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Association Between DNA Cleavage During Apoptosis and Regions of Chromatin Replication

Nikolai N. Khodarev,* Irina A. Sokolova, and Andrew T.M. Vaughan

Department of Radiotherapy, Loyola University Medical Center, Maywood, Illinois

Abstract We have addressed the association between the site of DNA cleavage during apoptosis and DNA replication. DNA double strand breaks were introduced into chromatin containing pulse labeled nascent DNA by the induction of apoptosis or autocleavage of isolated nuclei. The location of these breaks in relation to nascent DNA were revealed by Bal 31 exonuclease digestion at the cut sites. Our data show that Bal31 accessible cut sites are directly linked to regions enriched in nascent DNA. We suggest that these regions coincide with the termini of replication domains, possibly linked by strong DNA-matrix interactions with biophysically defined topological structures of 0.5 - 1.3 Mbp in size. The 50 kbp fragments that are commonly observed as products of apoptosis are also enriched in nascent DNA within internal regions but not at their termini. It is proposed that these fragments contain a subset of replicon DNA that is excised during apoptosis through recognition of their weak attachment to the nuclear matrix within the replication domain.J. Cell. Biochem. 70:604-615, 1998. © 1998 Wiley-Liss, Inc.

Key words: DNA replication; apoptosis; DNA cleavage; endonuclease; Bal 31; topological domains

Interphase chromatin and metaphase chromosomes are organized into domains, which include topologically constrained DNA of discrete sizes in complexes with different proteins [Dillon and Grosveld, 1994; Nickerson et al., 1995]. The sizes and possible organization of these domains have been reported using a variety of approaches leading to the description of several major classes, in the range between 20–50 kbp and 1.5- 3.0 Mbp [Mirkovitch et al., 1988; Saitoh and Laemmly, 1993]. The different sizes of these domains clearly reflect different levels of chromatin and/or chromosome structural organization, and models have been proposed to explain the structural hierarchy of these complex chromatin arrays [Fillipski et al., 1990; Cook, 1995; Jackson, 1995]. Most models utilize the nuclear matrix as an essential coordinating structure partitioning DNA into topological domains of different sizes and potentially, different functions. The high-level structural organization of chromatin and related DNA-nuclear matrix interactions are tightly connected with processes of DNA transcription, repair and replication [Dillon, Grosveld, 1994; Jackson, 1995]. In particular, associations of DNA replication origins and/or replication forks with the nuclear matrix have been demonstrated [Cook, 1995; Dijkwel and Hamlin, 1995). This association is strengthened by the correlation between the average size of mammalian replicons and replicon clusters with the average sizes of loops and supraloop elements [Nakamura et al., 1986; Jackson, 1995; Kunnev et al., 1997]. Additionally, the sizes of these structural/functional units also correspond to the sizes of high molecular weight fragments (HMW fragments), which have been detected during the early stages of apoptosis in virtually all cell systems tested [see Montague and Cidlowski, 1996, for review]. A further link between replication control and apoptosis has been shown through the activation of specific proteolytic cascades, triggered by the apoptotic program that targets specific components of DNA replication and repair associated with nuclear matrix structures. Among these are PARP [Kaufmann, 1989], the catalytic subunit of DNA-dependent protein kinase [McConnell et al., 1997], replication factor C and scaffold attachment factor A (SAF-A) [Gohring et al.,

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*Correspondence to: Nikolai N. Khodarev, Department of Radiotherapy, Loyola University Medical Center, Maywood, IL 60153. E-mail: nkhodar@luc.edu

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1997; Song et al., 1997]. A complete characterization of the relationship between DNA replication and apoptotic DNA fragmentation will also have to accommodate those systems where apoptosis may occur without DNA replication, such as in the majority of small lymphocytes [Clarke et al., 1993]. In this report, we address the association between the location of apoptotic DNA cleavage and DNA replication within chromatin. We used an approach based on pulse labeling of replicating DNA, combined with the insertion of dsbs in chromatin by either apoptosis or autocleavage of isolated nuclei. The presence of nascent DNA at the termini of such breaks was revealed by Bal 31 exonuclease cleavage. We show that Bal31 directly cleaves nascent DNA in control cells which we propose corresponds to excision of large DNA fragments that are functionally related to DNA replication domains. We suggest that the coincidence of dsbs recognized by Bal31 with sites of DNA synthesis occurs in matrix attachment regions (MARs) maintained by strong interactions with chromatin fibrils, defining supraloop chromatin domains of 0.5 − 1.3 Mbp, that we and others have described previously [Khodarev et al., 1997]. Further cleavage during apoptosis leads to the formation of 50 kbp fragments. However, such cleavage is not targeted to regions of replication as their termini are not enriched in nascent DNA. Nevertheless, such fragments may contain a subset of functionally active replicons in that these apoptotic fragments are enriched in nascent DNA within their internal structure.

MATERIALS AND METHODS

RPMI 1640 medium and antibiotic solutions were purchased from Sigma (St. Louis, MO), fetal calf serum was from Biologos (Naperville, IL). Aphidicolin and mimosine were purchased from Sigma and ^{3}H and ^{14}C-thymidine from NEN- Dupont (Boston, MA). Nuclease Bal 31 and appropriate buffer were purchased from Promega (St. Louis, MO), DNase I -from Worthington (Freehold, NJ), low melting agarose and electroelution-grade agarose were from FMS (Philadelphia, PA). Fish sperm DNA was from Boehringer Mannheim (Indianapolis, IN) and Proteinase K was from Oncor (Gaithersburg, MD).

