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ACCELERATED COMMUNICATION

Induction of Megabase DNA Fragments by 5-Fluorodeoxyuridine in Human Colorectal Tumor (HT29) Cells

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SUMMARY

Current evidence suggests that DNA fragmentation plays an integral role in mediating cytotoxicity that results from thymidine nucleotide depletion ("thymineless death"). Recently, Ayusawa *et al.* [*Mutat. Res.* **200**:221–230 (1988)] reported that dTMP starvation induces cellular processes that result in the release of 50–200-kilobase (kb) DNA fragments in FM3A cells, as detected by pulsed field gel electrophoresis (PFGE). The present study was undertaken to determine whether a similar DNA fragmentation process occurs in a human cell line in response to fluoropyrimidine treatment and, if so, to quantitate this process. When human colorectal tumor (HT29) cells were treated with 100 nM 5-fluorodeoxyuridine (FdUrd), this regimen induced the formation of high molecular weight DNA fragments, which were analyzed using three different PFGE protocols. Field inversion PFGE revealed that, in contrast to the discrete size range reported for FM3A cells, FdUrd-induced fragments in HT29 cells were broadly distributed from about 50 kb to sizes beyond the resolution of the field inversion mode (i.e., >600 kb). Analysis of these same samples by contour-clamped homogeneous electric field PFGE showed that the bulk of these fragments migrated in the 1–5-megabase region. In contrast, fragments from DNA that was broken randomly by γ-radiation appeared to be primarily in a zone corresponding to approximately 5–10 megabases. Equitoxic FdUrd and radiation treatments (100 nM FdUrd for 48 hr versus 10-Gy γ-radiation) each increased the fraction of DNA entering the gel from about 0.07–0.09 (untreated cells) to about 0.22–0.25. To a first approximation, the time course and quantity of DNA fragmentation induced by 100 nM FdUrd appeared to correlate with the loss of clonogenicity within the 48-hr period analyzed. These findings suggest that the processes responsible for DNA fragmentation in response to a thymineless state may be different in FM3A and HT29 cells, that in both cases the breaks caused do not appear to be located randomly with respect to the entire genome, and that these processes may be related to the chain of events by which temporary dTMP starvation is made into a lethal insult.

It is well established that, in the absence of exogenous thymidine, both eukaryotic and prokaryotic cells will die if they cannot synthesize thymidylate (1). This phenomenon of "thymineless death" can be induced by various means, including administration of the clinical antitumor agents 5-fluorouracil and FdUrd, whose common metabolite, FdUrd monophosphate, is a potent inhibitor of thymidylate synthase (2). Although it is predictable that DNA synthesis and cell growth will be inhibited while thymidine nucleotides are unavailable, it is not obvious how or why transient DNA synthesis inhibition should cause cells to permanently lose their capacity to proliferate.

Many studies have associated DNA strand breakage with thymineless death, and in the past decade some specific mechanisms have been put forth to account for the formation of these breaks. It has been demonstrated that antifolate-induced inhibition of thymidylate synthase activity results in incorporation of uracil into DNA in human cells (3, 4). Goulian and co-workers (5) have proposed that this misincorporation of uracil activates a futile repair cycle whereby uracil is excised only to be replaced by more uracil, generating sites of constant DNA excision/repair and, eventually, DNA strand breaks resulting from failure of the repair process. The concept that DNA damage and cytotoxicity are related to the ratio of dUTP/dTTP, rather than just the absolute level of dTTP itself, is supported by experiments in which these parameters were manipulated independently (6).

Although the uracil-excision scheme is an attractive one, other mechanisms may also play an important role in converting thymidine nucleotide depletion into DNA damage. By using thymidylate synthase-negative mutants of mouse FM3A cells, Ayusawa *et al.* (7) were able to induce a thymineless state in which no accumulation of dUMP and dUTP would be expected.

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ABBREVIATIONS: FdUrd, 5-fluorodeoxyuridine; CHEF, contour-clamped homogeneous electric field; FIGE, field inversion gel electrophoresis; PFGE, pulsed field gel electrophoresis; kb, kilobase; Mb, megabase.

