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Nonrandom Degradation of DNA in Human Leukemic Cells during Radiation-induced Apoptosis[1]

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ABSTRACT

In many cells, the process of apoptosis is accompanied by endonuclease-mediated double-strand cleavage of DNA between nucleosomes, resulting in the production of discrete fragments of 200 bp or multiples thereof. To address the question of whether this endonuclease attack occurs randomly or nonrandomly along chromosomes, we first constructed chromosome fluorescence *in situ* hybridization probes from the 200- and 400-bp fragments from γ-irradiated apoptotic human T cells along with similar-sized probes from randomly sheared DNA of nonirradiated cells. These probes were compared for their binding along normal human metaphase chromosomes after fluorescence *in situ* hybridization with and without the presence of unlabeled total human blocking DNA. The addition of blocking DNA to the apoptotic probes revealed a nonrandom pattern of hybridization that was not observed for the nonirradiated control probes. The most obvious areas of selective binding occurred around the centromeric and other heterochromatic regions along the chromosome arms, such as the long (q arm) of the Y chromosome.

The converse of this experiment was also carried out. DNA probes from heterochromatic and euchromatic regions of the human Y chromosome were hybridized onto slot blots of apoptotic ladder-sized and randomly sheared nonirradiated human T-lymphocyte DNA. The slot blot results showed that for an equal mass of ladder-sized apoptotic DNA and randomly sheared nonirradiated control DNA, the apoptotic DNA sample contains a relatively larger proportion of Y heterochromatin DNA sequences (approximately 2.5-fold). Together, these results indicate that apoptotis-mediated endonuclease attack does not occur randomly in the genome but occurs preferentially in heterochromatin.

INTRODUCTION

One of the earliest biochemical events often associated with apoptosis was described in a study by Wyllie in 1980 (1). In rat thymocytes undergoing glucocorticoid-induced apoptosis, a distinctive DNA "ladder" pattern was observed in agarose gels that showed specific banding at 200 bp and multiples thereof. The banding pattern was apparently due to endonuclease-mediated double-strand cleavage between nucleosomes. Although it is not seen in all cell types undergoing apoptosis, the presence of the ladder feature has long been recognized as a hallmark feature of apoptosis.

Whereas it is clear at the level of nucleosomes that apoptotic nuclease attack is not random, the question of randomness at higher levels of chromatin structure has received relatively little attention. For some nucleases, such as DNase I, it is known that transcriptionally active regions of DNA are far more sensitive to degradation than inactive or heterochromatic DNA (2), and this holds true even for condensed mitotic chromosomes (3). By analogy, we asked whether the so-called apoptotic nucleases are affected in their attack on DNA by chromatin structure in a way that is similar to or different from DNase I, or whether they act in a random fashion unrelated to higher-order chromatin structure.

In a 1994 review, Walter and Sikorska (4) summarized the various DNA fragmentation patterns observed in cells undergoing apoptosis. They reported that in addition to the internucleosomal DNA cleavage found in some apoptotic cell populations, DNA fragments of a size between about 0.4 and 1 Mbp and another fraction in the 30–50 kb size range also appear to be released during the apoptotic process (apparently independently of whether or not the internucleosomal ladder is observed; Ref. 4). Vodenicharov *et al.* (5) performed experiments that looked at the chromosomal *in situ* hybridization of nucleosomal ladder fragmented DNA from spontaneously apoptotic mouse F4N-S erythroleukemia cells. They constructed ^{3}H probes from a total apoptotic ladder population of DNA that ranged from about 0.2–2.0 kb. The group concluded from these *in situ* hybridization experiments that there were distinct "clusters" of selective binding at pericentromeric and peritelomeric locations along the mouse genome. At about the same time, we presented the results of similar studies with fluorescence-labeled probes.[3] However, the experimental design in both cases had a potentially serious flaw. We and others have shown that overall probe size itself can affect the pattern of signal hybridization along metaphase chromosomes (6–8). To control this potential problem, hybridization of randomly sheared DNA of similar size must be used as a standard for comparison to rule out the possibility that nonrandom labeling is simply a size-dependent artifact. Furthermore, early experiments did not use competitive blocking DNA. The purpose of using unlabeled competitive blocking DNA is to reduce the level of chromosomal hybridization of labeled probe sequences that are not enriched but are still present in the probe. Competitive blocking genomic DNA generally reduces the hybridization of probe sequences to a degree that depends on their relative concentration in the probe compared to that in the unlabeled blocking DNA. In our experiments, as described below, the appearance of a nonrandom consensus pattern of labeling was evident only after unlabeled genomic DNA was added to the hybridization mixture as a competitive blocking agent.

The rationale for this experiment was as follows. If the apoptotic process involving DNA degradation is entirely random, then labeled probe (*e.g.*, biotin) prepared from the ladder DNA after FISH[4] would produce uniform labeling along all chromosomes, barring any size-dependent artifact. Conversely, with the addition of unlabeled genomic competitive blocking DNA, the hybridization signal will be uniformly inhibited. On the other hand, if the apoptotic DNA degradation is highly targeted to a particular class of chromatin with different hybridization sequences than the genome average, then the labeled ladder probe would be correspondingly enriched in such sequences compared to the overall genome. Competitive blocking with genomic DNA would then less readily prevent hybridization of the "apoptotic favored" sequences to their targets along the chromosome.

An alternative approach for studying the differential or nonrandom attack of apoptotic nucleases is to prepare labeled probes from different regions of the genome with known differences in chromatin or

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[4] The abbreviations used are: FISH, fluorescence *in situ* hybridization; DOP-PCR, degenerate oligonucleotide primed PCR.

DNA properties such as heterochromatic and euchromatic regions and use these probes to compare relative degrees of binding to apoptotic ladder DNA *versus* randomly sheared DNA of the same size. In this case, the most appropriate comparison would be for euchromatic and heterochromatic regions of the same chromosome. Because the previous approach outlined above (FISH) indicated a preferential attack in heterochromatin in general, and we did, in fact, see copious binding of apoptotic ladder DNA to the classical satellite heterochromatic region of the Y chromosome q-arm but not the euchromatic p or centromere proximal region of the q-arm, this appeared to be an appropriate choice.

