THE ORGANIZATION OF REPEATED NUCLEOTIDE SEQUENCES IN THE REPLICONS OF MAMMALIAN DNA

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ABSTRACT Chinese hamster ovary cells were irradiated with 100–5,000 rads of X-rays, and inhibition of the initiation of replicons after irradiation was demonstrated by analyzing nascent DNA sedimented in alkaline sucrose gradients. The renaturation kinetics of DNA synthesized during 60 min of incubation after irradiation was compared with that of DNA synthesized during the 60 min after sham irradiation and with that of parental DNA. Nascent DNA from cells whose replicon initiation was inhibited renatured faster than nascent DNA from control cells in the C_0t range of repeated nucleotide sequences, suggesting that regions of the replicon not close to origins are enriched in repeated sequences and that regions close to origins are enriched in unique sequences. A class of repeated nucleotide sequences may be involved in the regulation of replicon initiation.

INTRODUCTION

Eukaryotic chromosomal DNA replicates in subunits called replicons (1, 2). Replication usually proceeds bidirectionally from initiation points called origins (2–4), and it may terminate by the simple fusion of neighboring replicons (5). Recently, it has been shown that X-radiation (100–1,000 rads) inhibits replicon initiation in several mammalian cell lines but has no detectable effect upon chain elongation or joining (6–9). It seemed possible to exploit this fact to study the organization of repeated nucleotide sequences in replicons because incubation of cells with radioactive DNA precursors during the inhibition of initiation preferentially labels regions that are not close to origins. To study replicon organization, we compared the renaturation kinetics of nascent DNA in X-irradiated Chinese hamster ovary (CHO) cells with that of nascent DNA in unirradiated (control) cells.

METHODS

Growth of Cell Cultures; Labeling of Parental DNA

CHO cell cultures were grown in 60-mm plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a humidified 5% CO_2 atmosphere in Eagle's minimal essential medium (MEM) supplemented with 15% fetal calf serum and the antibiotics penicillin and streptomycin. The cultures were checked periodically and found to be free of mycoplasma contamination. In some experiments, the cells were labeled for one cell generation (12–15 h) by replacing normal growth medium with MEM containing 0.02 μCi/ml [*methyl*-^{14}C]thymidine (50 mCi/mmol; New

England Nuclear, Boston, Mass.). After the labeling medium was removed, normal growth medium was returned to these cultures, and the cells were incubated for at least 60 min before irradiation.

X-Irradiation and Labeling of Nascent DNA

The cultures were irradiated at 23°C with 300-kvp X-rays from a G.E. Maxitron X-ray machine (2.0-mm Cu filter) (General Electric, Medical Systems Div., Milwaukee, Wis.) or in a walk-in incubator at 37°C with 110-kvp X-rays from a Faxitron machine (Hewlett-Packard Corp., McMinnville Div., McMinnville, Ore.). The dose rates were 200 rads/min (Maxitron) and 500 rads/min (Faxitron), as determined with lithium fluoride thermoluminescent dosimeters (Eberline Instruments Corp., Santa Fe, N. M.). The doses ranged from 100 to 5,000 rads. Samples were irradiated as monolayers in the Petri dishes in which they were grown and labeled; the cells were covered with 10 ml of MEM for irradiation. Unirradiated (control) cultures were treated the same as irradiated cultures, being kept at ambient temperature during the irradiation period.

Immediately after irradiation, the medium was removed from the cultures and fresh growth medium was added to them. In one experiment, labeling of nascent DNA was begun immediately; in the others, the cells were incubated for 15 or 30 min at 37°C before labeling was begun. To label DNA, 10–30 μCi/ml [*methyl*-^{3}H]thymidine (50–80 Ci/mmol; New England Nuclear Corp.) was added to the growth medium and the cultures were incubated at 37°C for 5 or 60 min. Labeling was terminated by removing the labeling medium and rinsing the cultures with ice-cold sodium saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate).