Cell Cultures

The human B-lymphoblastoid cell lines TK6 and WI-L2-NS have been described previously, and both share a similar genetic background [Amundsen et al., 1993]. The WI-L2-NS cell contains a point mutation within the p53 coding region, however, and shows reduced competence in accessing radiation induced apoptosis compared to TK6 [Zhen et al., 1995]. Cell cultures were maintained at 37°C in 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and $5x10^{-5}$ M 2-mercaptoethanol. For induction of apoptosis, cells were grown to a concentration of $5x10^{5}$ cells/ml, washed, and resuspended in fresh complete media at a concentration of 10^{6} cells/ml. Cells were then irradiated with γ rays at a dose rate of 1.3 Gy/min using a dual head ^{137}Cs Gammacell 40 irradiator (Nordion International, Kanata, Ontario, Canada). Cells were routinely checked for mycoplasma contamination using a PCR-based Mycoplasma detection kit according to the manufacturers instructions (ATCC, Rockville, MD).

Analysis of Apoptosis

For rapid measurement of the total number of dead cells, the trypan blue exclusion assay was performed as described elsewhere, which in this system gives an indication of the number of apoptotic cells produced [Wu et al., 1993]. For a more detailed determination of the mechanism of cell death, both morphological and DNA fragmentation based flow cytometry analyses were performed.

Flow Cytometry

Freshly prepared cells were pelleted, washed in PBS, and resuspended in a buffer containing PBS, 4 mg/ml BSA, 0.4% NP-40, 50 µg/ml propidium iodide. Flow cytometry analyses were performed using a FACStar^{Plus} (Becton Dickinson, Mountain View, CA) cytometer to determine those cells containing sub-G_1 content of DNA, which is consistent with apoptotic DNA fragmentation and subsequent elimination of soluble DNA products [Zhen et al., 1995].

DNA Gel Electrophoresis

To observe directly DNA fragmentation within the cells under study, $1x10^{6}$ cells were lyzed on ice for 2 h in 500 µl of buffer containing 10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% Triton X-100, followed by centrifugation at 24,000 x g for 20 min. The supernatant was treated with

RNase A, followed by proteinase K treatment (50 µg/ml, 37°C − 60 min). DNA was extracted with chloroform/isoamyl alcohol and precipitated with ethanol. The DNA was then dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 µg DNA loaded on 1.2% agarose gels and subject to electrophoresis in TAE buffer for 2 h at 100 V.

DNA Labeling

Cells were placed in fresh media at a concentration of 0.5×10^5/ml. ^{14}C-thymidine (59.7 mCi/mmol) was then added to a final concentration of 0.01 mCi/ml and cells returned to culture for a further 48 h. Typically cell cultures reached a concentration of 0.5×10^6 cells/ml. At this stage cells were centrifuged, washed, and resuspended in serum-free media; ^{3}H-thymidine (16.3 Ci/mmol) was added to final concentration of 0.1 mCi/ml and cells incubated for a further 10 min at 37°C followed by addition of aphidicolin (Sigma, St. Louis, MO) to final concentration of 5 µM. The cells were then washed twice in PBS containing the same concentration of aphidicolin. For samples that were to be irradiated, after 48 h of incubation with ^{14}C-thymidine the cells were transferred in isotope-free medium, irradiated at 6 Gy, and after different periods of time pulse-labeled with ^{3}H-thymidine as described above. Washed and pelleted cells were kept on ice at −20°C. Subsequent incorporation of radioactivity was measured in 3a70B scintillation cocktail (Research Products International, Mt. Prospect, IL) using a Beckman LS 6500 beta-spectrometer (Beckman Instruments, Fullerton, CA), calibrated for dual-mode counting (^{3}H/^{14}C) according to manufacturer's instructions. After purification of DNA (see above) from double-labeled cell cultures, no changes of ^{3}H/^{14}C-ratios were found in the samples with increasing amount of DNA, over the range 100 ng to 2 µg of DNA per sample (data not shown). Therefore, in subsequent experiments the direct measurement of ^{14}C was used as an indicator of total DNA, and the ratio of ^{3}H to ^{14}C used as a measure of the proportion of nascent to bulk DNA in the entire sample.

Field-Inverted Gel Electrophoresis (FIGE)

Cells (between $1–2 \times 10^6$) were centrifuged, washed, and resuspended in 100 µl PBS and mixed with an equal volume of 2% low melting agarose at 37°C. Subsequently the plugs were incubated in lysis buffer (1% lauroylsarcosine, 0.5M EDTA, 10 mM Tris, pH 7.5) in the presence of 0.4 mg/ml Proteinase K. Incubation was performed at 50°C for 24 h and plugs were then washed in washing buffer (0.5xTBE, 50 mM EDTA) at 4 °C. After rinsing in the same washing buffer, the plugs were loaded into 1.2% agarose gels, in 0.5 xTBE buffer. Separation of DNA fragments was performed at 100 V, 25 mA, 4°C, with the forward to reverse vectors 3 : 1 for times between 16 and 24 h (MJ Research, Watertown, MA). Gels were stained with EthBr, photographed under UV, and bands were electroeluted in 422 Electro-Eluter (Bio-Rad, Richmond, CA). Extracted DNA was mixed with fish sperm DNA to a final concentration of 50 µg/ml and used in subsequent nuclease experiments, as described below.

Isolation and Cleavage of Cell Nuclei

Cell nuclei were isolated as described previously, except that 5 µM aphidicolin was added to all buffers [Sokolova et al., 1995]. Freshly prepared nuclei were washed in TCS - 0.25 (50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 0.25 M sucrose) and resuspended in storage buffer (50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 0.25 M sucrose, 50% glycerol) or frozen at −20°C for subsequent DNA purification. DNA was purified from isolated nuclei essentially as described above for DNA laddering. For determining the amount of DNA in nuclear preparations, samples were dissolved in 0.1 N NaOH/0.1% SDS and DNA concentration calculated assuming that $1A_{260}$ = 50 µg DNA/ml. For nuclei cleavage by endogenous endonuclease(s), preparations were washed twice in TCMS buffer (50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.25 M sucrose) and incubated in the same buffer for between 5 and 90 min (see text). Digestion was stopped either by placing nuclei on ice and pelleting high molecular weight DNA in absolute ethanol at −20°C (for measurement of soluble radiolabeled DNA) or by mixing nuclei with an equal volume of lysis buffer/low melting agarose. Soluble DNA has been used as a measure of chromatin cleavage by various nucleases. It was calculated as $(^{14}C_s/^{14}C_t) \times 100$, where $^{14}C_s$ corresponds to ^{14}C counts (in dpm) in soluble fraction after precipitation of bulk DNA by ethanol, and $^{14}C_t$ corresponds to total ^{14}C counts (in dpm) per sample. For cleavage of nuclei with DNase I they were washed as above and transferred into TCMS buffer; DNase