Under these conditions they observed both cytotoxicity and DNA strand breaks, with only a minor amount of uracil being incorporated into DNA. Furthermore, a similar fragmentation profile was induced in wild-type FM3A cells upon treatment with 2-chlorodeoxyadenosine, an agent that induces deoxyribonucleoside triphosphate pool size imbalances independently of a direct effect on thymidylate synthase activity (8). The most striking feature of these studies was the demonstration that the DNA fragments formed appeared to be in a relatively discrete size range (50–200 kb). Based on these and other observations, a mechanism was proposed in which imbalance of deoxyribonucleoside triphosphate pools induces expression of a gene coding for an endonuclease, which is ultimately responsible for creating double-strand breaks (9).

Because one of the major therapeutic targets of agents that induce thymineless death is colorectal cancer and because the degree to which this fragmentation process may vary among cell types has not yet been reported, we initiated the present study in order to characterize the pattern of large DNA fragment formation in a human colorectal tumor (HT29) cell line treated with FdUrd.

Materials and Methods

Cell culture and drug treatments. HT29 cells were cultured as monolayers in McCoy's 5A medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), at 37°, in a humidified atmosphere containing 5% CO_2. These cells were tested for *Mycoplasma* at least once every 3 months. All experiments shown here were performed with cells shown to be *Mycoplasma* negative. FdUrd (Sigma) stocks were made in double distilled water and frozen at −20° for no longer than 2 months. FdUrd concentrations were verified by UV spectrophotometry [assuming ϵ = 6480 at 270 nm, in 0.1 N NaOH (10)]. All FdUrd treatments were performed with media supplemented with dialyzed fetal bovine serum. Medium was changed every 24 hr for incubations greater than 24 hr. For each experiment, cells were subcultured by trypsinization and plated at a density of 20,000 cells/cm² for cloning assays or 7,500 cells/cm² for [^{14}C]-thymidine labeling procedures. Cells were allowed to recover for at least two doubling times (one doubling time ≈ 22 hr) before the addition of drug or radioactivity. Cloning assays were performed by trypsinizing cells and replating appropriate dilutions in six-well culture dishes containing medium supplemented with 10 μM thymidine. Cells were fixed and stained after 12–14 days of incubation and were scored for colonies containing at least 50 cells. Prelabeling of cellular DNA for quantification of DNA fragmentation consisted of a 48-hr incubation with [^{14}C]thymidine (0.03 μCi/ml; specific activity, 56 mCi/ml; Morovek) followed by a 24-hr chase period before FdUrd treatment.

Preparation of cells for PFGE analysis. Cells were embedded in agarose blocks, as described previously (11). Briefly, cells were harvested by trypsinization, washed in Hanks' balanced salt solution (GIBCO), and resuspended to a concentration of 3 × 10^7 cells/ml. An equal volume of 2% low melting point agarose (BRL) prepared in Hanks' balanced salt solution and equilibrated at 45–50° was added, and the agarose-cell suspension was then poured into molds. Cells embedded in agarose were then digested in a solution containing 0.5 M EDTA, pH 8.0, 1% sarkosyl, and 1 mg/ml proteinase K (Sigma), for at least 24 hr at 50°. DNA agarose blocks were stored in 10 mM Tris·HCl, 50 mM EDTA, at 4°, until PFGE analysis.

PFGE analysis and quantification of DNA fragments. Agarose blocks containing approximately 4–5 × 10^5 cells were loaded into wells, which were then sealed in agarose. FIGE was performed using a Hoefer pulse controller (Hoefer Scientific), for which the ratio of forward and reverse pulse times was 3:1. CHEF gel electrophoresis was performed using the Hexafield gel electrophoresis apparatus (BRL), which contains a hexagonal array of electrodes having a reorientation angle of 120°. Size standards were λ DNA digested with *Hind*III, λ DNA ladder (Hoefer), and the chromosomes of *Saccharomyces cerevisiae* (Bio-Rad), *Candida albicans* (Hoefer), and *Schizosaccharomyces pombe* (Bio-Rad). Following gel electrophoresis, gels were stained in ethidium bromide and photographed. For quantification of DNA fragmentation, each lane was divided into 3-mm slices, which were then melted in 0.1 N HCl and analyzed by scintillation counting. Data for each slice are expressed as the percentage of total cpm contained in the corresponding lane.

