ALKALI-LABILE COLICINOGENIC FACTOR E1 DNA MOLECULES FORMED IN THE PRESENCE OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE

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SUMMARY

Plasmid ColE1 DNA of *E. coli* was used as a target DNA molecule to analyse the structural modification of DNA by N-methyl-N'-nitro-N-nitrosoguanidine. When the low concentration of this drug was used, both cell growth and overall DNA synthesis were neither stimulated nor inhibited, and plasmid DNA molecules were isolated as closed circles after replication. These molecules were stable for the ribonuclease treatment, but became susceptible to the alkaline hydrolysis. Such alkali-labile sites of ColE1 DNA were found in the parental strands and randomly distributed from the restriction endonuclease EcoR1 cleavage site.

INTRODUCTION

N-Methyl-N'-nitro-N-nitrosoguanidine(MNNG) is a well-known mutagen or carcinogen, which acts by methylating the chromosomal DNA (1). In the bacterial cells the mutagenic action is evident, and double mutations were frequently observed at the replication point (2). Similar observations were reported even if the low concentration of MNNG was used (3). On the animal cell, MNNG acts as a powerful carcinogen (4), and is known to create the alkali-labile site(s) in the chromosomal DNA (5). We have also extensively studied the effect of MNNG on various intermediary steps of mtDNA replication and found that the low concentration of MNNG brought about the relaxation of the closed-circular replicating DNA by single-strand cleavage, where the overall activity of DNA synthesis was neither stimulated nor inhibited (6).

As it is still ambiguous whether the structural modification of DNA by MNNG is directly relating to the mutation or carcinogenesis, ColE1 DNA of *E. coli* was used as a target molecule to analyse such correlation in the simple system. ColE1 DNA is a double-stranded supercoiled circle with a molecular weight of 4.2×10^6 daltons (7). With these characteristics, one can easily de-

tect a single-stranded cleavage of the DNA. Furthermore, the mechanism of ColE1 DNA replication has been studied in detail using cell lysate of *E. coli* (8), and the transformation experiment has been also established (9). ColE1 DNA is thus, a useful material for analysing a mechanism of mutation induced by chemical carcinogen.

In this paper, we present our findings that the closed-circular ColE1 DNA (ColE1 ccDNA) formed in the presence of low concentration of MNNG became alkali-labile, and such alkali-labile sites were randomly destributed in the parental strands of the molecule.

MATERIALS AND METHODS

1. *Bacterial strain and medium* — *E. coli* K12 A745(met, thy, ColE1$^+$), kindly supplied by Dr. Sakakibara, National Institute of Health, Tokyo, was cultivated in the OC medium containing 4 μg/ml thymine with shaking at 37°C (8). The growth was monitored with absorbance at 660 nm. DNA was labeled with [^{3}H]thymine(10 Ci/mmole, NEN Corp.) or [^{14}C]thymine(51 mCi/mmole). The doubling time of the log culture was about 30 min, under the conditions used.

2. *Preparation of closed-circular ColE1 DNA* — ColE1 ccDNA was prepared by the method of Clewel and Helinski (10) with slight modification. Cleared lysate, prepared from 10^{10} cells, was treated with 1 % SDS and extracted with saturated phenol, followed by dialysis against TES buffer(0.05 M Tris-HCl, pH 8.0, 0.05 M EDTA and 0.5 M NaCl).

The dialysed sample was subjected to the equilibrium centrifugation in CsCl-ethidium bromide. CsCl of 2.7 g and 600 μg of ethidium bromide were dissolved in 3 ml of the DNA solution. The centrifugation was performed in a Beckman SW50.1 rotor at 33,000 rpm for 40 hr at 20°C. After centrifugation, drops were collected from the bottom of the tube and 5 μl aliquote of each fraction was applied onto a filter paper disk(24mm, Toyo No.514), and washed twice with 5 % trichloroacetic acid(TCA). Filter paper disks were counted in a Beckman LS-250 liquid scintillation counter. Then, the ccDNA fractions were collected, and ethidium bromide was eliminated by tert-butanol, followed by dialysis against TES buffer.

