ABNORMAL SEQUENCE DISTRIBUTION IN DNA SYNTHESIZED BY ISOLATED NUCLEI FROM NORMAL OR MITOMYCIN C-TREATED HELA CELLS

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SUMMARY: The DNA synthesized by isolated nuclei from normal HeLa cells, or from cells previously treated with mitomycin C were compared by DNA-DNA reassociation kinetics with DNA synthesized in vivo. DNA synthesized in vivo was found to be 31% reassociated at Cot 50 whereas DNA synthesized in vitro by isolated nuclei was 40% reassociated at this Cot value. Isolated nuclei, from cells whose cellular DNA synthesis had been completely inhibited by mitomycin, synthesized DNA which was unusually repetitious. In these nuclei, the proportion of DNA hybridized by Cot 50 ranged from 45% to 60% of the total DNA. The abnormal distribution of DNA sequences formed by isolated nuclei is discussed in relation to DNA synthesis.

INTRODUCTION

DNA replication in isolated nuclei from various eukaryotic cells has been reported by many groups (1-7). The synthesis of small DNA fragments and the formation of replication "eyes" in DNA, as well as ATP and cytosol requirements for DNA synthesis have all been demonstrated in vitro. However, it is not known whether the DNA synthesized in isolated nuclei has the same relative distribution of DNA sequences as the DNA found normally in the intact cell. Since it is well known that the DNA of eukaryotic cells consists of classes of DNA, highly repetitious DNA, middle repetitious DNA, and unique sequence DNA (8-10), this question becomes of some interest.

This paper will report that DNA synthesized by isolated nuclei contains more repetitious DNA than DNA made in a normal cell. Furthermore, mitomycin C pretreatment of cells to stop DNA synthesis in vivo causes isolated nuclei from such cells to form a DNA in vitro which contains an unusually high percentage of middle repetitious DNA.

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MATERIALS AND METHODS

Chemicals: [^{3}H-methyl]TTP was purchased from Schwartz/Mann. [^{3}H-methyl] thymidine was obtained from New England Nuclear. Ribonucleoside triphosphates and deoxyribonucleoside triphosphates were obtained from Sigma Chemical Co. S_1 nuclease was prepared from α-amylase of Aspergillus oryzae (Sigma Chemical Co.) by the method of Wiegand et al. (11). Mitomycin C was obtained from Kyowa Hakko Kogyo, Tokyo.

Cell Culture: HeLa F cells were grown in monolayer culture as described previously (7). When the cells reached 80-90% confluency, the monolayer cells were washed with phosphate buffered saline (Gibco) and scraped off for collection. The cells were washed with 0.32 M sucrose, 2 mM $MgCl_2$, 1 mM potassium phosphate buffer (pH 6.8) (Buffer I).

Preparation of HeLa Cell Nuclei: The collected cells were suspended in 1.5 volumes of 1 mM potassium phosphate (pH 6.8) containing 0.01 M NaCl and 0.5 mM dithiothreitol. The cells were broken in a Dounce homogenizer with 10 strokes and centrifuged at 1000 x g for 8 min. Pelleted nuclei were washed with 0.32 M sucrose containing 1 mM $MgCl_2$, 1 mM potassium phosphate (pH 7.0), 0.5 mM dithiothreitol, and 0.3% Triton N101 by Dounce homogenization with 5 strokes and centrifuged at 1000 x g for 8 min. The washed nuclei were suspended in Buffer I containing 0.5 mM dithiothreitol and stored in liquid nitrogen.

Standard Reaction Mixture for Nuclear DNA Synthesis: Reaction mixtures (100 µl) contained 5 µmol of Tris-HCl (pH 8.1), 0.4 µmol of $MgCl_2$, 0.25 µmol of ATP, 5 nmol of CTP, UTP, and GTP each, 0.25 µmol of dithiothreitol, 10 nmol of [^{3}H]dTTP (1-9 μC_i/nmol), dCTP, dGTP and dATP and 1-3 x 10^6 nuclei. The mixture was incubated at 37° for 20 min. To measure the incorporation of dTMP into acid-insoluble materials, 80 µl aliquots of the reaction mixture was removed and placed onto the glass filter Whatman GF/C. The glass filters were washed with trichloroacetic acid as described by Bollum (12). The radioactivity on the glass filter was measured in 0.3 ml NCS (Amersham/Searle) and 10 ml of LSC complete (Yorktown Research).

To isolate the DNA from the reaction mixture, a ten times larger reaction mixture was used and the DNA was prepared from the nuclei as previously described (13).

Isolation of DNA from Cells: HeLa cells were labeled with [^{3}H]thymidine at 2 μC_i - 20 μC_i/ml for 20 min or 24 h or with [^{32}P]H_3PO_4 at 10 mC_i/l for 18 h and the nuclei were isolated from the cells as described above. DNA was purified from nuclei as described previously (13).