Velocity Sedimentation Analysis of Nascent DNA

After the cells were labeled, they were harvested into 2 ml of ice-cold SSC. 0.5 ml of each cell suspension and 0.5 ml of lysis solution (0.5 N NaOH, 0.02 M EDTA, 0.2% NP40 [Shell Chemical Co., Houston, Tex.]) were layered onto 30 ml of preformed 5–20% alkaline sucrose gradients (0.10 N NaOH, 0.9 M NaCl, 0.01 M EDTA). Lysis was carried out for 3 h at 25°C and the gradients were centrifuged at 27,000 rpm for 3 h in a Beckman L2-65B ultracentrifuge (SW 27 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). A hole was made in the bottom of each centrifuge tube and about 25 equivalent-weight fractions were collected. 100 μg calf thymus DNA was added to each fraction and the DNA was precipitated by adding 0.5 ml of a solution of ice-cold 6 N HCl and 6% sodium pyrophosphate. Each fraction was then filtered through GF/C filters (Whatman, Inc., Clifton, N. J.) soaked with 4% perchloric acid. The filters were washed sequentially with ice-cold 4% perchloric acid, 70% ethanol, 95% ethanol, and 100% ethanol, and then dried. ^{14}C and ^{3}H radioactivity in each fraction was determined in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Isolation and Purification of DNA

Cells used in renaturation experiments were lysed in 0.1% sodium dodecyl sulfate and the lysates were incubated at 37°C in the presence of 50 μg/ml bovine pancreatic ribonuclease (heat-treated; Calbiochem, San Diego, Calif.) for 2 h and then in the presence of 500 μg/ml self-digested pronase (Calbiochem) for 12 h. The DNA was extracted twice with two volumes of SSC-saturated phenol and precipitated with 2–3 vol of ethanol - 0.15 M sodium acetate. The precipitates were pelleted in a centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.) at 11,000*g* for 20 min, and the pellets were suspended in 0.5–1.0 ml of SSC. DNA solutions were then dialyzed against 2.0-liter volumes of SSC for 24 h. The purity of the DNA was checked by the measurement of absorbance at 260 and 280 nm. A_{260}/A_{280} was always 1.80–1.90. DNA from nonradioactive cultures was prepared according to the same procedures. The recovery of DNA was checked after each step of the purification procedure, including the elution from hydroxyapatite (see next section). The amount of DNA recovered was 90% or greater

and the $^3H/^{14}C$ ratios were constant after all steps. Thus, neither ^3H-labeled nascent DNA nor ^{14}C-labeled parental DNA was purified selectively.

Determination of Renaturation Kinetics of Nascent (^3H-Labeled) and Parental (^{14}C-Labeled) DNA

The amount of tracer (^3H or ^4C) DNA that hybridized with unlabeled DNA was determined by a hydroxyapatite binding method. Appropriate volumes of purified unlabeled DNA, ^{14}C-labeled DNA, and ^3H-labeled DNA were combined and degraded to 6.5S pieces by adding 3.0 N NaOH to a final concentration of 0.3 N and heating at 100°C for 20 min (10). The 6.5S DNA was then dialyzed against 0.125 N sodium phosphate buffer (pH 6.8), denatured by heating at 100°C for 10 min, and allowed to renature during incubation at 60°C for times yielding the appropriate C_0t values (11). Incubation volumes were 1.0–2.0 ml. The DNA solutions were covered with layers of mineral oil and the tubes were tightly stoppered during incubation. At appropriate times, 50-μl portions of the solutions containing renaturing DNA were removed and mixed with 0.5 ml of wet hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.) and 4.0 ml of 0.05 M phosphate buffer in conical centrifuge tubes. The DNA and the hydroxyapatite were mixed thoroughly and incubated at room temperature for 15 min, after which the mixture was centrifuged at 1,000 rpm for 1 min at room temperature. The supernatants were removed, and 4.0 ml of 0.125 M sodium phosphate buffer kept at 55°C was added to each tube and mixed with the contents. The tubes were incubated at 55°C for 5 min, after which the buffer layer was separated by centrifugation and collected and the 0.125 M phosphate buffer procedure was repeated. Double-stranded DNA was released from the hydroxyapatite by two washes with 4.0 ml of 0.40 M phosphate buffer. 1-ml portions of each phosphate buffer wash were added to 15 ml of PCS liquid scintillation solution (Amersham/Searle Corp., Arlington Heights, Ill.) and the ^3H and ^{14}C radioactivity was determined in a liquid scintillation spectrometer. The ^3H and the ^{14}C radioactivity released by incubation with 0.40 M phosphate buffer represents the amount of double-stranded nascent and parental DNA in each sample. The data are expressed for each C_0t value as the percentage of hybridized DNA—i.e., 100 × [(cpm in 0.40 M phosphate buffer)/(cpm in 0.125 M phosphate buffer + cpm in 0.40 M phosphate buffer)].