Irradiation conditions. In order to generate DNA containing breaks truly located at random sites (and to avoid complications of DNA repair following radiation), intact cellular DNA was first obtained by suspending control cells in agarose blocks and digesting them with proteinase K, as for the drug-treated cells. These blocks, containing DNA that was at this point presumably intact and biologically inert, were irradiated on ice at 1 Gy/min, using an AECL Theratron 80 (^{60}Co). Dosimetry was carried out using a Baldwin Farmer ionization chamber connected to an electrometer system that was directly traceable to a National Bureau of Standards calibration.

Results and Discussion

Our initial characterization of DNA fragmentation employed FIGE for analysis of HT29 cells treated with 100 nM FdUrd. As previously discussed, FdUrd induces DNA fragments in a discrete size range of 50–200 kb in FM3A cells (12). However, as seen in Fig. 1, the DNA fragmentation pattern observed in HT29 cells consists of a much broader size range, beginning at 50 kb and increasing to a size that is not resolvable by this system. This is verified by the compression of the upper range of *S. cerevisiae* marker chromosomes, which are greater than 600 kb in length. Cells that had been irradiated were also included in this experiment, to represent specimens in which

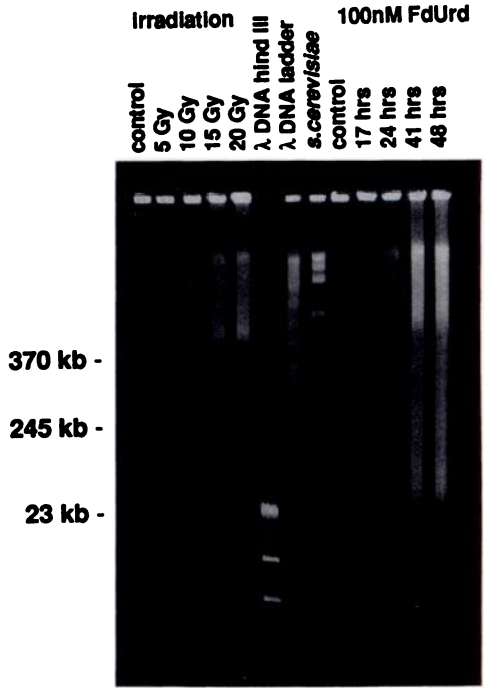

Fig. 1. FIGE analysis of DNA fragmentation induced by irradiation or FdUrd treatment. Agarose cell blocks, containing DNA given various doses of γ-radiation or HT29 cells treated with 100 nM FdUrd for the indicated time periods, were processed as described in the text. Samples were electrophoresed for 18 hr at 240 V in a 1% agarose gel (24 cm). Forward and reverse pulses were ramped at 3–36 sec and 1–12 sec, respectively. Size standards shown are *Hind*III-digested λ DNA, λ DNA ladder, and chromosomes of *S. cerevisiae*.

double-strand breaks were distributed in a presumably random pattern. Under these conditions, only a small amount of DNA appeared to enter the gel from the irradiated samples. From this first experiment we observed that the response of HT29 cells (i.e., the size distribution of DNA fragments formed) was qualitatively quite different from that reported for the FM3A line. However, due to the upper limits of resolution of FIGE, we could not determine from this analysis whether there was a difference in the size distribution of larger (i.e., >600 kb) fragments upon treatment with FdUrd versus γ-radiation. We, therefore, chose to continue our studies using a CHEF gel electrophoresis system, which is capable of resolving DNA in the megabase size range. In addition, we began to use [^{14}C] thymidine-prelabeled cells, in order to quantitate DNA fragmentation and to more precisely compare radiation-generated and FdUrd-induced DNA fragmentation in HT29 cells.

One of the drawbacks of pulsed-field techniques is the limited range of resolution given by any single combination of voltage and switching interval. However, this can be circumvented by altering the switching conditions during the run, either by a continuous change of the switching interval (ramping) or by an abrupt change in both voltage and switching interval. Using conditions previously described for the latter approach (13), we were able to resolve both the *S. pombe* standards (3.5–5.7 Mb) and most of the *S. cerevisiae* standards (0.2–2 Mb). When samples equivalent to those shown in Fig. 1 were analyzed by this two-phase CHEF procedure, the distributions observed for the radiation- and drug-treated cells were quite different (Fig. 2). Of the radiation-induced fragments that migrated into the gel, most appeared to be larger than the highest *S. pombe* standard, even at the greatest radiation dose (20 Gy). In contrast, the majority of the FdUrd-induced fragments were concentrated in a region near the smallest *S. pombe* standard. Another, more broad, distribution was also present throughout the region of resolved *S. cerevisiae* markers, in these samples.