Because Y classical satellite and Y-specific euchromatic painting probes are commercially available, we used these for hybridization to slot blots of extracted ladder-sized DNA. Here, the idea was to extract apoptotic ladder-sized DNA from preparative gels and transfer a known mass (say L ng) and serial dilutions ($L/2$, $L/4\ldots$) to a membrane in a slot blot apparatus. The comparison standard was to transfer a known mass (say R ng) of similar-sized randomly sheared genomic DNA, also with serial dilutions ($R/2$, $R/4\ldots$), from nonapoptotic control cells. In both instances, ^{32}P-labeled Y classical satellite heterochromatic DNA or ^{32}P-labeled Y-specific euchromatic probes would be hybridized to the blots, and after autoradiography, a comparison could be made of the ratio $(R{:}L)_{het}$ of randomly sheared bound signal (R) to apoptotic ladder DNA bound signal (L) for the labeled heterochromatic Y classical satellite probe and the ratio $(R{:}L)_{euch}$ for the labeled euchromatic Y-specific probe. Here we would again anticipate that nonlabeled competitive blocking genomic DNA from female-derived cells, which do not contain a Y chromosome, would be useful to help increase differential binding.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. MOLT-4 (ATCC CRL 1582) and Jurkat clone E6-1 (ATCC CRL TIB 152) were obtained from Dr. Helen Evans (Case Western Reserve University, Cleveland, OH). MOLT-4 T lymphoblasts were originally isolated from a 19-year-old male who was receiving treatment for acute lymphoblastic leukemia. Jurkat clone E6-1 and T lymphoblasts were originally derived from a male patient with acute leukemia. Both cell lines tested negative for the EBV nuclear antigen (9). The cell lines were grown in suspension and maintained in RPMI 1640 containing 2 mM L-glutamine (Irvine Scientific) supplemented with 10% fetal bovine serum (Summit Biotechnology), 0.100 g/liter penicillin G (Sigma), and 0.100 g/liter streptomycin sulfate (Sigma). The cultures were maintained in a 5% CO_2, 37°C incubator. Cells were plated into T-175 flasks (Greiner), and cell densities were not allowed to exceed 5×10^5 cells/ml to ensure asynchronous log-phase growth.

The *in situ* hybridization studies were originally performed using the MOLT-4 cell line. After the completion of these experiments, however, the cells began to exhibit a marked decrease in proliferation rate and no longer demonstrated an appreciable amount of radiation-induced apoptosis. These observations were confirmed by Dr. Helen Evans, the original supplier of the cell line to our laboratory, and we found a similar problem with two separate samples obtained from the American Type Culture Collection. There are several possible explanations for the apparent change in cell line characteristics, but we subsequently switched to another T-lymphocyte cell line, Jurkat, and repeated the chromosome work in a comparison study. Because the results of the *in situ* hybridization experiments described above were qualitatively similar, we proceeded with the remainder of the study using Jurkat cells.

The low passage number (passage 6–8), normal human fibroblast cell line AG1521A (National Institute on Aging, Aging Cell Culture Repository AG01521A) was used to prepare normal human mitotic chromosome spreads. AG1521A cells were derived from male nonfetal foreskin tissue. These cells grow in monolayers with a doubling time of about 24 h and were sustained in α-MEM (Life Technologies, Inc.) supplemented with 15% fetal bovine serum, 0.100 g/liter penicillin G, and 0.100 g/liter streptomycin sulfate. AG1521A cultures were also maintained in a 5% CO_2, 37°C incubator. After thawing, the cells were rinsed in medium, plated into a T-25 flask (Greiner), and allowed to attach for a 24-h period. The next day, the culture was seeded into a T-75 flask (Greiner), and after the cell density reached about 75% confluence the cells were passed into six T-75 flasks. These cultures were then used to prepare mitotic cell chromosome spreads.

Two plasmid constructs, pBSY and DYZ1, were used as probes for the identification of Y euchromatin and heterochromatin, respectively. The pBSY plasmid obtained from J. W. Evans and M. S. Kovacs (Stanford University, Stanford, CA) contains Y euchromatic sequences from the p arm and approximately one-third of the centromere proximal portion of the q arm of the Y chromosome. This library is contained in the BlueScript plasmid, and the sequences are flanked by the T3 and T7 priming elements for amplification of the insert by PCR. The DYZ1 construct was obtained from the American Type Culture Collection (ATCC 57498) and contains the complete 3564-bp Y heterochromatic (Y classical satellite) repetitive sequence from the remaining two-thirds of the Y chromosome q arm. The insert is within the pBR325 plasmid and was cloned into the plasmid at an *Eco*RI site. The bacteria containing plasmids were cultured in Luria-Bertani broth for 16 h at 37°C before DNA isolation.

Exposure of Cells to Ionizing Radiation. To induce apoptosis, log-phase, asynchronous populations of MOLT-4 or Jurkat cells were irradiated in T-175 flasks at room temperature using a Shepherd and Associates Mark I ^{137}Cs unit at a dose rate of 3.94 Gy/min. After irradiation to a total dose of 6 Gy, the cells were returned to the 5% CO_2, 37°C incubator.

Apoptotic and Genomic DNA Isolation and Probe Preparation. DNA from the 6 Gy-irradiated MOLT-4 or Jurkat cells was extracted using either a standard phenol:chloroform (10) or high salt method (11) 24 h after the dose. The reasons for choosing this sampling time are discussed later. Unirradiated populations of both samples were obtained using an identical protocol. Large-scale preparative agarose gels were performed with both classes of DNA to ensure ample recovery for probe preparation and the presence of DNA laddering in the apoptotic samples. The preparative gels contained 2% agarose (Life Technologies, Inc.) in 0.5× Tris-borate EDTA. The DNA was separated on a horizontal gel apparatus by running these samples for 2.0 h at 200 V followed by 60 V for 45 min. Bands representing about 200 and 400 bp from the DNA ladder of apoptotic cells were excised from the gel using a clean scalpel. The DNA from these bands was recovered by using Qiagen's Qiaquick gel extraction kit or by electrobridge. The high molecular weight DNA from the nonirradiated samples was also removed and isolated using the same protocols. This larger-size DNA was then cut with DNase I or sonicated to encompass a size range of 200–600 bp. This sizing manipulation was necessary to ensure that hybridization was not size dependent. After recovery and cutting to the desired size, DNA from both populations was photobiotinylated using Vector's photo-labeling kit. After the biotinylation process, confirmation of biotin incorporation was performed using the nonradioactive Blugene detection system (Life Technologies, Inc.).