I was added, and incubations were carried out at 37°C between 5 and 30 min (see text). Soluble products were determined after ethanol precipitation, as described above. For Bal 31 cleavage, nuclei were washed twice in TCMS buffer and the pellet carefully resuspended in 0.4 M NaCl, 10 mM Tris, pH 8.0, 1 mM $CaCl_2$, and 2 mM $MgCl_2$. Bal 31 was added and incubations were carried out between 5 and 60 min. Soluble DNA was determined, as above. Samples of purified DNA were treated identically except that carrier DNA was added to samples (fish sperm DNA, see above; final concentration 50 μg/ml) and enzyme/substrate ratios were changed. These ratios are as follows: for purified DNA Bal 31–0.05 units/μg DNA, DNase I − 0.01 μg /μg DNA. For nuclei − Bal 31–0.15 units/μg DNA, DNase I − 0.7 μg/μg DNA.

Statistical significance of data was estimated by the Student's *t*-test. All error bars shown on graphs are standard deviations.

RESULTS

Irradiation of TK6 But Not WI-L2-NS Cell Lines Is Accompanied by Apoptotic Death and High Molecular Weight DNA Fragmentation

Irradiation of TK6 cells leads to a dose-dependent increase in cell death as determined by the trypan blue-exclusion assay (see Fig. 1A). Parallel analysis of cellular morphology and DNA fragmentation as viewed by flow cytometry confirmed that the mechanism of cell death was through apoptosis, as previously observed [Zhen et al., 1995]. By plotting the proportion of sub G1 material visualized by flow cytometry against trypan blue exclusion a linear correlation was found ($r^2 = 0.77$ − 13 measurements). Thus for routine use, trypan blue staining was taken as an indicator of apoptosis. Quantitatively, 24 h after irradiation with 6 Gy, 36% of the TK6 population showed signs of apoptosis, whereas only 15.5 % of the WI-NS-L2 population showed the same features. It has been shown that the p53 status of these two cell lines correlates with the ability to undergo DNA damage dependent apoptosis, wild-type p53 facilitates access to apoptosis in TK6, restricted in the mutant p53 WI-NS-L2 cell line [Zhen et al., 1995; Carrier et al., 1996]. Further, the fate of these cell cultures after irradiation were examined by FIGE for the presence of high molecular weight DNA fragmentation (Fig. 1B). After irradiation, the dose-dependent formation of 50 kbp fragments in TK6 cells was observed without any detectable signs of this DNA fragmentation in the WI-L2-NS cells. By varying the conditions of FIGE [Walker et al., 1993], it was possible, however, to detect formation of 200–400 kbp fragments in both the TK6 and WI-L2-NS cell lines (data not shown).

Purified DNA and Isolated Cell Nuclei Are Randomly Cleaved by DNase I

Cells were uniformly labeled with ^{14}C-thymidine and pulse-labeled with ^{3}H-thymidine as described in Materials and Methods. Samples of nuclei or purified DNA were subject to direct cleavage by DNase I. Figure 2A shows that isolated DNA is cleaved by DNase I with the formation of soluble products; however, the relationship of these products to regions of DNA replication appears to be random as the ratio of ^{3}H to ^{14}C was unchanged between 5 and 30 min of incubation. The same results were obtained using isolated nuclei as substrate for DNase I except that the fraction of solubilized DNA was approximately fourfold lower under comparable incubation conditions (Fig. 2B). Therefore, we concluded that both in isolated nuclei and purified DNA, DNase I cleavage does not recognize nascent DNA. For purified DNA, the most likely interpretation is that this treatment led to random endonucleolytic cleavage of DNA sequences, as described previously [Lown et al., 1986]. For nuclei cleavage we suggest that formation of DNase I hypersensitive sites does not co-localize with sites of DNA replication (see below).

Bal 31 Can Recognize and Cleave Nascent DNA From Both Nuclei and Purified DNA Preparations

Bal 31 digestion of purified DNA led to a 2.5-fold enrichment of solubilized DNA by pulse-labeled (nascent) sequences both in TK6 and WI-L2-NS cell lines; no significant differences in the amount of eluted DNA were appreciated between the two lines (Fig. 3). Thus in the purified DNA preparation used here, nascent DNA is associated with Bal 31 accessible DNA double strand breaks. A similar conclusion was reached using isolated nuclei as the substrate, although as for DNase I digestion, the total amount of DNA released was reduced compared to soluble DNA (Fig. 4A,B). Therefore, we concluded that isolated nuclei from these cell