^{14}C-labeled DNA (double-stranded) and denatured ^{14}C-labeled DNA (single-stranded) were eluted from hydroxyapatite several times. Typically, 90% of the double-stranded DNA was retained on the hydroxyapatite during the 0.125 M phosphate buffer step and was then released by 0.40 M phosphate buffer; 90% of the single-stranded DNA was released by 0.125 M phosphate buffer. The first wash, with 0.05 M phosphate buffer, always released very small amounts (3% or less) of the radioactivity, which represented DNA that did not bind to hydroxyapatite.

RESULTS

Replicon initiation in CHO cells (indicated by the small fragments represented by radioactivity in fractions 5–10) was inhibited by X-radiation in a dose-dependent manner (Fig. 1), being almost completely abolished by 5,000 rads. This inhibition was evident for up to 60 min after irradiation (9). Chain elongation and joining, however, proceeded at essentially normal rates. If they had not, the radioactivity at high S values in profiles of DNA from irradiated cells would have been less than that at high S values for the control profiles (see ref. 9 for a complete discussion of this point). Comparison of the ^3H profiles (nascent DNA) with the ^{14}C profile (parental DNA) indicates that no DNA degraded during lysis and sedimentation. If it had, the profile for ^{14}C-labeled DNA would have been in the low-molecular-weight region instead of in its character-

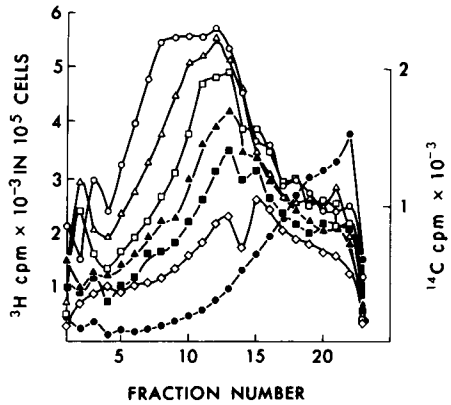

FIGURE 1 Inhibition of replicon initiation by various doses of X-radiation, as indicated by alkaline sucrose gradient profiles of DNA from CHO cells incubated with [^{14}C]thymidine (to label parental DNA), irradiated with 100–5,000 rads, and incubated with [^{3}H]thymidine (to label nascent DNA) for 5 min beginning at 15 min after irradiation. Sedimentation is from left to right. Bottom fractions (containing 10–20% of total radioactivity due to wall effects) are omitted from the figure. ^{3}H-labeled (nascent) DNA: (○), 0 rads (control); (△), 100 rads; (□), 500 rads; (▲), 1,000 rads; (■), 2,500 rads; (◇), 5,000 rads. ^{14}C-labeled (parental) DNA: (●).

istic position. At 20 min after irradiation of cells with the highest dose, the incorporation of labeled precursor into DNA was only 40% of the control value, which indicates that the effect of X-radiation was primarily on cells already in S-phase at the time of irradiation, rather than on G_1 cells prevented from entering S-phase. The latter explanation requires a faster exit of cells from S to G_2 than can reasonably be expected.

The renaturation kinetics of DNA synthesized by CHO cells in the first 70 min after irradiation (i.e., the irradiation and the subsequent incubation period) was compared with that of DNA synthesized by sham-irradiated control cells in the same time period. In nascent DNA from X-irradiated cells, there was a larger proportion of repeated sequences (5–10%) than in either nascent DNA from unirradiated controls or in parental DNA (Fig. 2). This result was obtained in seven independent experiments. Parental DNA and control nascent DNA renatured with essentially the same kinetics. Nascent DNA from irradiated cells renatured with the same kinetics as that of parental DNA or nascent DNA from control cells up to C_0t values greater than 10^{-2} (see Fig. 2, insert). Therefore, the difference in the populations of nascent DNA molecules from X-irradiated and control cultures was in a class of sequences repeated a very large number of times, but fewer times than foldback DNA or satellite DNA. Because cells were incubated with [^{3}H]thymidine during X-ray-induced inhibition of replicon initiation, ^{3}H-labeled origins were underrepresented in nascent DNA from unirradiated cells. The data suggest, therefore, that a higher proportion of repeated nucleotide sequences is characteristic of replicon regions that are not close to origins, and it follows that regions near origins must contain, on the average, higher proportions of unique sequences than the DNA as a whole.