Chromosome Y Region-specific Probe Preparation. Y chromosome euchromatic DNA was obtained from the pBSY plasmid library, which contains sequences from the p arm and the centromere proximal third of the q arm. This library was incorporated into a BlueScript plasmid so that the inserts could be amplified using a T3/T7-primed PCR reaction. The Y heterochromatic DNA, also known as Y classical satellite, is composed of repetitive DNA representing the remaining two-thirds of the long arm of the Y chromosome. This 3564-bp sequence repeat was obtained as an insert in the pBR325 plasmid, inserted at the *Eco*RI site. Without any well-designed primer sites contained in the construct, a different PCR amplification scheme was used. Amplification was performed by DOP-PCR (originally described by Telenius *et al.* Ref. 12) using a partially degenerate primer (5′-CCGACTCGAG*NNNNNN*ATGTGG-3′, in which N = A, C, G, or T) in the PCR reaction mixture. We confirmed that the amplified and labeled sequences did contain the Y heterochromatin classical satellite DNA of interest by DNA FISH analysis on AG1521A target chromosome spreads (data not shown).

DNA from the overnight-cultured plasmid was recovered using Qiagen's QIAprep spin miniprep kit. The FISH and slot blot analysis using the pBSY plasmid DNA were performed after T3- and T7-primed PCR amplification of the insert. FISH experiments with the DYZ1 insert sequence used a DOP-PCR-amplified product. Slot blot analysis with the DYZ1 sequence incorporated only the specific 3564-bp heterochromatic Y sequence that was excised from the plasmid using *Eco*RI restriction enzyme digestion, separated from the vector by gel electrophoresis, and isolated by elution from the gel.

PCR. Recovered DNA from the pBSY plasmid was amplified using T3- and T7-primed PCR. A 50-μl reaction mixture consisted of the following: 1× PCR reaction buffer [60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$ (pH 8.5), 0.2 mM deoxynucleotide triphosphate solution, 0.3 μM T3 and T7 primers (Promega), pBSY DNA (3 μl), and H_2O (to obtain a 50-μl final volume]. A wax bead (Perkin-Elmer) was placed over the reaction mixture, and the tube was heated to 90°C and then cooled to 4°C. Taq polymerase (2.5 units; Perkin-Elmer) was added on top of the wax bead, and the tubes were placed in a Perkin-Elmer thermal cycler. Hot-start PCR was performed using a single 5-min denaturation step at 94°C, followed by a 30-cycle program of 1.5 min at 94°C, 1.5 min at 58°C, and an extension step at 72°C for 2 min. The PCR product was then run on a 1.5% agarose gel and visualized under ethidium bromide staining. A 3-μl aliquot of the amplified sequence was then subjected to a second round of PCR amplification with the addition of 3 μl of digoxigenin 11-dUTP (Boehringer Mannheim) to the 50-μl reaction volume (thus decreasing the amount of H_2O by 3 μl). The same 30-cycle protocol was then used to label the pBSY insert.

A random priming method of PCR was used to generate quantities of the DYZ1 product. This method, termed DOP-PCR, followed that initially described by Telenius *et al.* (12) in principle. A 20-μl reaction tube contained 1× thermal sequence buffer (Amersham), 26 mM Tris-HCl, 6.5 mM $MgCl_2$ (pH 9.5), 0.2 mM deoxynucleotide triphosphate solution, 4 μM DOP primer, 3 μl of DYZ1 DNA, 8 units of thermal sequenase (Amersham), and H_2O to yield a volume of 20 μl. The tubes were placed in a Perkin-Elmer thermal cycler, and an initial six-cycle primer program was performed after an initial denaturation step at 94°C. The protocol used during this program was 94°C for 1.5 min, 30°C for 1.5 min, and a 4-min ramp function up to 72°C, followed by an additional 3-min extension period at 72°C. Fifty cycles were then performed using a denaturation step of 94°C for 1.5 min, annealing at 54°C for 1.5 min, and a 3-min extension at 72°C. The samples were then run on a 1.5% agarose gel and visualized under ethidium bromide staining.

A 3-μl aliquot of amplified product was subjected to a digoxigenin 11-dUTP-substituted PCR reaction. The reaction mixtures and PCR program were identical to those used when labeling the pBSY insert, except that 4 μM DOP primer was used instead of the T3 and T7 primers.

FISH. Metaphase chromosome spreads were prepared by fixing mitotic AG1521A normal human fibroblast cells on cold, wet slides using a 3:1 mixture of methanol:acetic acid. The procedure used for FISH analysis was similar to that originally described by Pinkel *et al.* (13) and by Lichter *et al.* (7). Briefly, target slides were immersed in three sequential washes of 70%, 90%, and 100% ethanol for 2 min each to ensure dehydration. The slides were then denatured for 2.5 min at 72°C in a solution of 70% formamide in 2× SSC. After denaturation, the slides were washed again in a series of 70%, 90%, and 100% ethanol for 3 min under gentle agitation. Probe hybridization mixtures containing either apoptotic or nonapoptotic DNA, salmon sperm (carrier DNA), 2× SSC, 50% formamide, and 10% dextran sulfate in a 40-μl final reaction volume were denatured for 20 min at 84°C. In cases where competitive blocking DNA (human total genomic DNA) was added to the hybridization mixture, the 20-min denaturing step was followed by a 1-h prehybridization incubation at 37°C.