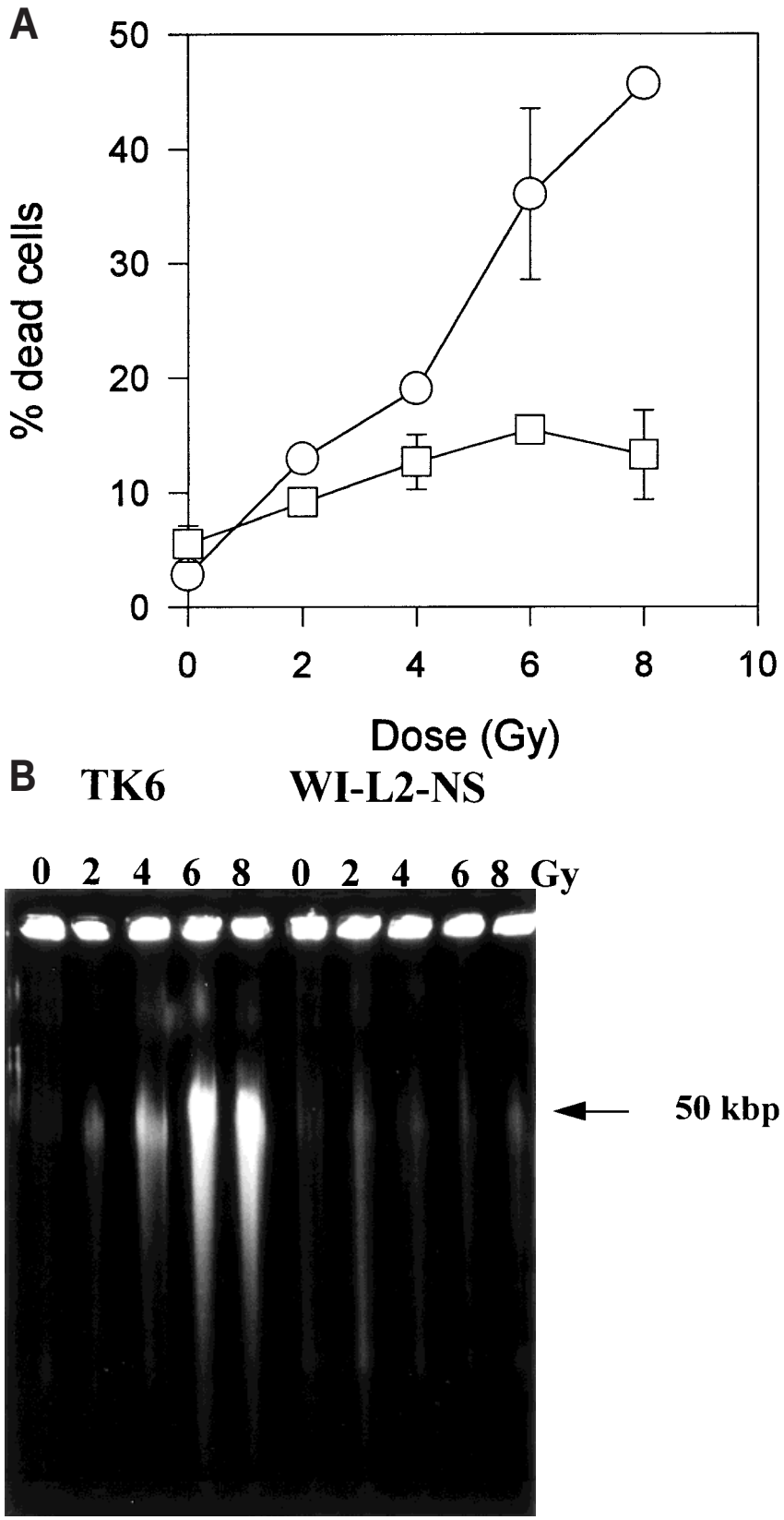

Fig. 1. **A**. Accumulation of dead cells, measured by Trypan blue exclusion, as a function of radiation dose in apoptotic sensitive cells, TK6 (circle) or resistant, WI-L2-NS (square). **B.** Measurement of DNA fragmentation in the same cells exposed to radiation doses between 2 and 8 Gy and analyzed 24 h later. Only in TK6 cells is significant fragmentation leading to 50 kbp products observed.

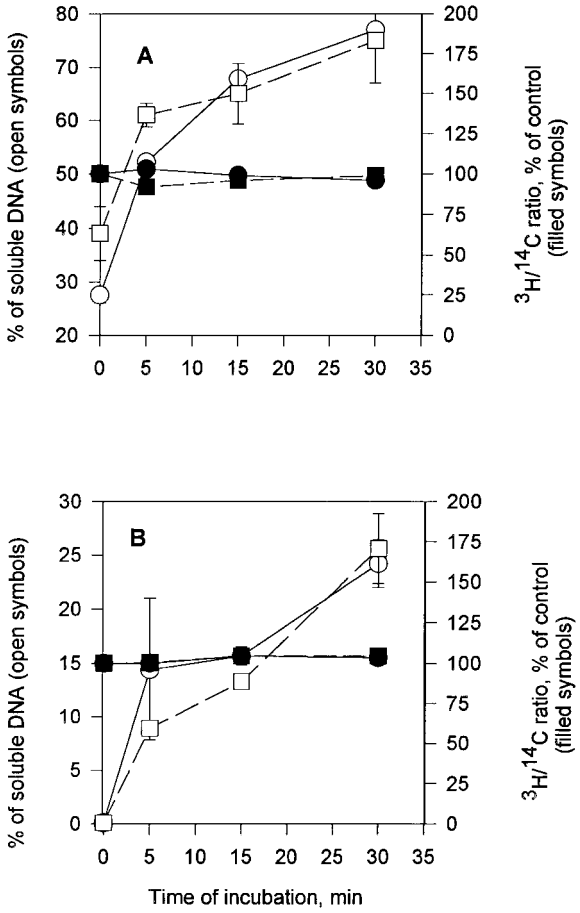

Fig. 2. Random cleavage of nascent sequences in purified DNA (**A**) and isolated nuclei (**B**) by DNase I. Shown is % soluble DNA (see Materials and Methods) released by DNase I (left axis) TK6 (blank circle) and WI-L2-NS (blank square) compared to the ratio of nascent to bulk DNA released (right axis) TK6 (filled circle) or WI-L2-NS (filled square).