DNA-DNA Reassociation Kinetics: The rate of the reassociation of DNA was followed by the methods of Britten et al. (14) or S_1 nuclease digestion (11). In the former method the DNA was sheared by placing it at 100° for 15 min in 0.33 N NaOH and then neutralized with 2 M NaH_2PO_4. After reassociation at 65° the single-stranded DNA and double-stranded DNA were separated on a hydroxylapatite column (0.8 ml) at 60° with stepwise elutions of 4 ml of 0.15 M sodium phosphate buffer (pH 6.8) containing 0.4% sodium dodacyl sulfate (SDS) followed by 4 ml of 0.40 M of sodium phosphate buffer with 0.4% SDS. The DNA in these fractions was precipitated with carrier salmon sperm DNA (750 µg) by the addition of trichloroacetic acid to 10% and the precipitate was collected on glass filters. The glass filters were washed 3 times with 5 ml of 5% trichloroacetic acid and dried in vacuum at 60°.

In measurements of reassociation kinetics by S_1 nuclease, the DNA sheared under the above conditions was neutralized with HCl and was brought to 0.75 M NaCl, 10 mM Tris-HCl (pH 7.8) and 1 mM EDTA. The DNA reassociation in 10 µl

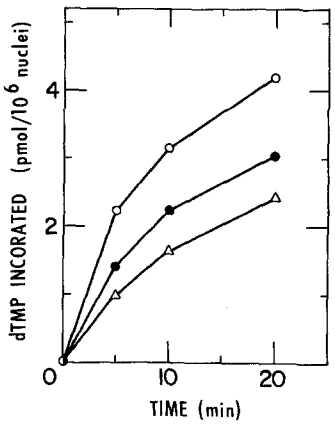

Fig. 1. The Effect of Mitomycin C Treatment on Nuclear DNA Synthesis. The reaction mixture for DNA synthesis by isolated nuclei was the same as described in "Materials and Methods." Nuclei were prepared from cells which were either not exposed to mitomycin C (○), or treated with mitomycin C (50 μg/ml) for 1 h (●) or 3 h (Δ).

aliquots was performed as described above and followed by S_1 nuclease digestion in a mixture (128 μl) containing 6.25 μmol sodium succinate (pH 4.8), 0.105 μmol $ZnCl_2$, 4 μg calf thymus DNA, 12.5 μmol NaCl, and 9 units S_1 nuclease. The mixture was incubated at 40° for 70 min. Two 50 μl aliquots were removed and placed on glass filters to measure both the total radioactivity of the added DNA and the radioactivity remaining as acid-insoluble reassociated DNA as described above (12). Cot (mol/l x sec) was corrected for the salt concentration effect (14).

RESULTS

DNA Synthesis by Nuclei Isolated from Mitomycin-treated or Normal, Untreated HeLa F Cells: When HeLa cells were exposed to mitomycin C (50 μg/ml), the rate of DNA synthesis in these cells at 1 h and 3 h after addition of mitomycin C was 25% and 3% respectively of the untreated control (data not shown). Nuclei were isolated from such cells, either untreated or treated with mitomycin C and their DNA synthetic capacity was measured *in vitro* (Fig. 1). In contrast to the cells exposed to mitomycin C, nuclei obtained from these cells for 1 h or 3 h had 72% and 58%, respectively, of the TMP incorporation of the control nuclei. These results suggest that isolated nuclei are, in part, free of the restraints on DNA synthesis which are present in whole cells treated with mitomycin C.

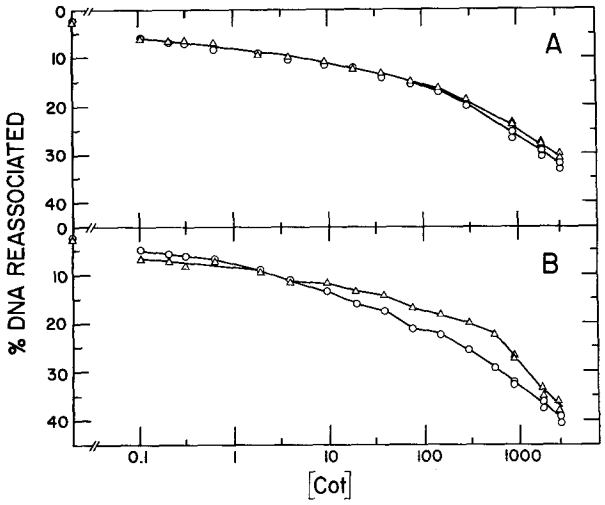

Fig. 2. Reassociation Kinetics of DNA Synthesized by Isolated Nuclei. The mixture for DNA reassociation contained 550 µg DNA/ml of HeLa DNA, 0.75 M NaCl, 10 mM Tris HCl (pH 7.8), and 1 mM EDTA. The reassociation kinetics was measured by S_1 nuclease digestion as described in "Materials and Methods."