After "quick spinning" the hybridization mixtures to centrifuge any condensed water droplets from the tube lids or sides, the probe solution was added to the target slide, covered with a 22 × 50 mm coverslip, and sealed with rubber cement. The slides were then placed in a humidified chamber and allowed to hybridize overnight at 37°C. The next day, the slides were rinsed in a solution of 50% formamide in 2× SSC for 7.5 min at 48°C, followed by a rinse in 2× SSC for 15 min at 48°C. When biotinylated DNA probes were used, fluorescent signal development was performed using an avidin-FITC antiavidin detection system. Fluorescent signal development in studies where digoxigenin-labeled probes were used incorporated an incubation with a direct labeled FITC antidigoxigenin molecule (Oncor).

Fluorescence Microscopy. Before viewing the slides, 30 μl of counterstaining solution containing antifade, 4′,6-diamidino-2-phenylindole, and propidium iodide were placed onto the slide. The slides were viewed under a Zeiss Axioscope fluorescence microscope, and all photographs were taken using a Sony UP-5000 video printer unit.

Slot Blot Hybridization. After spectrophotometric determination of DNA concentrations, serial dilutions of the apoptotic ladder-sized DNA samples along with the sheared nonapoptotic DNA were loaded into the slot blot apparatus and DNA was bound by vacuum filtration to charged nylon filter membranes as described by Sambrook *et al.* (10). Negative control (turtle) DNA that should not bind to the probes and positive control DNAs (the unlabeled probes themselves) were also loaded in known quantities onto the slot membranes. Samples of the T3/T7 PCR-amplified pBSY insert and recovered *Eco*RI-digested DYZ1 construct were random primed labeled with ^{32}P. First, the labeled probes (at a concentration of about 1×10^6 cpm/ml in 10 ml of probe solution) were hybridized onto the filters in the absence of competitive blocking DNA. After autoradiography, the filters were stripped and reprobed with the addition of 15 μg (30 μl of a 0.5 μg/μl stock solution) of total genomic HeLa (human female) DNA to the hybridization mixture to serve as blocking DNA. The filters were once again exposed to X-ray film for autoradiography and development. The film density exposures were then captured using a Kodak digital camera. The images were analyzed for pixel signal intensity using a Kodak imaging software package.

RESULTS

Apoptotic Ladder-sized DNA. To induce apoptosis, a dose of 6 Gy of γ-irradiation was used, and DNA was extracted from the cells 24 h after exposure. This time period yielded the greatest proportion (38%) of apoptotic cells for the MOLT-4 cells as measured by the terminal 1 deoxynucleotidyl transferase-mediated nick end labeling assay (14–16) in our laboratory (data not shown). For earlier times of 6, 12, and 18 h after a 6-Gy dose, the proportion of cells undergoing apoptosis was 3%, 6%, and 10%, respectively. This is consistent with measurements of the time course of radiation-induced apoptotic cell frequencies with TK6 cells using the comet assay (17) and with MOLT-4 cells using a dye exclusion test (18), although the latter study shows a higher proportion of cells undergoing apoptosis (around 80%) at 24–32 h after a 6-Gy dose. It is also noteworthy that total cell counts in the cultures did not decrease during the first 24 h after the 6-Gy dose, indicating that cells were not being selectively lost during the postirradiation incubation. The 6-Gy dose was also used for the Jurkat cell line, and the degree of nucleosomal laddering present was similar to that of the MOLT-4 cell line.

FISH. The first experiments were performed in the absence of competitive blocking DNA in the hybridization mixtures. The results, which are illustrated in Fig. 1, *A* and *B*, showed that there was uniform painting along mitotic AG1521A chromosomes for both the nonapoptotic and apoptotic DNA probes. To ascertain whether ladder-sized DNA from apoptotic cells contained a random sample of all DNA classes, including various repetitive and unique or low copy number DNAs, unlabeled total human DNA was added to the hybridization mixture as competitive blocking DNA, as shown in Fig. 1, *C* and *D*. If the probes and blocking DNA contained the same proportion of all DNA sequences across the genome, the blocking DNA would be expected to uniformly eliminate the binding and fluorescence signal along the chromosomes. Fig. 1*C* shows that unlabeled total genomic blocking DNA uniformly eliminated the signal from labeled, randomly sheared DNA. Here, in addition to 100 ng of labeled probe, 1 μg of unlabeled human total genomic DNA (AG1521A) was present in the 40-μl hybridization mixture. The elimination of the signal would be the expected result, due to the fact that a 100-ng, labeled, sheared total genomic probe is being blocked by 1 μg of total genomic DNA. Fig. 1*D* illustrates the results when blocking DNA was added to the hybridization mixture containing ladder-sized DNA from apoptotic cells. In the sample from which this photograph was taken, 100 ng of labeled apoptotic ladder probe and 1 μg of total genomic DNA were present in the 40-μl hybridization mixture; *i.e.*, in exactly the same proportion described above (Fig. 1*C*) for the labeled, randomly sheared, nonapoptotic DNA probe with unlabeled blocking. The result demonstrates that there is nonuniform hybridization to the mitotic chromosomes. Binding to centromeric regions occurs in some