3. *Neutral and alkaline sucrose density gradient centrifugation* — About 200 μl of DNA sample was layered on the 4.5 ml neutral sucrose gradient (5-20%) in 0.025 M Tris-HCl buffer, pH 8.0, containing 0.01 M EDTA and 0.5 M NaCl (10). Centrifugation was carried out in a Beckman SW50.1 rotor at 45,000 rpm for indicated time at 5°C. The tube was, then, punctured and drops were directly collected on filter paper disks. The radioactivity was measured, as described above. In the case of alkaline sucrose density gradient centrifugation, 0.6 ml of 60 % sucrose was placed in the bottom as a cushion, and sucrose gradient of 5-20 % in 0.26 M NaOH was made. The alkali-treated sample was layered on the gradient and centrifugation was performed at 45,000 rpm for 5 hr at 5°C.

RESULTS

1. *Formation of ColE1 DNA in the presence of low concentration of MNNG* — Growth of the cell and the overall synthesis of DNA observed by the uptake of labeled thymine in the presence of 1 μg/ml MNNG are summarized in Fig. 1. Under

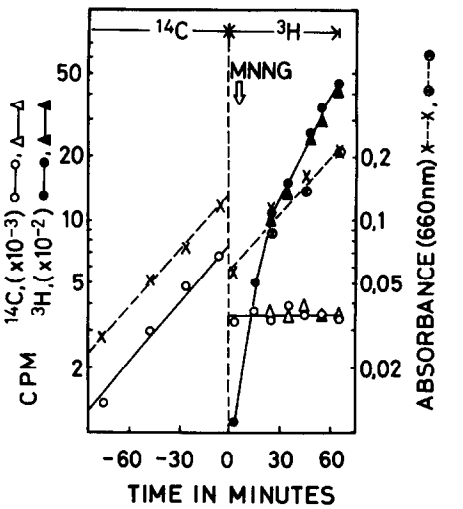

Fig. 1. Effect of MNNG on the cultivation of *E. coli*. The cells were grown in the OC medium containing [^{14}C]thymine(0.5 μCi/ml) with shaking at 37°C. At 0.1 of absorbance at 660 nm, cells were harvested, washed once with glucose- and thymine-free OC medium and suspended in the fresh OC medium containing [^{3}H]thymine(0.5 μCi/ml), followed by incubation. After 5 min, MNNG(1 μg/ml) was added to a half of the culture. The rest was the control without MNNG. From these cultures, 0.1 ml aliquotes were taken at the indicated times and mixed with cold 5 % TCA. The precipitate was collected by Whatman GF/C(25mm) filter and washed with cold 5 % TCA and with ethanol. Filters were counted in a toluene based scintillation fluid. Absorbance at 660 nm with (⊗--⊗) or without (x--x) MNNG. Incorporation of [^{14}C]thymine with (Δ—Δ) or without (○—○) MNNG. Incorporation of [^{3}H]thymine with (▲—▲) or without (●—●) MNNG.

the conditions described in the legend, both growth of the cell and incorporation of [^{3}H]thymine into the cell in the presence of MNNG were almost the same as those of the control. This indicates that the growth of the cell and the overall DNA synthesis are not affected by this concentration of MNNG. These data are consistent with the previous results reported by Sanchetzs and Ormedo (3).

As depicted in the legend of Fig.2, pronase-treated cell lysate was directly subjected to equilibrium centrifugation in CsCl-ethidium bromide (11). The radioactivity of the closed-circular DNA fraction was measured and the ratio of [^{3}H] to [^{14}C] is shown in Fig.2. Synthesis of ColE1 ccDNA was almost unaffected by 1 μg/ml of MNNG, although there was a little delay in its synthe-