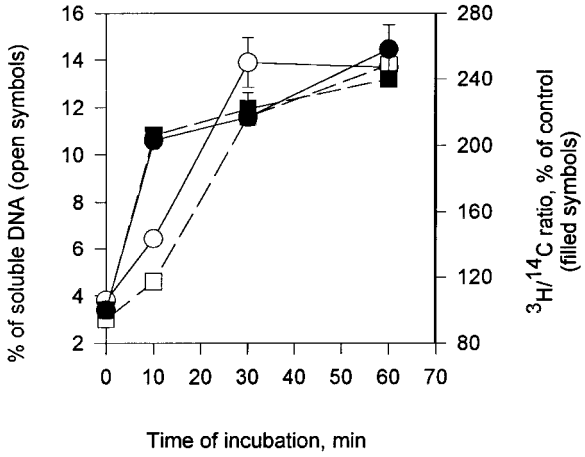

Fig. 3. Specific cleavage of nascent sequences in purified DNA by nuclease Bal 31. TK6 and WI-L2-NS cells were pulse-labeled and DNA purified as described. Bal 31 was added in amounts of 0.15 units/μg DNA and incubation was performed during either 10, 30,or 60 min. Symbol notation, TK6 (blank circle) and WI-L2-NS (blank square) and ratio of nascent to bulk DNA given by TK6 (filled circle) or WI-L2-NS (filled square).

lines contain regions linked to DNA replication that are targets for Bal 31 nuclease. Due to the specificity of Bal 31 cleavage, these sites are presumably dsbs, susceptible to the exonucleolytic cleavage of Bal 31, but not the endonucleolytic action of DNase I (see Discussion).

Apoptotic Chromatin Fragmentation Decreases Bal 31-Dependent Cleavage of Nascent DNA

To determine if DNA undergoing apoptotic fragmentation is cleaved at sites associated with DNA replication, both cell lines were irradiated and used 24 h later when apoptotic DNA fragmentation was evident in TK6, but to a much lesser extent in WI-L2-NS (see above). Nuclei isolated from apoptotic TK6 cells contained a five fold increase in Bal 31 cleavable DNA compared to those from WI-L2-NS cells (Fig. 4C). This result was thought to be due to the presence of Bal 31 accessible double strand breaks inserted in TK6 nuclei as a result of apoptotic cleavage events. Further analysis showed that in apoptotic nuclei from TK6 the proportion of nascent DNA released during the Bal 31 digestion was significantly decreased compared to both nuclei from irradiated WI-N2-LS cells and control unirradiated cells of either type (compared D with B, Fig. 4). This was thought to be due to the presence of an excess of apoptotic DNA double strand breaks being inserted in DNA at sites distant to that of nascent DNA. Addition of Bal 31 produced digestion of these sites in addition to those sites of replication described above, producing a relative reduction in the proportion of newly replicated DNA in the Bal 31 soluble fraction.

To verify this possibility a model system of nuclei autodigestion was used where isolated cell nuclei are incubated under conditions which favor activation of Ca^{2+} and/or Ca^{2+}/Mg^{2+} metallo-dependent nuclear endonucleases. These enzymes have been linked to apoptotic fragmentation and have been used to model the chromatin cleavage stage of apoptosis [Filipski et al, 1990; Montague and Cidlowski, 1996]. For both cell lines cleavage of chromatin DNA under these conditions led to an increase in the number of sites that are recognized and cleaved by Bal 31 nuclease (Fig. 5). After 30 min of