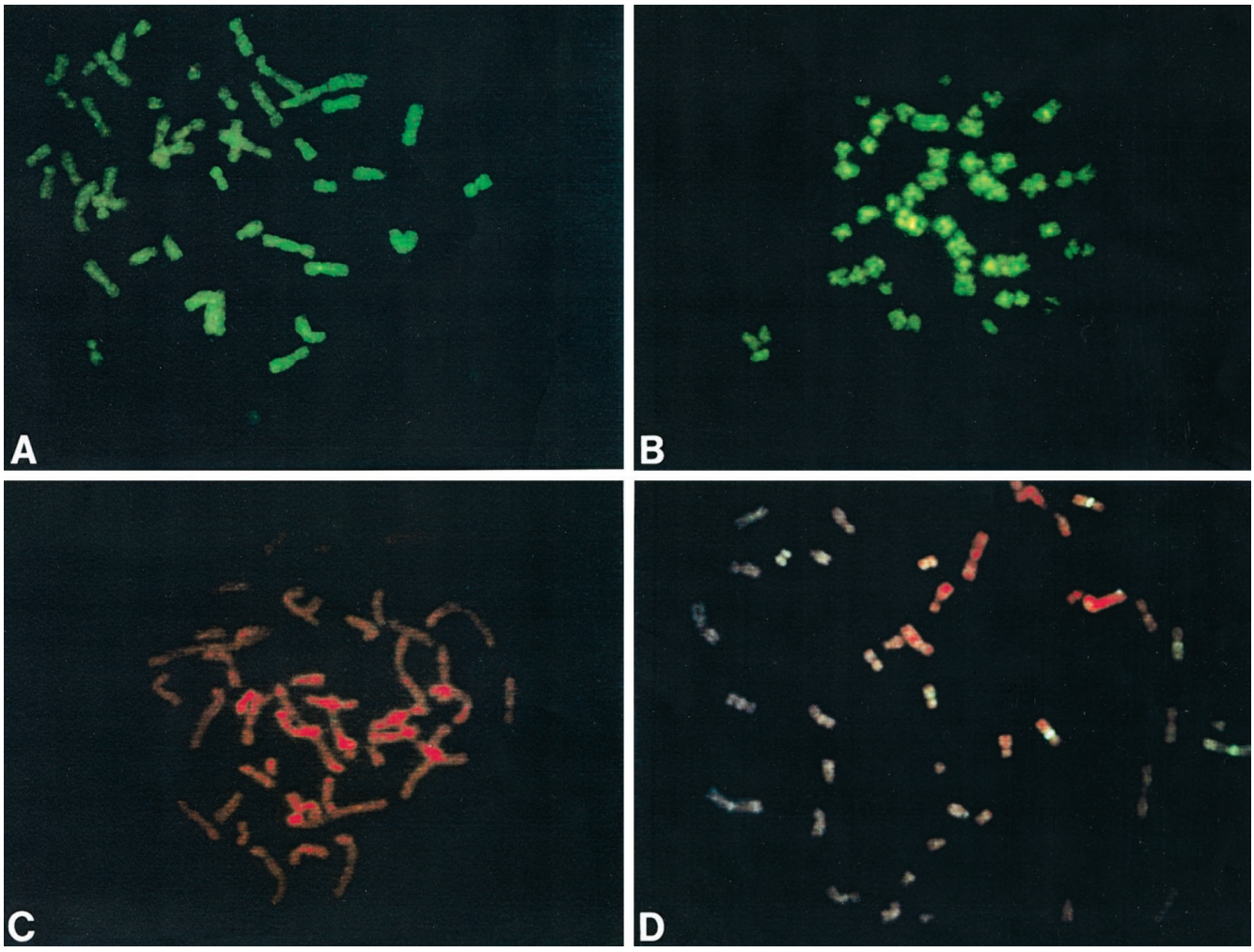

Fig. 1. Photographs taken after the hybridization of biotin-labeled probes containing either nonapoptotic DNA randomly sheared to 200–600 bp (*A* and *C*) or apoptotic DNA from the 200- and 400-bp ladder (*B* and *D*) onto AG1521A metaphase chromosomes. In *A* and *B*, approximately 100 ng of labeled probe were present in the hybridization mixture (reaction volume, 40 μl) without the addition of competitive blocking DNA. The panels show uniform labeling along the mitotic chromosome for both probe classes, although increased signal was seen in some centromeres of B (indicated by FITC, *green* signal). In *C* and *D*, hybridization of labeled probes was carried out as described for *A* and *B*, but with the addition of 1 μg of unlabeled total genomic blocking DNA. *C* shows that after the addition of unlabeled total genomic blocking DNA to nonapoptotic DNA probes randomly sheared to 200–600 bp, the signal (present in *A*) was completely suppressed (counterstained with propidium iodide, *red* signal). When the same concentration of total unlabeled genomic blocking DNA was added to a 100-ng mass of apoptotic DNA probes from the 200- and 400-bp ladder (*D*), nonrandom hybridization is observed along the chromosomes.

chromosomes, along with the presence of banding within the arms of some chromosomes. These results indicate that these probes are enriched in certain sequences, and, as a result, they are not suppressed to the same degree by the presence of unlabeled total genomic DNA.

To determine whether there was a consensus pattern of labeling, photographs of seven mitotic spreads from this blocking experiment were analyzed. Individual chromosome images from the photographs were cut out and rearranged as karyotypes to enable the construction of a histogram showing the fluorescent signal occurrence along the chromosomes. This histogram is shown in Fig. 2. Without banding, all chromosomes could not be identified unequivocally; therefore, karyotypes were arranged according to the original Denver classification, *i.e.*, groups A–G. Where a specific chromosome could be identified with reasonable certainty, it was paired with its homologue and assigned the appropriate number (1–3 and Y). The remaining chromosomes, as mentioned above, were combined according to the Denver classification grouping (B–G group). The X chromosome was placed in the C group, along with chromosomes 6–12. Bars were placed along the ideogram corresponding to the signal location along the chromosome. One bar represents the fluorescent signal at the same location on both homologues (in the case of the A group) or where signal was present in two or more different chromosomes at the same location in a given chromosome group (chromosome groups B–G). The figure shows that painting occurred nonuniformly along the chromosomes, with selective areas of binding around the centromeres and other regions of the chromosomes, such as the heterochromatic long arm of the Y chromosome. It should be noted that the photographs used to construct Fig. 2 were taken from *in situ* hybridization experiments with the MOLT-4 cell line, but similar results were seen for Jurkat cells that were used as described below for slot blot hybridization experiments.

