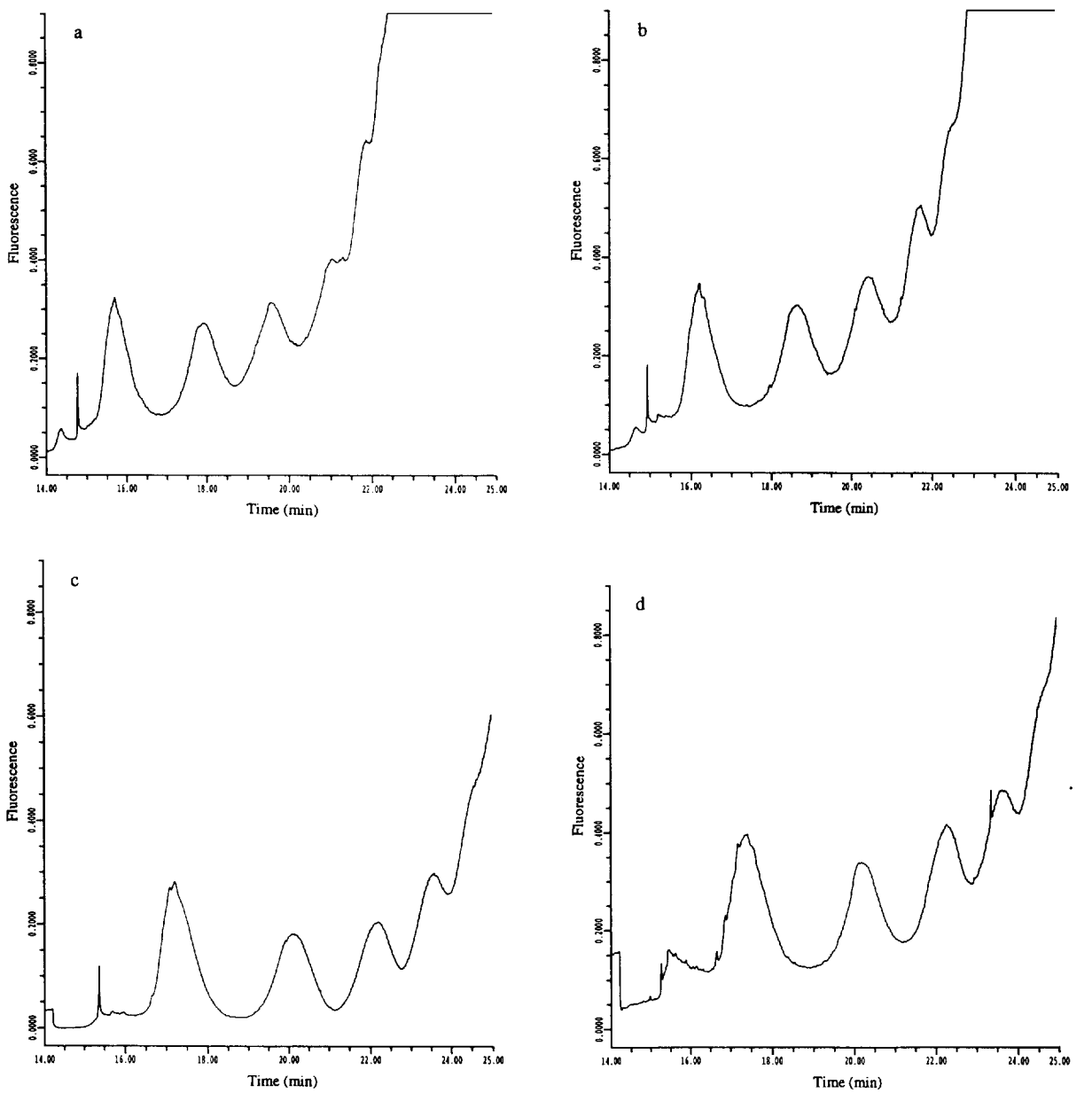

Fig. 6. DNA ladder region for a digest of $1 \cdot 10^5$ VP-16-treated thymocytes analysed using various concentrations of YO-PRO-1: (a) 100 n*M*; (b) 200 n*M*; (c) 400 n*M*; (d) 800 n*M*.

3.3. Thymocyte digestion conditions

A method similar to that first reported by Sorenson et al. [17] as an in-well digestion method on slab gels was adopted as the thymocyte digestion procedure prior to CE. The thymocytes were lysed and digested with proteinase K and RNase in a single incubation step and the whole cell digests used for CE analysis. A useful feature of this method is the elimination of a DNA extraction step prior to electrophoresis which minimises sample handling. In the

digestion procedure reported here, RNase and proteinase K are co-incubated with little detriment to the final electropherogam. The sequential incubation of RNase then proteinase K (2 h RNase, 2 h proteinase K at 37°C) or proteinase K then RNase, yields electropherograms with negligible differences compared to that for a digest obtained when to the two are co-incubated (data not shown).