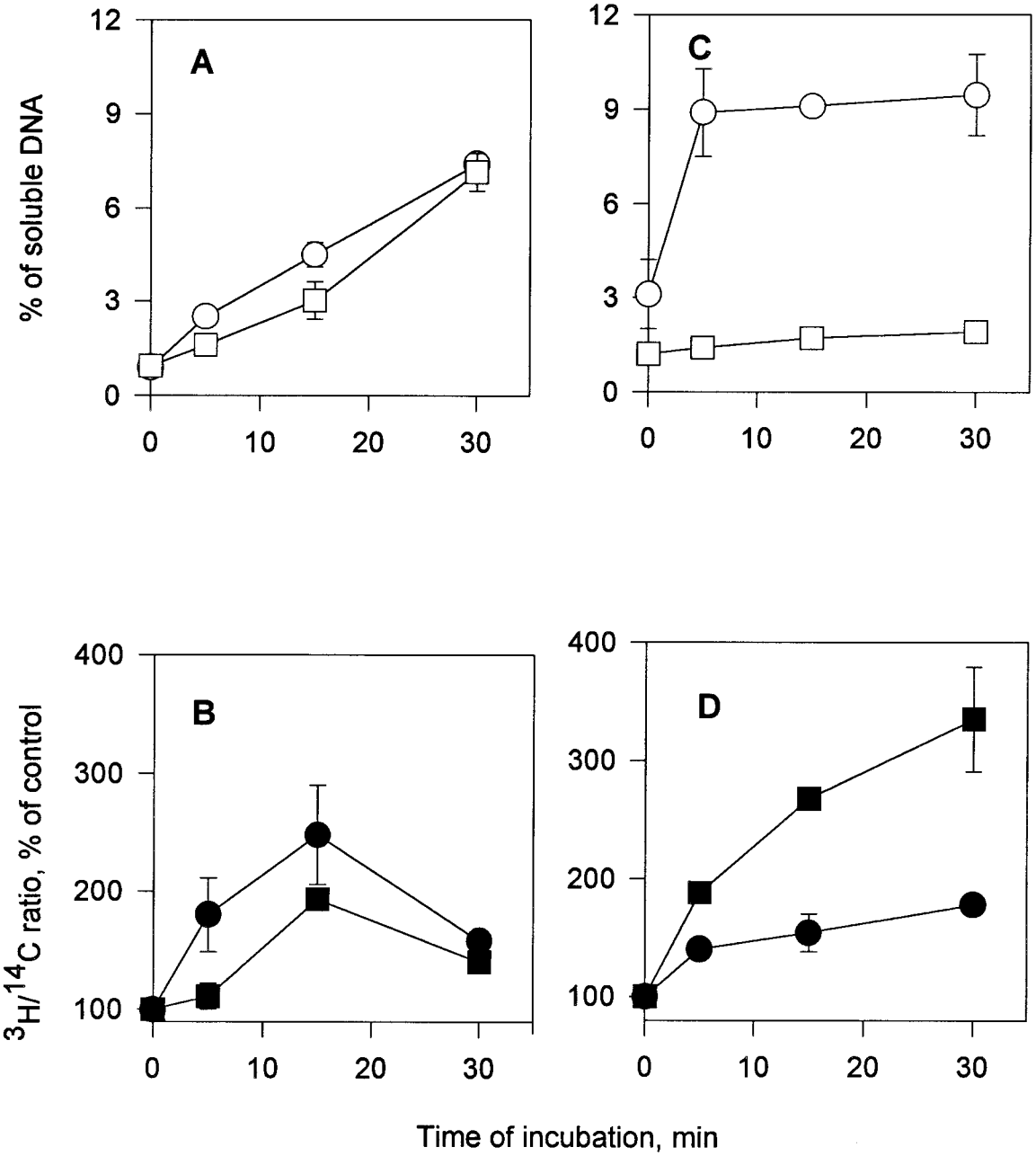

Fig. 4. Cleavage by Bal 31 of bulk DNA compared to nascent DNA in isolated nuclei taken from either control or irradiated cells. In **A and B** data from control cells; **C and D** data from cells 24 h after irradiation with 6Gy. Symbol notation TK6 (blank circle) and WI-L2-NS (blank square) and ratio of nascent to bulk DNA given by TK6 (filled circle) or WI-L2-NS (filled sqaure).

autodigestion, the amount of Bal 31 solubilized DNA increased about 3-fold compared with unincubated nuclei (Fig. 5A). The amount of nascent DNA in the solubilized fraction decreased from an initially high level in control nuclei to levels comparable to that observed in apoptotic nuclei (Fig. 5B,C). Thus endonucleolytic cleavage of chromatin both in apoptotic cells and in isolated cell nuclei occurs at sites distant from replicating DNA.

Distribution of Nascent DNA in Apoptotic Chromatin Fragments After Electrophoretic Separation

As discussed above, only TK6 cells showed the appearance of 50 kbp fragments after irra-

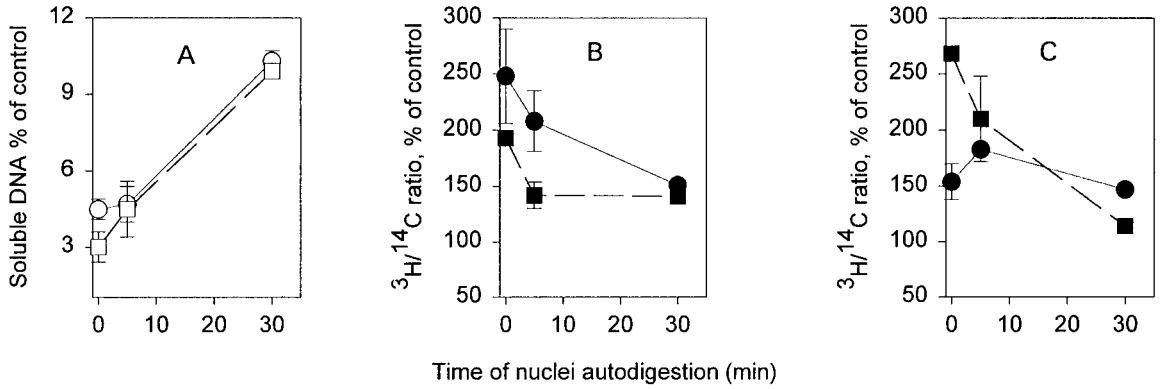

Fig. 5. Autodigest of chromatin in isolated nuclei leads to the introduction of dsbs, unrelated to nascent sequences. Control or irradiated TK6 and WI-L2-NS cell lines were pulse-labeled with ^{3}H-thymidine for 15 min as before. Nuclei were isolated, washed twice in TCMS buffer,and incubated in the same buffer to allow autodigestion for 0, 5,and 30 minutes (see text). Immediately after autodigestion nuclei were exposed to Bal 31 cleavage for 15 min. **A**. Total solubilized DNA. Ratio of nascent DNA to bulk solubilized from either control (**B**) cells or irradiated (**C**) cells. Symbol notation - TK6 (circle) and WI-L2-NS (square).

diation (see above) and therefore only this line was studied here for distribution of nascent DNA in these fragments. TK6 cells were irradiated and pulse labeled as described in Materials and Methods and subjected to FIGE analysis. Gels were stained with EthBr and incremental 1 cm slices excised from different stained regions of the gel (see Fig. 6). DNA was subsequently eluted from the gel slices and the radioactivity content determined as described in Materials and Methods. Figure 6 shows that the 50 kbp fragments produced were enriched with nascent DNA. Both larger and smaller DNA fragments as determined by their position in the gel had nascent DNA content comparable with control material. To pursue the nature of nascent DNA enrichment in the 50 kbp fragments, the DNA from this region was isolated by electroelution and subject to exonucleolytic digestion by Bal 31 (Fig. 7). No increase in $^3H/^{14}C$ ratios was detected in fractions treated with enzyme over a 30-min period. This indicates that the 50 kbp fragments do not contain replicating DNA at their ends, but must therefore contain significant amounts of nascent DNA within their internal regions.

DISCUSSION

In this report, we address the relationship between apoptotic high-molecular weight DNA fragmentation and the distribution of DNA replication regions. The rationale to investigate these relationships is provided by the size similarity between chromatin elements involved in DNA replication (replicons and replicon clusters) and the 50 and 300–1000 kbp fragments produced during apoptotic cleavage [Filipski et al., 1990; Cook, 1995; Kunnev et al., 1997]. More recently, the reversible nature of HMWDNA fragmentation has been described and this finding may depend on the nature of chromatin structures that are disrupted by this cleavage [Beer et al., 1995; Solovyan et al., 1997]. Additionally, it has been reported that apoptosis targets specific components of the DNA replication process for proteolytic digestion but the functional significance of this is unclear [Kaufmann, 1989; Song et al., 1997; Gohring et al., 1997]. It has been shown that irradiation suppresses the DNA replication process, mostly due to inhibition of DNA replication initiation [Lavin et al., 1989]. If irradiation induced apoptosis specifically targets the regions of DNA replication, this may in part explain the shutdown of nuclear replication and specific alterations within the cell cycle after application of apoptotic stimuli [Neiman et al., 1994; Sanchez and Elledge, 1995].