Slot Blot Analysis. Quantification and comparison of the degree of hybridization of DNA associated with Y euchromatin and heterochromatin to defined masses of apoptotic ladder-sized (200–1200 bp) DNA *versus* genomic DNA from unirradiated cells sheared to a similar size range were carried out by a slot blot analysis. Apoptotic ladder-sized DNA in the 200–1200-bp size range was cut out and eluted directly from preparative gels and concentrated. The majority of the total DNA from these samples was of a much higher molecular weight and either did not migrate into the gels or migrated much more slowly than the 200–1200-bp range of sizes isolated. The Y euchromatic labeled probe was prepared from the pBSY plasmid library by

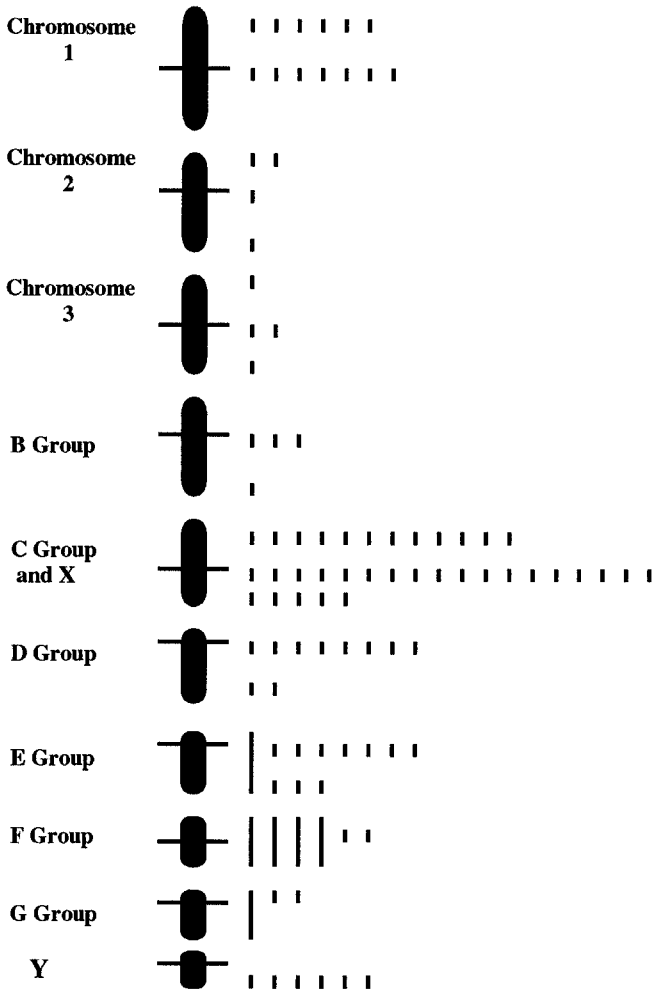

Fig. 2. A histogram shows the presence of the fluorescent signal of apoptotic ladder-sized DNA probes hybridized onto normal human metaphase spreads. Photographs from the FISH experiments were analyzed by cutting the chromosomes out of the pictures and grouping them based on the Denver classification system because Giemsa banding to help with identification was not possible after the FISH procedure. A *bar* was placed at the location where the signal occurred at the same location for both homologues (A group chromosomes 1, 2 and 3) or where signal was present in two different chromosomes at a similar location in the same chromosome group (chromosome groups B–G). This shows the consensus labeling over seven different metaphase cells as described in the text.

first amplifying the inserts with T7- and T3-primed PCR and then labeling ^{32}P by random priming. The Y heterochromatic probe was labeled with ^{32}P in a similar fashion, but the probe was prepared directly from the plasmid isolated in quantity from bacteria, excised from the plasmid with *Eco*RI, and separated and eluted from the gels after electrophoresis.

After preparing target and probe DNA, serial dilutions were then prepared for the apoptotic ladder-sized DNA, sheared nonirradiated DNA, a negative control DNA (turtle DNA), and one positive control sample (either pBSY or DYZ1), depending on the probe used. The individual samples were denatured in 0.4 M NaOH and applied to membrane filters through a slot blot apparatus under a vacuum.

Preliminary trials yielded information on the concentration limits that could be visualized during reasonable exposure times on the autoradiography film. Separate and simultaneous hybridization experiments were performed with ^{32}P-labeled Y euchromatic pBSY and Y heterochromatic DYZ1 inserts, and the autoradiographs are reproduced in Fig. 3. To obtain baseline measurements of binding, no competitive blocking DNA was added to the hybridization mixtures. The *left panel* of Fig. 3 represents the scanned film probed with the Y euchromatic pBSY DNA, whereas the *right panel* of Fig. 3 shows hybridization of the Y heterochromatic DYZ1 DNA. *Lanes 1* and *2* on both panels show serial dilutions (the quantities loaded are listed on the *left side* of the films) of the apoptotic ladder-sized DNA and of the sheared nonirradiated Jurkat cell DNA, respectively. *Lane 3* contains the positive control samples (the same DNA as the probe), and the quantities loaded are listed on the *right*. Finally, the negative control (turtle DNA) samples were loaded in *Lane 4*, with the quantities of the serial-diluted samples recorded on the *left side* of the panels.

Digital images of the films were taken, and pixel intensity values were analyzed. The purpose of these measurements was to compare the intensity ratios of binding to the apoptotic to nonapoptotic DNA samples for the heterochromatic *versus* euchromatic probes. There was a 1:1 signal ratio of the bands in *Lanes 1* and *2* probed with the DYZ1 DNA; when the same mass of DNA is probed with the pBSY DNA, the ratio of signaling is approximately 1:1.4.

To determine whether the signal distribution would be affected by eliminating any nonspecific hybridization, total genomic HeLa DNA was added as a competitive blocking agent. Because HeLa cells were derived from a cervical carcinoma, *i.e.*, of female origin, they do not contain a Y chromosome. Therefore, HeLa DNA would not competitively hybridize (block) Y-specific sequences. The filters used to derive the autoradiographs shown in Fig. 3 were stripped and reprobed with the same activity of labeled probe, but this time, 15 μg of blocking DNA (30 μl of a 0.5 μg/μl stock solution) were denatured together with the probe mixture, added to the membranes, and hybridized overnight. After autoradiography (Fig. 4), the films were