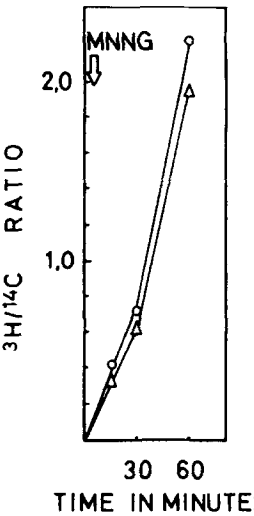

Fig. 2. Effect of MNNG on the ColE1 DNA synthesis. From the culture, as described in Fig. 1, 10 ml aliquotes were taken at 10, 30 and 60 min, and DNA synthesis was terminated by 20 mM KCN. Cells were precipitated, washed with 0.8 % NaCl in 0.2 M Tris-HCl, pH 8.0 and resuspended in 1.5 ml of 0.02 M Tris-HCl, pH 8.5, 0.025 M EDTA. The suspension was,then, incubated at 0°C for 20 min with 0.15 ml of lysozyme(4 mg/ml). The lysozyme-treated cell was mixed with 1.5 ml of 0.75 % sarkosyl and lysed at 65°C for 8 min. Pronase E(2 mg/ml) of 0.15 ml was added to the lysate and incubated for 60 min at 37°C. This pronase-treated lysate was vigorously mixed with vortex mixer, then centrifuged in CsCl-ethidium bromide (11). After centrifugation, the radioactivity of ccDNA fraction was measured. Ratios of [^{3}H] to [^{14}C] were plotted. MNNG added (Δ—Δ) and the control without MNNG (o—o).

sis. This may indicate that a sensitivity of ColE1 DNA synthesis to MNNG is slightly different from that of the chromosomal DNA.

2. *Alkali-labile closed-circular ColE1 DNA formed in the presence of MNNG* — As it is interesting to know whether a structural modification occurs at the parental strand of ColE1 DNA, following experiments were carried out. The cell, labeled with [^{3}H]thymine(5 μCi/ml), was washed once at mid-log phase(2~4 x 10^8 cells/ml) and resuspended in the thymine-free OC medium. MNNG was, then, added to the culture with final concentration of 1 μg/ml and incubation continued for further 60 min. The ColE1 ccDNA, prepared as described in MATERIALS AND METHODS, was centrifuged in both neutral and alkaline sucrose density gradients. As shown in Fig.3a, a single peak of the ccDNA(23s), which was stable

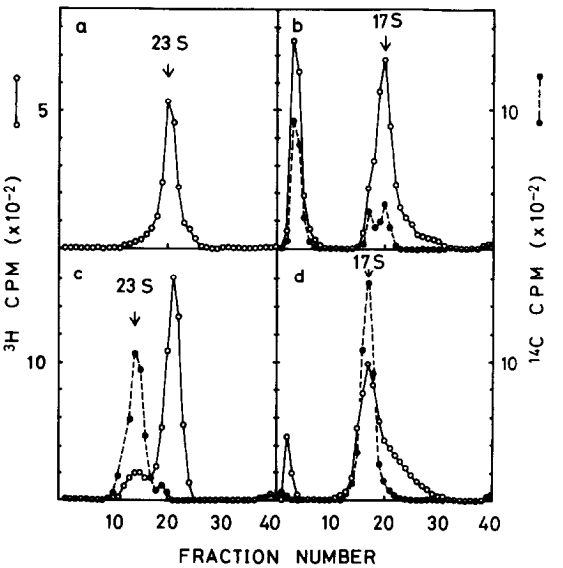

Fig. 3. Neutral and alkaline sucrose gradient centrifugation of MNNG-treated ColE1 ccDNA. [^{3}H]Thymine-labeled cells were harvested, as described in the RESULTS. The cleared lysate was prepared according to the legend of Fig. 2, and MNNG-treated ColE1 ccDNA was extracted as shown in MATERIALS AND METHODS. (a) MNNG-treated [^{3}H]ccDNA was centrifuged in 5-20 % neutral sucrose gradient at 45,000 rpm, for 180 min, at 5°C (o—o). (b) MNNG-treated [^{3}H]ccDNA was dialysed against pH 12.0 for 180 min, at 37°C and then subjected to 5-20 % alkaline sucrose density gradient centrifugation (o—o), as described in MATERIALS AND METHODS. As the internal marker, the ribonucleotide-containing [^{14}C]ColE1 ccDNA (8) was added. Peaks at 17th and 20th fractions corresponding to single-stranded circular(19 s) and linear(17 s) DNAs, respectively (o—o). MNNG-treated [^{3}H]ccDNA in 0.1 M Tris-HCl, pH 7.6, containing 0.5 mM EDTA and 10 mM $MgCl_2$ was digested with restriction endonuclease EcoR1 for 30 min at 37°C (12). (c) EcoR1-digested DNA (o—o) was centrifuged in 5-20 % neutral sucrose density gradient with the 23 s internal marker of [^{14}C]ColE1 ccDNA (●—●), separately prepared in the control culture. (d) EcoR1-treated DNA was dialyzed against alkali and centrifuged in 5-20 % alkaline sucrose density gradient (o—o). As the 17 s internal marker, EcoR1-digested [^{14}C]ColE1 DNA was added (●—●).