The generation of HMW DNA fragmentation precedes the commonly observed DNA laddering stage where multiples of the nucleosomal units are formed. HMW fragmentation of DNA has been shown to contain at least three products, separately identifiable by size ~1 Mbp, 300–500 kbp, and 50 kbp; it is possible that the latter are precursors of oligonucleosome sized fragments [Walker et al., 1993]. However, the precise relationship between HMW fragments produced by the apoptotic process is not known. It has been suggested that the 300–400 kbp

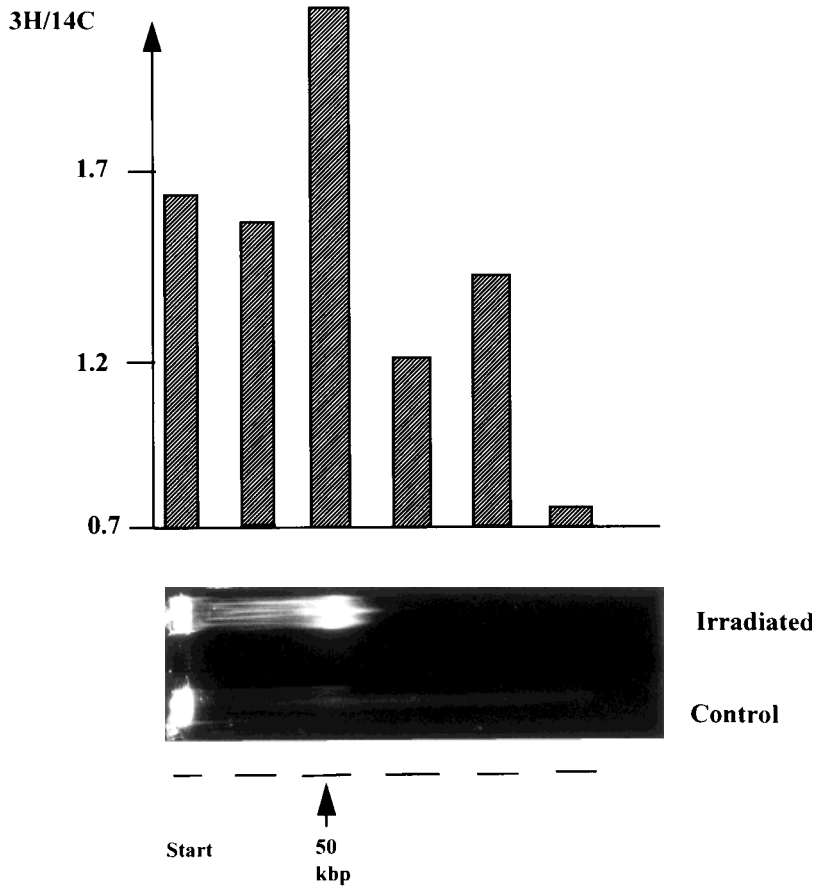

Fig. 6. Distribution of the nascent sequences in FIGE-electrophoresis. TK6 cells were irradiated with 6 Gy and pulse-labeled, as described in Materials and Methods followed by field inversion electrophoresis (bottom panel). Shown above is the proportion of nascent to bulk DNA corresponding to each portion of the separated DNA. Only in the 50 kbp region was elevated levels of nascent DNA seen.

fragments are not the precursors of 50 kbp structures, such that each class may have separate morphological and biochemical origins requiring unique endonuclease(s) for their generation [Sestili et al., 1996]. In the present work we studied the cleavage of control and apoptotic cell nuclei by DNase I, Bal 31 and endogenous Ca^{2+}/Mg^{2+}-dependent endonuclease (CMDE) in an attempt to determine the relationship between apoptotic DNA fragmentation and the DNA replication process.

DNase I cleaved both cell nuclei and purified DNA with no selectivity for nascent DNA, as shown by the ratio of pulse labeled DNA (^3H) to bulk labeled DNA (^{14}C) in solubilized DNA fractions (Fig. 2). These results are consistent with the reported absence of sequence specificity of DNase I cleavage of purified DNA [Lown et al., 1986]. Specifically, these data show that in our experimental system, hypersensitive chromatin sites that are targets for DNase I action do not coincide with nascent DNA sites. This was not the case when the nuclease Bal 31 was used to similarly digest pulse labeled nuclei or purified DNA. Bal 31 is a nuclease with mostly exonucleolytic mode of cleavage and is active at both the 3′ and 5′ ends of double stranded DNA [Lu and Gray, 1995]. Both in untreated TK6 and WI-L2-NS cells, this enzyme preferentially released nascent DNA from isolated nuclei and purified DNA; thus the cut sites that are targets for this enzyme contained nascent DNA in their flanking regions. The existence of dsbs in these regions may be correlated with the formation of 0.3–1.3 Mbp DNA fragments that are reversibly generated in a variety of systems [Beere at al., 1995; Solovyan et al., 1997]. Alternatively, the presence of such breaks in the DNA may be connected with the basal level of apoptosis, which is observed in both cell cultures without application of specific stimuli (5–7% of these cell populations, see Fig. 1).