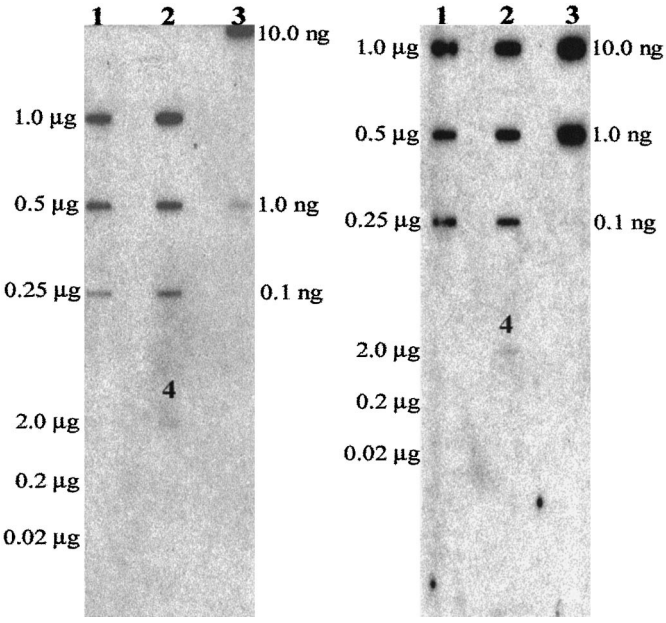

Fig. 3. Slot blots were probed with the labeled pBSY PCR insert that covers the euchromatic p arm and the centromere proximal q arm of the human Y chromosome (*left panel*) and with the DYZ1 insert that covers the larger heterochromatic block in the q arm of the same chromosome (*right panel*) in the absence of competitive blocking DNA. Both blots were probed with an initial activity of 1×10^6 cpm of ^{32}P-labeled probe. *Lanes 1, 2,* and *4* (*bottom portion below Lane 2*) are identical for both panels. *Lane 1* is apoptotic ladder sized-DNA; *Lane 2* is sonicated DNA from nonirradiated cells. Positive and negative controls are shown in *Lanes 3* and *4*, with the latter directly below *Lane 2*. *Lane 3* in the *left panel* is the PCR product of the euchromatic pBSY insert probed to itself, and *Lane 3* in the *right panel* is the heterochromatic DYZ1 insert probed to itself. *Lane 4* is total genomic negative control (turtle) DNA. Mass amounts listed on the *left side* of the panels correspond to *Lanes 1, 2,* and *4*, whereas the mass listed on the *right side* of the panels corresponds to *Lane 3*. Although the two different probes were of equal total activity (dpm) per unit volume during the hybridization, they did not have the same specific activity (dpm/μg DNA). Therefore, the appropriate comparison is the ratio of signals in *Lanes 1* and *2* for the euchromatic probe (*left panel*) *versus* the same ratios for the heterochromatic probe (*right panel*). The quantitative ratios are given in the text.

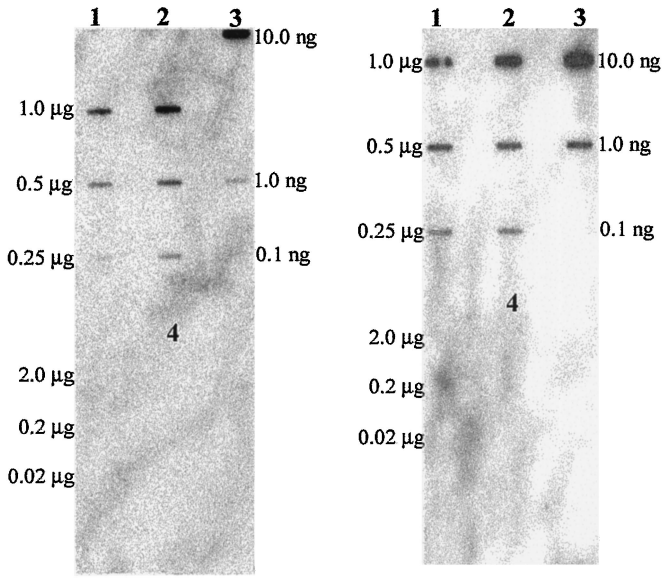

Fig. 4. Slot blots were probed with the labeled euchromatic Y pBSY PCR insert (*left panel*) and the heterochromatic DYZ1 insert (*right panel*) with the addition of 15 μg of total HeLa (female-derived) DNA to the hybridization mixture as competitive blocking DNA. Both blots were probed with an initial activity of 1×10^6 cpm of ^{32}P-labeled probe. *Lanes 1, 2,* and *4* (*bottom portion below Lane 2*) are identical for both panels. *Lane 1* is apoptotic ladder sized-DNA; *Lane 2* is sonicated DNA from nonirradiated cells. Positive and negative controls are shown in *Lanes 3* and *4*, with the latter being directly under *Lane 2*. *Lane 3* in the *left panel* is the PCR product of the euchromatic pBSY insert probed to itself, and *Lane 3* in the *right panel* is the heterochromatic DYZ1 insert probed to itself. *Lane 4* is the total genomic negative control DNA (turtle). Mass amounts listed on the *left side* of the panels correspond to *Lanes 1, 2,* and *4*, whereas the mass listed on the *right side* of the panels corresponds to *Lane 3*. As with Fig. 3, the appropriate comparison is the ratio of signals in *Lanes 1* and *2* for the euchromatic probe (*left panel*) *versus* the same ratios for the heterochromatic probe (*right panel*). The quantitative ratios are given in the text.

scanned for quantification of probe binding. Because these are the same actual membranes used for the previous analysis, the quantities of target DNA in the various slot positions are labeled as described in Fig. 3. After the images were captured and analyzed, we again found a 1:1 ratio of binding of the heterochromatin DYZ1 probe to apoptotic ladder DNA relative to total sheared nonapoptotic DNA. However, the ratio of hybridization binding to apoptotic to nonapoptotic target when probed with the euchromatic pBSY DNA was now 1:2.5 as opposed to the 1:1.4 ratio seen previously without the competitive blocking DNA. These ratios reflect a greater relative reduction or inhibition of hybridization of Y euchromatic probe by unlabeled HeLa competitive blocking DNA. The numerator and denominator of these ratios are not informative by themselves, nor are the individual ratios. It is the comparison of the ratios, particularly the way the comparison changes with competitive blocking, that indicates enrichment of Y heterochromatic-associated DNA degraded by the apoptotic process relative to Y euchromatic DNA. In other words, the nucleosomal ladder DNA present in radiation-induced apoptotic cells is enriched in Y heterochromatic elements.