The inclusion of proteinase K in the digestion mixtures was critical, since its omission resulted in a substantially depleted level of fluorescence (Fig. 7). Only a fraction of the mononucleosome fragment was detected and there was a complete absence of other fragments and high-molecular-mass DNA. Failure to remove protein from the complexes with DNA would obviously have a profound effect on the migration of DNA in this system from the perspectives of both charge and fragment (or complex) size. Conversely, although omission of RNase from the digestions had little effect on the electropherograms (Fig. 7), it was retained in the digestion mixtures to avoid possible detection of RNA by YO-PRO-1.

Contamination of the digestion mixture by exogenous nucleic acid was substantially eliminated by the use of filter-sterilised buffers. However, in many instances low-molecular-mass nucleic acid contamination was evident in both the RNase and proteinase K solutions which migrated just before the mononucleosome fraction with some overlap. The suitability of the incubation time of thymocytes at 37°C with RNase and proteinase K was assessed by determining the total spatial area (integrated peak area divided by migration time) for the dinucleosome to tetranucleosome peaks inclusive following incubation for 1, 2, 3 and 4 h. Over this incubation period the variation in peak spatial area was only 7%.

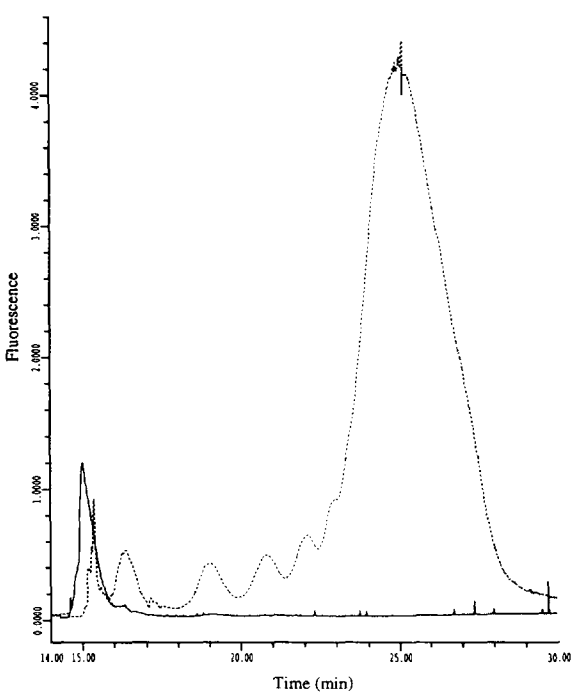

Fig. 7. The effect of excluding proteinase K or RNase from the digestion of $1 \cdot 10^5$ VP-16-treated thymocytes on the appearance of the electropherogram: (—) Proteinase K absent; (· · ·) RNase absent.

4. Discussion

In this study immature rat thymocytes were used since apoptosis in this cell type has been extensively studied from both morphological and biochemical perspectives [12,13,23]. Studies on DNA fragmentation in these cells are at a relatively advanced stage of characterisation and they are known to undergo internucleosomal fragmentation [9,12,13,24,25]. In addition, the conditions known to induce apoptosis in these cells are well known and this was induced to varying degrees using the synthetic glucocorticoid dexamethasone and the topoisomerase II inhibitor etoposide (VP-16). The CE method reported in this paper uses whole cell digests for the analysis of DNA laddering and enables ladder pattern recognition. In addition the data indicate that some useful quantitative information can be obtained and a preliminary comparison between apoptosis quantitated by flow cytometry and in terms of DNA laddering by CE show close agreement.

In this CE method we used the sensitive monomeric cyanine nucleic acid dye YO-PRO-1 as the DNA detection reagent for several reasons. The dye has a very high affinity for

nucleic acid, with a partition coefficient between DNA and 10% v/v aqueous ethanol of $8 \cdot 10^6$ [26]. Although YO-PRO-1 can bind to RNA and single stranded DNA, its affinity and fluorescence are much higher for dsDNA [26]. YO-PRO-1 also has excitation characteristics compatible with the 488 nm argon ion laser and has negligible intrinsic fluorescence in solution thus minimising the background fluorescence signal and increasing sensitivity [24]. The use of YO-PRO-1 as a DNA detection reagent in CE has been reported for the analysis of restriction enzyme digests and polymerase chain reaction-amplified DNA from forensic samples [6]. The high mass sensitivity of CE in combination with YO-PRO-1 means that we were able to detect DNA laddering using 1000–2000 fold fewer cells than used for slab gel electrophoresis. These very low cell numbers suggest that CE analysis of DNA ladder fragmentation can be applied to limited cell sample sizes.