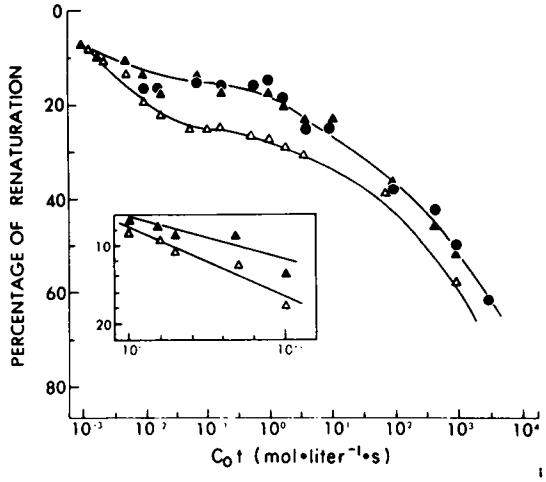

FIGURE 2 Renaturation kinetics of DNA from CHO cells labeled with [^{14}C]thymidine, X-irradiated with 1,000 rads, and incubated with [^{3}H]thymidine for 60 min beginning at 10 min after irradiation. Before being labeled with [^{3}H]thymidine, both irradiated cultures and unirradiated controls were incubated for 10 min at 37°C to prevent the labeling of regions around the origins of replicons that had initiated just before irradiation. The cells were harvested and their doubly labeled DNA was isolated, mixed with a great excess of unlabeled DNA, and incubated at 60°C for various times. The fraction of each radioactive tracer that renatured with unlabeled DNA during the different incubation times was determined by hydroxyapatite binding. ^{3}H-labeled (nascent) DNA: (▲), 0 rads, 60 min incubation (control); (△), 1,000 rads, 60 min. ^{14}C-labeled (parental) DNA: (●). The insert contains data from the renaturation in the C_0t range 10^{-3} to 10^{-2}

DISCUSSION

The fiber autoradiography experiments of Hand (12) and of Hori and Lark (13) suggested that the control of mammalian replicon initiation is exercised over clusters of replicons. This hypothesis is supported by radiation inactivation data obtained in this laboratory that led to the postulate that a single radiation hit within one cluster completely blocks the initiation of all the replicons therein. Clusters of replicons seem to be the same entities as the supercoiled subunits found in the chromosomes of mammalian cells (14, 15), because the D_0 (1/exp) for inactivation by X-irradiation of the steep component of DNA synthesis is about 500 rads, which represents a target molecular weight of 10^9 (6, 16), and because the average molecular weight of supercoiled subunits is about 10^9 (14, 15). In addition, Gellert et al. (17) have recently shown that in *Escherichia coli*, DNA supercoiling is required for the replication of col E_1 and lambda DNA, and the results of Lovett et al. (18) indicate that the binding of a relaxation protein to supercoiled DNA precedes the initiation of the R6K plasmid replicon in *E. coli*. It is therefore possible that the functional subunit for mammalian DNA replication—the cluster—must be supercoiled before initiation can occur, as suggested by Povirk and Painter (19).

The maintenance of the correct structure for replicon initiation may involve the way in which unique and repeated nucleotide sequences are organized within replicons or

clusters of replicons. In the present experiments, we found that nascent DNA from X-irradiated cells, whose replicon initiations were blocked, contained a greater proportion of repeated sequences than normally initiating cultures (controls). This difference must have resulted from X-ray-induced inhibition of initiation itself. It could not have been caused by "pool effects" because several different methods (6, 8, 20) have shown that up to 1,000 rads of X-rays have no effect upon the incorporation of exogenously supplied thymine precursors into DNA—i.e., there is no expansion of precursor pools. Therefore, the difference in renaturation kinetics between nascent DNA from initiation-inhibited and normally initiating cultures must indicate that regions near origins have, on the average, more unique sequences per unit length than other regions of the replicon.

The functions of unique and repeated sequences are not completely understood. Britten and Davidson (21) have suggested that moderately repeated sequences are involved in the regulation of transcription. Other functions for this class of nucleotide sequences have been proposed (22, 23). The results reported here, together with those of Cook and Brazell (24), indicate that a class of repeated sequences may indirectly regulate the initiation of replicons—perhaps by keeping replicon clusters in a required conformation. The finding that the site of regulation of replicon initiation is far removed from the actual origin of polymerization and involves a large number of sequences within the replicon cluster is compatible with the evidence that implicates the cluster as the unit of regulation of mammalian DNA replication (12, 13, 19). The organization of eukaryotic DNA into unique and repeated sequences may therefore be used, in a manner yet to be elaborated, to regulate DNA replication as well as to control gene expression.

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