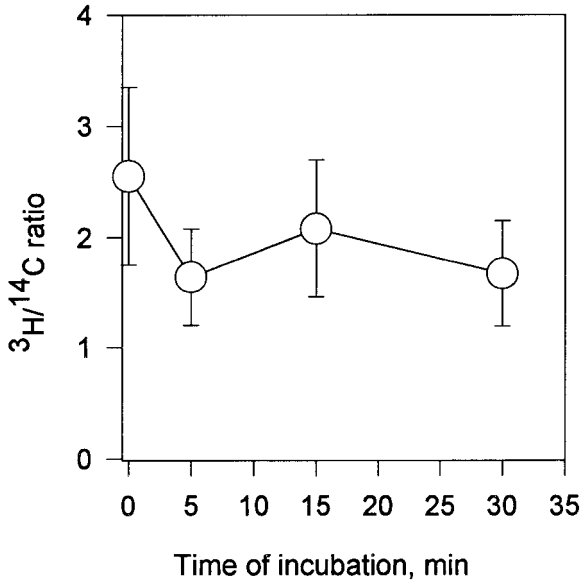

Fig. 7. 50 kbp fragments do not contain nascent DNA in flanking sequences. The 50 kbp fragments shown in Figure 6 were electroeluted, mixed with fish sperm carrier DNA, and cleaved by Bal 31 nuclease, total nascent ^3H and bulk DNA ^{14}C counts were determined and the ratio between them calculated. No significant change in ratio was noted over a 30 min Bal 31 digestion period indicating that nascent DNA was not specifically located at the fragments termini.

However, for these control cultures no accumulation of 50 kbp and nucleosomal material has been detected and DNA fragments that may be extracted from isolated nuclei are of higher molecular weight (data not shown). The development of extensive apoptosis in TK6 cells after irradiation leads to the formation of 50 kbp fragments and low molecular weight material (Fig. 1). Exposure of these apoptotic nuclei to Bal 31 led to an increase in soluble DNA (Fig. 4) compared to both control nuclei and nuclei taken from similarly irradiated but nonapoptotic WI-L2-NS cells. This indicates an increase in the number of dsbs in the apoptotic TK6 nuclei. The majority of these breaks induced by apoptosis were, however, not targeted to the sites of DNA synthesis as the digested DNA contained a decreased proportion of nascent DNA compared to control material. Two possibilities were considered to explain these data. First, the distribution of apoptotic cuts does not target regions enriched in replicating DNA. Second, the apoptosis machinery selectively excises nascent DNA sequences, subsequently reducing the proportion of newly synthesized DNA remaining in the cell nuclei. In an attempt to resolve these two possibilities, a model system of nuclei autodigestion was employed that permits greater control over both the pulse labeling and nuclease excision processes. Here, extracted nuclei were allowed to autodigest their DNA using endogenous endonuclease(s) activated by buffers supporting Ca^{2+} and Mg^{2+}-dependent endonuclease (CMDE) digestion. This approach has been used by others to model the fragmentation phase of apoptosis [Filipski et al., 1990; Cain et al., 1995]. The autodigest of isolated nuclei, shown in Figure 5, indicates an accumulation of Bal 31 cleavable sites leading to the release of DNA. The progress of chromatin cleavage was, however, always associated with a decrease in the proportion of nascent DNA released. These results show that during chromatin solubilization within the autodigest procedure, DNA cuts introduced into chromatin by endogenous CMDE are not localized within regions of DNA replication. Under the conditions of nuclei incubation used, chromatin cleavage led mostly to the accumulation of 50 kbp fragments, which is consistent with the data from cells undergoing radiation-induced apoptosis. We also observed accumulation of solubilized DNA during incubation of nuclei without treatment by Bal 31; however, the amount of this DNA did not exceed 1–2% of total and was not the major cause of observed differences in $^3H/^{14}C$ ratios in solubilized DNA (see above).

The initial premise of this study was that the 50 kbp DNA fragments produced during apoptosis are equivalent to replicon structures. Thus it was possible that such DNA regions might contain within them newly replicated DNA, even though the nucleases studied here do not cut at these locations. Analysis of the 50 kbp fragments for the presence of nascent DNA showed that compared to all other DNA extracted from apoptotic cells by electrophoresis, the 50 kbp band was most enriched in nascent DNA (Fig. 6). Subsequent digestion of 50 kbp fragments in vitro by Bal 31, which targets their termini, showed no specific enrichment for nascent DNA (Fig. 7). Thus we concluded that in the 50 kbp fragments, nascent sequences are located internally and might reflect the correspondence of 50 kbp fragments with actively replicating structures (replicons). The digestion of nascent DNA in control nuclei by Bal 31, however, does recognize sites of nascent DNA and this may be at the level of replicon clusters or domains. Based on investigations of nuclear DNA supercoiling, we have

previously suggested that two types of chromatin-nuclear matrix interactions determine supranucleosomal chromatin folding [Khodarev et al., 1997]. These are the strong and weak interactions with the nuclear matrix. Strong interactions between DNA and the nuclear matrix lead to the formation of topologically fixed structures, which may be identified by the loss of their supercoiling after insertion of a single strand break. We have shown that these topological domains are approximately 0.5 and 1.3 Mbp in size, which is consistent with other data reported in the literature [Khodarev et al., 1997]. Weak interactions lead to the formation of smaller (~ 50 kbp) structures that are fluid in organization, thus they may appear during genome functioning such as replication and transcription and seem not to have unique interactions with the nuclear matrix. The presence of such structures may explain the easy transfer of torsional stress between adjacent loops [Milner et al., 1993], the remote action of some enhancers [Barton et al., 1997], and other effects involving the cross-talk between loops [Marshall et al., 1996; Cook, 1997]. We propose that the initial excision of nascent DNA studied here represents targeting of regions linked to the initiation of DNA synthesis in matrix-attached replicating factories [Cook, 1995; Jackson, 1995]. Such structures can topologically restrain tracts of DNA between 0.5–1.3 Mbp and may include 5–20 replicons [Jackson, 1995; Kunnev, 1997]. In terms of apoptotic fragmentation it appears the units of supercoiled loop DNA, including active replicons that are subunits of such structures, are targets for chromatin disassembly. Therefore the organization of this fundamental repeat structure of eukaryotic DNA organization is a critical factor that separately impacts processes as diverse as replication and apoptotic fragmentation. Experiments are now in progress to map these regions within individual genomic loci.

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