Although on first inspection a major portion of the fragments in the FdUrd-treated samples in Fig. 2 seem to represent a relatively narrow band, it has been shown that CHEF protocols may contain "compression zones," within which resolution is very poor (14). Because the *C. albicans* standards were not resolved under these conditions, it seemed likely that the cluster of FdUrd-induced fragments near the smallest *S. pombe* marker was due to compression rather than to the existence of a discrete population of fragments. This interpretation is supported by the gel shown in Fig. 3, in which a CHEF protocol was used that did partially resolve the *C. albicans* chromosomes. Analysis using this procedure resulted in a relatively disperse distribution of FdUrd-induced fragments throughout the gel.

The apparent differences between FdUrd-induced and irradiation-generated DNA fragmentation patterns in Fig. 2 were confirmed by quantitative analysis of these distributions. In each case, about 7–9% of the label was found in the lane in control samples, increasing to 30% at the highest radiation dose and 25% at the longest FdUrd exposure. Fig. 4A demonstrates that fragments generated by irradiation were distributed within the upper one third of the gel, with peaks at slices 4–5 (upper compression zone) and slices 9–10 (lower compression zone). In contrast, few of the FdUrd-induced fragments appeared in the upper compression zone (Fig. 4B). Rather, these fragments migrated either in a peak at the lower compression

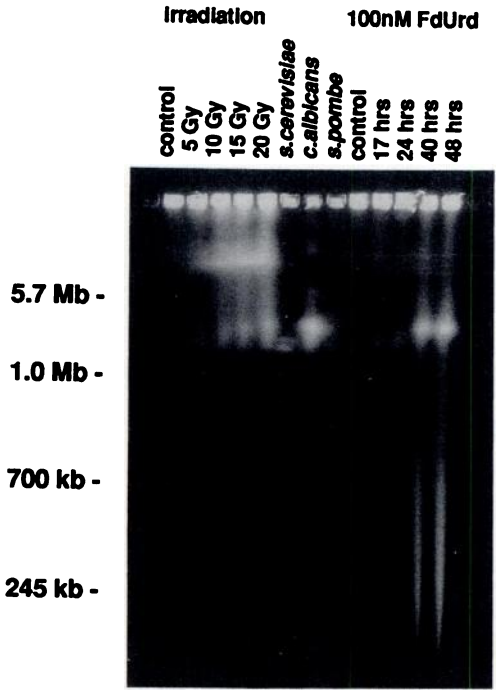

Fig. 2. Analysis of radiation- and FdUrd-induced fragments by CHEF gel electrophoresis. I. Portions of the samples used in Fig. 1 were analyzed in a 0.7% agarose gel (14 cm) in two phases, using a BRL Hex-a-field CHEF apparatus. The first phase consisted of a 45-min switching interval for 48 hr at 36 V, at 14°; the second phase consisted of a 140-sec switching interval for 44 hr at 60 V, at 14°. Size standards shown are the chromosomes of *S. cerevisiae, C. albicans* (not resolved), and *S. pombe*. Size designations (in Kb or Mb) indicate the positions of selected markers, which did not photograph well.

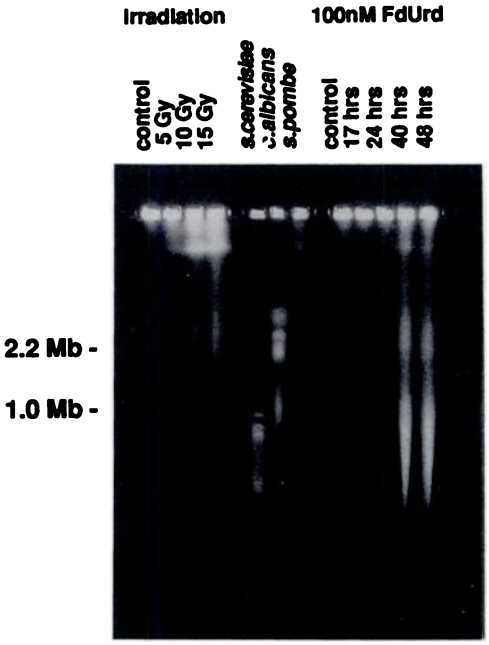

Fig. 3. Analysis of radiation- and FdUrd-induced fragments by CHEF gel electrophoresis. II. These samples are portions of those used in Figs. 1 and 2. In this case, the 1–4-Mb zone of compression was partially resolved by alteration of the switching conditions. The first phase consisted of a 20-min switching interval for 48 hr, followed by a second phase consisting of a 6-min switching interval for 22 hr. Both phases were run at 45 V, at 14°. Under these conditions, the *C. albicans* chromosomes were partially resolved.