(A) Δ, [^{32}P]HeLa DNA made *in vivo* (18 h labeling period); 0, [^{3}H]HeLa DNA made for 24 h *in vivo*. Each point had a total of 2900 [^{32}P]cpm and 3800 [^{3}H]cpm in the incubation.

(B) Δ, [^{32}P]HeLa DNA made *in vivo*; 0, [^{3}H]HeLa DNA made in isolated nuclei with [^{3}H]dTTP. Each point had a total of 2500 [^{32}P]cpm and 6500 [^{3}H]cpm. Points shown on the ordinate were determined at 0 time.

Reassociation Kinetics of the DNA Synthesized by Isolated Nuclei from Normal and Mitomycin-treated HeLa F Cells: Reassociation kinetics of the DNA synthesized by isolated nuclei were compared with *in vivo* labeled DNA obtained from growing cells (Fig. 2). For our control studies, we have measured the reassociation of both a HeLa DNA pulse labeled *in vivo* for 24 h with [^{3}H] thymidine, and a preparation of [^{32}P] labeled HeLa DNA obtained from cells incubated for 18 h with inorganic [^{32}P] PO_4. To eliminate any possible errors, these two DNA preparations were mixed and the reassociation kinetics of the [^{3}H] and [^{32}P]DNA were determined simultaneously in the same solution. These results are shown in Fig. 2A where it can be seen that the rate of reassociation of the 24 h [^{3}H] labeled DNA made *in vivo* is identical to that of [^{32}P]DNA from cells labeled continuously for an 18 h period. Though not

shown, the reassociation rate of [^{3}H]DNA made in a 20 min pulse label of growing HeLa cells is also the same as DNA labeled for 18 or 24 h. However, when [^{3}H]DNA made in isolated nuclei, during a 20 min incubation period *in vitro*, is examined, a clear difference from normal HeLa DNA is noted. This is shown in Fig. 2B in which normal, *in vivo* labeled [^{32}P]DNA was mixed with a sample of [^{3}H] labeled DNA made by isolated HeLa nuclei. The presence of the [^{32}P] HeLa DNA in these reassociation studies acts as an internal control. Fig. 2B shows that the [^{3}H]DNA made by isolated nuclei shows an abnormally high reassociation of sequences in the Cot range from 5 to 500 indicating the presence of excess amounts of labeled middle repetitious DNA sequences. Below Cot 5 or above Cot 500, the *in vitro* synthesized DNA reassociates at the same or slightly lower rate than normal HeLa DNA.

The experiments shown in Fig. 2 were performed using S_1 nuclease treatment, which destroys single-stranded DNA, to measure the rate of reassociation of the DNA to a double-stranded form. These experiments were repeated using hydroxylapatite separation of the single- and double-stranded DNAs to measure the reassociation kinetics. Fig. 3A shows the reassociation curves of these DNAs from the Cot 0.045 to Cot 217. In these experiments the reassociation curve of DNA synthesized by normal nuclei crossed that of the normal, *in vivo* synthesized DNA at a Cot of about 10. Below Cot 10, the reassociation rate of DNA synthesized *in vitro* by normal nuclei is about that of *in vivo* synthesized DNA. However, above Cot 10, the reassociation rate of the *in vitro* synthesized DNA is significantly quicker than that of natural HeLa DNA made *in vivo*, so that 5-10% more of the labeled *in vitro* DNA acts as a middle repetitious DNA. This is in agreement with the data shown in Fig. 2.

The synthesis of an anomalous DNA is more dramatically illustrated when nuclei from mitomycin C-treated cells are incubated *in vitro*. We have examined two separate preparations of DNA made by isolated nuclei obtained from cells pretreated for 3 h with mitomycin C. The reassociation of the first of these DNA samples is shown in Fig. 3A. The reassociation curve of DNA made by mito-