for the ribonuclease treatment(1 mg/ml at 37°C for 60 min), was observed in the neutral sucrose gradient. While in the alkaline sucrose density gradient, the radioactivity was split into two distinct peaks (Fig.3b). The rapidly-sedimenting peak(55 s) was the alkali-resistant MNNG-treated ColE1 DNA. The slowly-sedimenting peak(17 s) was a single-stranded linear form of the full-sized ColE1 DNA, having a small shoulder at the position of the single-stranded circular DNA(19 s). The slowly-sedimenting peak in Fig.3b was about 50 % of the total DNA, under the conditions used. On the other hand, the ColE1 ccDNA

isolated from the control culture was completely resistant to this alkaline condition (data not shown). This clearly indicates that a part of the ColE1 ccDNA molecules, formed in the presence of low concentration of MNNG, became alkali-labile on their parental strands.

3. *Position of the alkali-labile site* — Since restriction endonuclease EcoR1 from *E. coli* cleaves only one specific site in the ColE1 DNA (12), ccDNA, used in the previous experiment of Fig.3a, was completely digested with EcoR1 and subjected to the neutral sucrose density gradient centrifugation. As shown in Fig.3c, the main peak of the [^{3}H]ColE1 DNA was found at 17 s as compared with the internal marker of [^{14}C]ColE1 ccDNA(23 s), indicating that MNNG-treated ColE1 DNA became double-stranded linear form by the EcoR1 digestion.

After EcoR1 digestion, MNNG-treated DNA was analysed by alkaline sucrose density gradient centrifugation. In this experiment, EcoR1-digested [^{14}C] ColE1 DNA from the control culture was added as the internal marker. As shown in Fig.3d, fairly many counts were tailed to the lower molecular-weight fractions, although the single-stranded linear DNA was observed as a 17 s peak. Total counts in the lower molecular-weight fractions were about 50 % of the [^{3}H]DNA. This amount is comparable to the single-stranded linear ColE1 DNA in Fig.3b. Results in Fig.3d indicate that the alkali-labile sites are randomly distributed from the EcoR1 restriction site. In addition, a small amount of the [^{3}H]DNA observed at the cushion is possibly corresponding to the 23 s [^{3}H] molecules in Fig.3c, which may be resistant to the EcoR1 digestion.

DISCUSSION

From the results described above, it is likely indicated that MNNG creates a structural modification on the parental strand of ColE1 ccDNA, resulting in the formation of alkali-labile sites, which are randomly distributed from the EcoR1 restriction site in the molecule. Since MNNG is well-known to methylate the N-3 position of adenine and the N-7 and O-6 positions of guanine in the chromosomal DNA (13), it is easily expected that some purine base(s) in the ColE1 DNA molecule is methylated in the presence of low concentration of

MNNG, and an alkali-labile apurinic site is subsequently formed by depurination of methylated DNA (13). However, an increase of the open-circular DNA was not observed in the cleared lysate in this case (data not shown). This observation is distinctly different from the case of mtDNA exposed to MNNG in the *in vitro* replication system (6). As the alkali-labile site was formed on the prelabeled parental strand of ColE1 ccDNA, it is suggested that such modification reaction is a premutational event and not dependent on the DNA replication. This possibility is examined by preliminary studies on density-shift experiments using bromouracil and inhibitor experiments by nalidixic acid. Results will be published elsewhere. Recently Kimball and Setlow indicated that the synthesis of DNA is essential for the fixation of mutation (14). It is, therefore, interesting to know a repair mechanism of alkali-labile ccDNA, and to analyse the product of replication of the alkali-labile ColE1 DNA as a template.

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