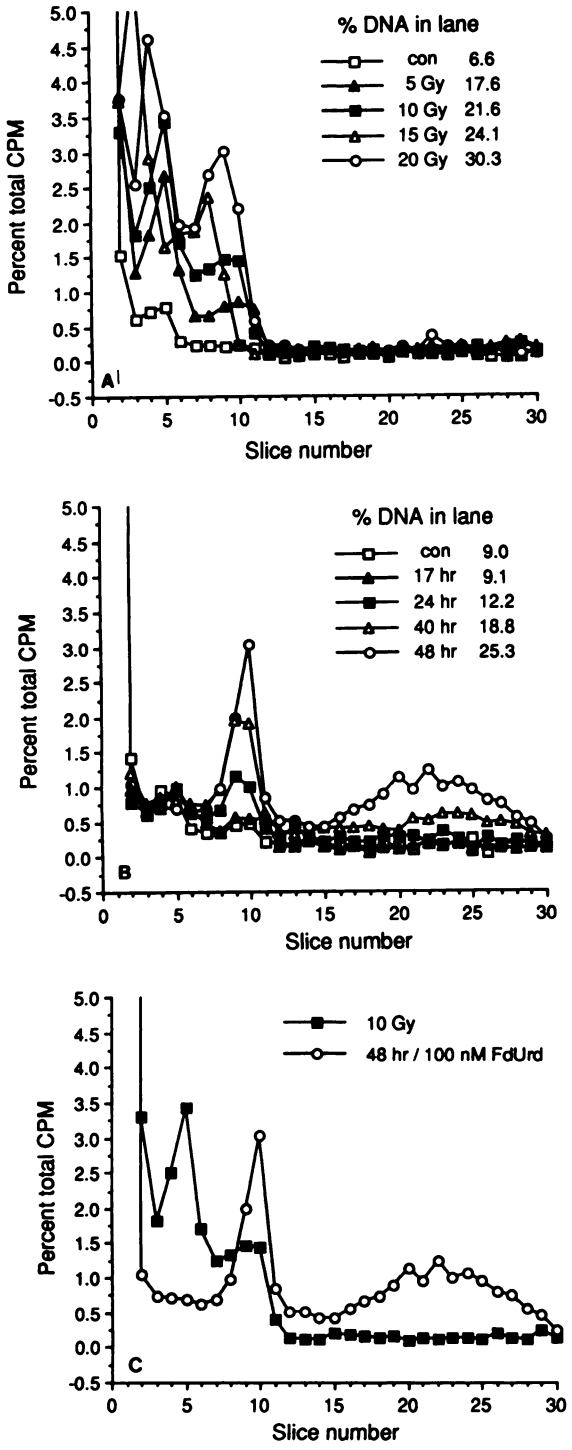

Fig. 4. Quantity and distribution of DNA fragments induced by FdUrd or irradiation. Cellular DNA was prelabeled with [^{14}C]thymidine for 48 hr and then chased for 24 hr before irradiation or FdUrd treatment. Following CHEF gel electrophoresis, each lane (Fig. 2) was cut into 3-mm slices, and the quantity of ^{14}C was determined by scintillation counting. Data for each slice are expressed as the percentage of total cpm found in the corresponding lane. The results presented here are from one of two quantitative experiments, in which equivalent results were obtained. A third experiment using unlabeled DNA also appeared to give similar results, as judged by ethidium bromide staining. A, Pattern of DNA fragmentation generated by 0, 5, 10, 15, or 20 Gy. B, Pattern of DNA fragmentation induced by 100 nM FdUrd for 0, 17, 24, 41, or 48 hr. C, Comparison of DNA fragmentation due to 10 Gy of radiation and 48 hr of 100 nM FdUrd treatment, conditions that both resulted in about 25% of the radioactivity migrating into the gel. % *DNA* in lane percentage of cpm found in the lane of each sample. The total cpm for each sample are as follows. A, irradiated samples: 0 Gy, 8186; 5 Gy, 6448; 10 Gy, 6653; 15 Gy, 6951; 20 Gy, 7529. B, FdUrd-treated samples: 0 hr, 5816; 17 hr, 8933; 24 hr, 9271; 41 hr, 12575; 48 hr, 10669. *con*, control.