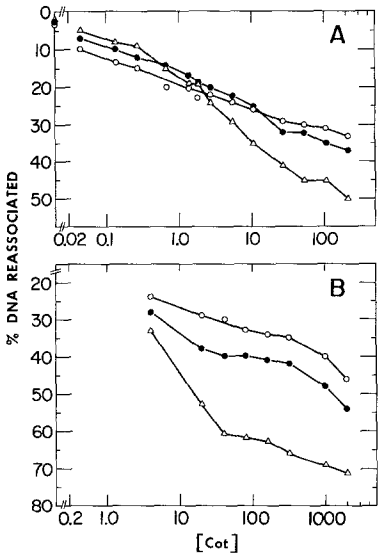

Fig. 3. The Reassociation Kinetics of DNA Synthesized by Isolated Nuclei. HeLa F cells were cultured with or without mitomycin C (50 µg/ml) for 3 h and then nuclei from these cells were isolated. DNA synthesis in isolated nuclei was accomplished as described in "Materials and Methods." The reassociation kinetics was measured by the hydroxylapatite method. The DNA in A and B were from separate preparations: (A) The DNA reassociation incubations contained 1600 µg/ml of the HeLa DNA and 0.75 M Na^+ from Cot 1.8 to 218 or 40 µg/ml of HeLa DNA from Cot 0 to 5.4. In the latter case, the DNA concentration was brought to 1600 µg/ml with salmon sperm DNA. Each incubation point contained either 8500 cpm of [^{3}H]DNA made *in vitro* by normal nuclei, or 2400 cpm of [^{3}H]DNA made *in vitro* by nuclei from mitomycin-treated cells. (B) 600 µg/ml DNA and 0.75 M Na^+ were used in DNA reassociation from Cot 4.08 to Cot 1958. Each incubation point contained 25,000 cpm of [^{3}H] *in vivo* DNA, or 15,000 cpm of [^{3}H]DNA made by normal nuclei, or 3500 cpm of [^{3}H]DNA made by mitomycin-treated nuclei.

0 - DNA labeled *in vivo* for 20 min with [^{3}H]thymidine; ● - DNA synthesized by nuclei from untreated cells; Δ - DNA synthesized by mitomycin-treated nuclei.

mycin-treated nuclei *in vitro* crosses that of normal *in vivo* DNA at Cot 5. Below Cot 5 the reassociation rate of the former again seemed similar to normal DNA, but in the Cot range from 5 to 50 the DNA made by nuclei from mitomycin-treated cells reassociated almost 2 times faster than normal DNA. The amount of reassociated DNA at zero time (Cot < 0.04) with the DNA made *in vitro* by mitomycin nuclei equaled 3% of the total DNA. This is the same as that found with the *in vivo* and the *in vitro* synthesized DNAs from normal cells or nuclei derived therefrom. The reassociation kinetics of the second of these DNA preparations synthesized by mitomycin-treated nuclei is shown in

Fig. 3B which covers the Cot range from 4 to 1958. As before, the amount of reassociated DNA at Cot 50 was 31% of the total DNA for in vivo DNA and 40% for DNA synthesized in vitro by normal nuclei. However, 62% of the DNA made by mitomycin-treated nuclei in vitro was reassociated by Cot 50. It is apparent from Fig. 3B that about 30% of the DNA made in vitro by nuclei from mitomycin C-treated cells reassociates in the Cot range from 5 to 50. By contrast, only 5-10% of normal HeLa DNA reassociates within this Cot range.

DISCUSSION

This study has shown that DNA synthesized by isolated nuclei can be distinguished by its reassociation kinetics from normal DNA synthesized by growing cells. The reassociation rate of various DNA preparations labeled for different time intervals (20 min or 24 h) in vivo showed the same kinetics, indicating that DNA was randomly labeled in vivo even within a 20 min labeling period. The DNA synthesized in vitro by nuclei from HeLa F cells shows a significant increase in its reassociation rate in the Cot range from 5 to 500. Above Cot 500, the slope of the Cot curves are roughly the same. The very high amount of moderately fast reassociating DNA made by nuclei from mitomycom C-treated cells cannot be ascribed to the cross-linkage of DNA which is induced by mitomycin (15), since the Cot value at zero time was the same as normal DNA. As further controls, we have also carried out reassociation at two different DNA concentrations (40 μg/ml and 1600 μg/ml) and obtained the same reassociation rate constants. [^{3}H]thymidine labeled DNA obtained directly from cells exposed to mitomycin also had the same reassociation curve as control DNA. This finding indicates that any cross-linkage of DNA with mitomycin could not be seen in the reassociation kinetic measurements of the sheared DNA under the conditions used in these experiments.

Isolated nuclei from HeLa cells preferentially synthesize some or all of the middle repetitious DNA sequences at a higher rate than the unique sequences. The preferential synthesis of middle repetitious DNA in isolated nuclei may represent a meaningless abnormal DNA synthesis or perhaps the syn-

thesis of a repeated DNA present in an initiation or temination region. In this respect, it has been found that major amounts of repetitious sequences in eukaryotic chromosomal DNA are evenly interspersed among the unique sequence DNA (16). Assuming that HeLa cells have at least 10^4 replicons (17), the molecular weight of an average replicon would be about 100 million and one would assume that repetitious DNA would be present in these units of replications. If repetitious DNA is involved in the control of DNA synthesis *in vivo*, such involvement may be magnified *in vitro* and be reflected in the results presented in this paper.

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