DISCUSSION

In addition to the findings of this study, there are a few published investigations on the types of DNA contained in the 200-bp ladder region of gels from cells undergoing apoptosis. One laboratory reported on DNA fragmentation during apoptosis in rat chloroleukaemia cells (19). Here, apoptosis was induced by exposing the cells to doses of UV radiation. The DNA was run on agarose gels, and the nucleosomal ladder fragments were collected. These fragments were inserted into plasmid vectors, and 100 clones were isolated, and the inserts were sequenced. They found that there was a statistically significant increase in the relative proportion of long interspersed nuclear elements, short interspersed nuclear elements, rat microsatellite DNA [$(CA)_n$], and polypurine/polypyrimidine repetitive domains compared to the proportions expected when a random sample was taken across the entire genome. However, these results alone would not entirely predict the metaphase chromosomal localization we observed. For example, short interspersed nuclear elements are enriched in the quinacrine dark bands, and long interspersed nuclear elements are enriched in the quinacrine bright bands.

As mentioned above, a more recent study analyzed the nucleosomal DNA fragments appearing in mouse F4N-S erythroleukemia cells undergoing spontaneous apoptosis. This group concluded that the nucleosomal ladder DNA is heterochromatic (and repetitive) in nature (5). In the cell culture they studied, approximately 8% of the cells were spontaneously apoptotic. The results of their *in situ* hybridization experiments with radioactively labeled probes indicated a clustering of signal around the pericentromeric and peritelomeric regions of the mouse metaphase chromosomes, regions known to be heterochromatic in nature. In spite of the differences in experimental approach, their conclusions are similar but not identical to those reached in the present study, although certain potential artifacts were not ruled out. By eliminating the effect of potential probe size-dependent nonrandom binding and by using competitive blocking DNA, we were able to confirm some of the general conclusions of the work cited above and to extend the resolution of the nonrandom *in situ* hybridization binding of degraded DNA from apoptotic cells along metaphase chromosomes (7, 8).

It is worth considering an alternative explanation of our findings. If apoptosis began early after radiation exposure in a significant portion of cells and progressed gradually throughout the 24-h period before collection, it might be argued that the only DNA remaining undigested or partially digested would be that most resistant to attack by apoptotic nucleases. Because we found apoptotic degraded ladder DNA at 24 h to be enriched or to preferentially contain heterochromatic DNA, this scenario would argue for the reverse interpretation, *i.e.*, that DNA associated with euchromatin is preferentially much more sensitive to apoptotic nucleases and is entirely or mostly digested by 24 h. The low proportion of radiation-induced apoptotic cells, which was only 3%, 6%, and 10% respectively at 6, 12, and 18 h after irradiation, coupled with the relatively high proportion of 38% at 24 h, argues against this. Because we did not observe any loss of cells during this time period, the increasing proportion of apoptotic cells seems likely to be cumulative, so that an additional 30% of the total cell population was undergoing apoptosis in addition to the 10% of cells already in this state at 18 h. Thus, at 24 h, the recently apoptotic cells outnumbered older apoptotic cells by something on the order of 3:1. If more recent apoptotic cells show a more rapid degradation in euchromatic DNA, this should predominate in the 24-h samples, but it did not.

In addition to the *in situ* hybridization experiments performed, we labeled available probes from known euchromatic and heterochromatic regions of the same chromosome. By incorporating these probes in slot blot experiments, we were able to demonstrate that DNA in the multiple 200-bp apoptotic ladder region consists of relatively more Y heterochromatic than Y euchromatic DNA sequences compared to total human genomic DNA. We were able to make these conclusions by simultaneously hybridizing these Y-specific sequences onto DNA slot blots from sheared DNA derived from a nonapoptotic cell population that encompassed a similar size range profile as the apoptotic DNA, and we enhanced this selective binding by competitive blocking with genomic DNA. Vodenicherov *et al.* (5) also reported that DNA from the apoptotic ladder region is heavily methylated. These conclusions were reached after cutting both apoptotic ladder DNA and

control nonapoptotic DNA with restriction endonucleases and analyzing the respective digestion patterns. Finally, through both Northern analysis and low copy number gene analysis, Vodenicherov *et al.* (5) concluded that the apoptotic ladder from these mouse F4N-S erythroleukemia cells was also poorly transcribed.

Our conclusions regarding nonrandom DNA degradation in radiation-induced apoptotic human cells are similar in most respects to the conclusions from the two previous reports on either spontaneous (5) or UV light-induced apoptosis (19) in rodent lymphoblastic leukemia cell lines.

It is also interesting to note that our present study and both other reports are at variance with the suggestion that DNase I may be involved in the apoptotic DNA laddering process, as pointed out in the review by Khodarev *et al.* (20), because this enzyme characteristically shows preferential attack on transcriptionally active euchromatic DNA and not heterochromatic DNA (2, 3, 21, 22).

The question of why DNA degradation associated with apoptosis in these cells preferentially attacks heterochromatic DNA elements is an interesting one. In the abovementioned sequence studies by Luokkamaki *et al.* (19), they found that in 100 cloned and sequenced fragments of apoptotic ladder DNA, there were no specific known restriction recognition sequences common to the fragments. Another possibility might involve DNA methylation or histone acetylation status. With DNA that is transcriptionally active or potentially active (*i.e.*, euchromatin), histones associated with these sequences are hyperacetylated, a condition that inhibits the higher-order folding of nucleosomal arrays (23). Conversely, histones associated with transcriptionally inactive DNA, or heterochromatin, are hypoacetylated. It is possible that folding itself may facilitate apoptotic nuclease cleavage. On the other hand, heterochromatin is often characterized by extensive methylation at cytosine residues in CpG dinucleotides, whereas euchromatin is not. This has been shown specifically for the heterochromatin in the q arm of the Y chromosome as well as other heterochromatic satellite DNA (24, 25). The function of these features has often been associated with gene transcription and recognition control. The results of our studies suggest the possibility that these modifications may play a role in the recognition of nucleosomal DNA to be cut during apoptosis. Centromeres play a key role in the segregation of sister chromatids to daughter cells during mitosis, and one feature of the centromeres is the associated heterochromatin. Breakage of DNA near the centromeres would maximize genetic loss for any cells attempting to divide because acentric fragments do not segregate in an orderly way during mitosis. If heterochromatin breakage is an Achilles' heel, it may not be coincidental that the apoptotic nucleases most effective in ensuring cell death preferentially attack heterochromatin.

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