The data obtained yield some useful information regarding the biochemistry of the internucleosomal DNA cleavage. The endonuclease involved in nucleosomal fragmentation during thymocyte apoptosis probably cleaves DNA at various points on the linker DNA to generate a family of nucleosomal fragments differing by only a few base pairs and hence appearing as a broad distribution on electrophoresis. A recent study using mouse thymocytes indicate that the formation of ladder fragmentation is due to the action of a single stranded rather than double stranded DNA cutting activity [27]. The apparent DNA double strand cleavage arises from the dissociation of DNA around multiple single-stranded cuts on opposite DNA strands. Besides the confirmation of fragment size, the co-injection data (Fig. 2) indicate that the nucleosomal fragments span about 50 bp either side of the estimated nucleosome size. The nucleosomal core particle contains 140 bp and species variability in nucleosome size arises from variations in the length of the linker DNA. If the endonuclease involved in ladder fragmentation in apoptosis has no sequence specificity for DNA cleavage, then the most accessible part of the DNA, the central portion of the linker region, would be the predominant cleavage point on steric accessibility grounds. The data in Fig. 2 indicate that this is likely; the least populated fragment sizes around the mononucleosomal fragment are close to the core particle (130–150 bp) and the adjacent nucleosomal particles (approximately 230–250 bp). Compared to slab gel electrophoresis, the CE method is more amenable to this type of co-injection analysis.

Cell treatment prior to analysis by electrophoresis is important to free DNA from complexes with protein and RNA and to minimise noise on the electropherograms. Several factors need to be considered when preparing the samples such as the ease and speed of preparation, the yield of DNA, and whether the handling generates artefacts. Several DNA preparation procedures have been reported in the literature for analysing low-molecular-mass DNA generated during apoptosis for which the final step is commonly analysis of the DNA in 1–2% agarose slab gels followed by ethidium bromide staining. Although there are variations in the details of the techniques the majority of workers choose one of the three main protocols. First, cell lysis in the presence of proteinase K followed by DNA extraction and RNase digestion [18,19,28]. Second, cell lysis and DNA extraction followed by sequential digestion with RNase and proteinase K [29,30]. Third, cell lysis and sequential digestion with RNase and proteinase K followed by DNA extraction [31]. Rarely are proteinase K and RNase co-incubated. It is assumed that RNase will be a substrate for the non-specific proteinase K and consequently will lose activity. The studies reported here imply this is not the case, but since whole cell digests are being used it is possible that cell-derived protein substrates for the proteinase K are in excess and thus RNase is a quantitatively minor substrate.

Although DNA ladder fragmentation patterns are frequently used as a marker of apoptosis, it is suggested to be a relatively late event and its formation is often dependent on the cell type and the stimulus used to induce apoptosis [9,16,32,33]. It is possible that in cell systems which do not show evidence of internucleosomal DNA cleavage the event is quantitatively minor

and the failure to detect DNA laddering is due to the relatively poor sensitivity of the currently used electrophoresis methods. Other criteria, related to DNA fragmentation, for detecting apoptosis have been developed, such as the application of pulsed field, or field inversion gel electrophoresis (FIGE) to examine cells for earlier, large (50–300 kbp) DNA fragments [33,34]. These larger fragments probably result from DNA strand breaks introduced via nuclease-sensitive sites arising from higher order packing of the DNA into loop (50 kbp) and rosette (300 kbp) structures [23,34]. The analysis of DNA fragments by FIGE is lengthy and the ability to detect ladder fragmentation and large DNA fragments in a single, relatively rapid, electrophoretic analysis would be of considerable use in the study of DNA fragmentation in apoptosis. The CE method described here is capable of separating a 9.4-kbp fragment from a 23.1-kbp fragment in the *Hind*III digest of λ phage within 30 min (data not shown). However, the resolution of a 23.1-kbp from a 48.5-kbp (intact λ phage DNA) fragment was not achieved. The separation of large DNA fragments in the kbp range has been reported, using polyacrylamide-coated capillaries and linear polyacrylamide as the sieving matrix and also by pulsed field CE [35,36]. We are currently developing CE methodology to analyse for internucleosomal cleavage and large DNA fragments in a single run using cellular systems undergoing apoptosis.

5. Conclusions

In this paper we have demonstrated that CE using replaceable dilute polymer solutions can be applied to the analysis of DNA ladder fragmentation in apoptotic thymocytes. The analysis can be successfully performed on whole cell digests, obviating the need for a DNA extraction step, and the resultant electropherograms allow the identification of DNA laddering. The relative degree of apoptosis based on the comparison of DNA laddering by CE agrees with that seen for slab gel electrophoresis and with flow cytometric values presented in the literature. Also, the CE method is very sensitive compared to slab gel electrophoresis, is somewhat less labour intensive, and can be automated.

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