TABLE 1

Loss of clonogenicity due to FdUrd treatment

HT29 cells were exposed to the indicated concentrations of FdUrd for either 17, 24, or 48 hr; after which clonogenicity was assayed as described in the text. Data represent the mean of at least two experiments, in which four to six determinations were made at each concentration. The standard error is provided for experiments performed three or more times. Clonogenicity is expressed as the decimal fraction of control plating efficiency, which is typically 0.55–0.70.

Treatment	Clonogenicity		
	17 hr	24 hr	48 hr
	surviving fraction		
10 nM FdUrd	0.81 ± 0.03	0.42 ± 0.08	0.28
100 nM FdUrd	0.55 ± 0.03	0.20 ± 0.05	0.03 ± 0.01
100 nM FdUrd + 10 μM thymidine		0.82 ± 0.06	1.04 ± 0.09

zone (slices 9–10) or as a broad distribution throughout the lower zone of resolution (slices 16–29).

The main issue we wished to address by inclusion of the radiation-treated samples was whether the distribution of fragments formed following FdUrd is random, as we assume is the case for those caused by γ-radiation. One approach to this question is to compare samples from each group in which the same fraction of DNA was released into the gel. The fragments migrating into the gel represent only a subset of all DNA in the sample, due to the inability of pieces beyond a given size limit to enter the gel. We are, therefore, only able to compare the lower tail of each distribution. However, if the overall distributions of the two types of sample are shaped similarly, then we would expect the tails to be shaped similarly as well. By comparing the 10-Gy radiation and the 48-hr FdUrd treatments (Fig. 4C), we had two samples in which 25% of the total DNA in the cell block entered the gel (about 15–18% in excess of the background signal observed in controls), but in which the size distributions of fragments within the gel were quite different from one another.

A second way of normalizing the two types of treatments is in terms of biological effect, i.e., cytotoxicity. Table 1 demonstrates the effects of FdUrd treatments on loss of clonogenicity of HT29 cells. In our hands, the treatment discussed above, (100 nM for 48 hr) reduces survival by about 2 log. In previous experiments we have determined that the radiation dose required to reach that degree of cytotoxicity in HT29 cells is about 8 Gy (15). Therefore, in this instance, FdUrd and radiation treatments that elicit the same degree of cell kill also cause about the same fraction of DNA to be fragmented to a size small enough to enter the CHEF gel. Whether this relationship will hold over a wide range of circumstances remains to be investigated.

The mechanism responsible for the formation of megabase DNA fragments in FdUrd-treated HT29 cells is, at this time, a topic for speculation. The dissimilarity between the FdUrd- and radiation-induced distributions indicates that double-strand breaks caused by FdUrd are not located randomly with respect to the entire genome. However, this finding does not rule out the possibility that they could occur randomly within a certain subpart of the genome, e.g., those regions that are

physically exposed by virtue of their involvement in transcription or replication. It is, therefore, possible that the uracil excision/misrepair cycle mentioned earlier could play a role in the generation of the megabase fragments we observed.

It is also possible that an enzymatic activity associated with the replication fork could be responsible for double-strand breaks. This idea was presented as an explanation for the size of fragments that were found in the FM3A system (50–200 kb), which is approximately that of a replicon. The finding that newly replicated regions of DNA were preferentially represented in fragmented FM3A DNA (compared with DNA remaining in the well) is consistent with this concept (12). Although FdUrd-induced fragments from HT29 cells are considerably larger than 200 kb, it could be that these cell lines differ in their pattern of replicon activity or in the probability that a break will occur in any individual replicon.

DNA fragmentation upon FdUrd treatment has also been ascribed to "programmed cell death" or apoptosis (16). Although the fragments observed in this process are orders of magnitude smaller than the ones reported here, it is conceivable that the large pieces are precursors of the smaller ones. Based on the relatively slow time course with which megabase fragments arise in HT29 cells, however, we do not believe that this is the most likely explanation in this case.

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