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Abstract

To study chromatin structure at the sites of DNA replicated in permeable cells, deoxyribonuclease I (DNase I) sensitivity of newly replicated DNA in permeable mouse sarcoma cells was compared with that of newly replicated DNA in intact cells. About 35% of the DNA replicated in permeable cells was hypersensitive to DNase I, and the remaining DNA showed the same DNase I sensitivity as that of parental chromatin DNA. The sensitivity of DNA replicated in permeable cells was higher than that of DNA newly replicated in intact cells, and was close to that of DNA replicated in the presence of cycloheximide. The sensitivity of DNA pulse-labeled with [3H]deoxythymidine triphosphate by replication in permeable cells was reduced significantly by chasing with cold deoxythymidine triphosphate. The present results suggest that chromatin structure at the sites of DNA replicated in permeable cells is similar to that at the sites of DNA replicated in living cells in the absence of protein synthesis, and that some structural change (possibly toward the maturation) of newly replicated chromatin occurs after the DNA replication in permeable cells.

KEYWORDS: DNase I sensitivity, DNA replication, chromatin replication, permeable cells (mouse sarcoma)

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Deoxyribonuclease I Sensitivity of DNA Replicated in Permeable Mouse Sarcoma Cells

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To study chromatin structure at the sites of DNA replicated in permeable cells, deoxyribonuclease I (DNase I) sensitivity of newly replicated DNA in permeable mouse sarcoma cells was compared with that of newly replicated DNA in intact cells. About 35% of the DNA replicated in permeable cells was hypersensitive to DNase I, and the remaining DNA showed the same DNase I sensitivity as that of parental chromatin DNA. The sensitivity of DNA replicated in permeable cells was higher than that of DNA newly replicated in intact cells, and was close to that of DNA replicated in the presence of cycloheximide. The sensitivity of DNA pulse-labeled with [^{3}H]deoxythymidine triphosphate by replication in permeable cells was reduced significantly by chasing with cold deoxythymidine triphosphate. The present results suggest that chromatin structure at the sites of DNA replicated in permeable cells is similar to that at the sites of DNA replicated in living cells in the absence of protein synthesis, and that some structural change (possibly toward the maturation) of newly replicated chromatin occurs after the DNA replication in permeable cells.

Key words : DNase I sensitivity, DNA replication, chromatin replication, permeable cells (mouse sarcoma)

Several permeable cell systems for studying DNA synthesis in mammalian cells have been developed and shown to be useful for analytical studies of certain steps of DNA replication (reviewed in Ref. 1) and repair (see Ref. 2). To evaluate the permeable cell systems from the standpoint of chromatin replication, it is necessary to study chromatin structure in regions of newly synthesized DNA.

Chromatin structure has been analyzed effectively by using nucleases, such as staphylococcal nuclease and DNase I, as probes (1, 3-8). Staphylococcal nuclease primarily attacks the internucleosomal region and has been used to study the correlation between DNA synthesis and repeating nucleosome structure (1, 3, 8-19). DNase I which attacks both between and within nucleosomes is used as a sensitive probe of transcriptionally-competent chromatin and also unspecified gross chromatin structure (1, 3, 7, 8, 10, 19, 20).

Previously we compared staphylococcal nuclease sensitivity between DNA syntheses in intact cells and in permeable mouse ascites sarcoma cells (14). In this paper, chromatin structure in the regions replicated in permeable mouse ascites sarcoma cells was studied by using DNase I as a probe in comparison with that in the regions replicated in intact cells or repaired in bleomycin-treated permeable cells.

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Materials and Methods

DNase I (grade II) was obtained from Boehringer-Mannheim Co., staphylococcal nuclease from Worthington Biochemical Corp., and cycloheximide from Sigma Chemical Co. All other chemicals were obtained as described previously (21). Ascites sarcoma (SR·C3H/He) cells were collected from the ascites fluid of mice 3 days (in rapid growth phase) or 10 days (in stationary phase) after inoculation of 0.05 ml ascites per mouse (22).

The following procedures were conducted at 0-4℃ unless otherwise indicated. For replicative DNA synthesis in intact cells, ascites cells in rapid growth phase were suspended in a spinner culture medium and distributed in assay tubes at 2×10^6 cells per tube. After addition of $[^3H]$deoxythymidine ($[^3H]$dThd; 2.5 μM/0.6 ml, 1 Ci/mmol) and adjusting the volume to 0.6 ml, the suspension was incubated at 37℃ (22). For *in vitro* DNA synthesis, cells were permeabilized by treatment with buffer B (0.25 M sucrose, 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0) supplemented with Triton X-100 at 0.0175 % (Triton-buffer B)(23). For replicative DNA synthesis, permeable cells (2×10^6 cells) suspended in 0.4 ml of Triton-buffer B were mixed with 0.2 ml of a replicase substrate mixture consisting of 0.1 M Tris-HCl, 7 mM $MgCl_2$, 0.24 M NaCl, 7.5 mM ATP, 0.15 mM dATP, 0.15 mM dCTP, 0.15 mM dGTP and 7.5 μM $[^3H]$dTTP (0.5 Ci/mmol), adjusted to pH 8.0 at 25℃ (23). The suspension was incubated at 37℃ for 10 min. For bleomycin-induced unscheduled (repair) DNA synthesis, permeable cells prepared from cells in stationary phase were incubated in the same assay mixture as that for replicative DNA synthesis except that bleomycin A_2 was added at 200 μg/0.6 ml (0.22 mM), and ATP was omitted (21). Incubation was conducted at 37℃ for 60 min. For treatment with DNase I, $[^3H]$dThd or $[^3H]$dTMP-incorporated cells were washed thrice with 0.5 ml of Triton-buffer B. The washed cells were suspended in 0.5 ml of Triton-buffer B supplemented with 25 μg DNase I. The suspension was incubated at 37℃ for various intervals. After incubation the suspension was chilled at 0℃, and a half volume of 3 M perchloric acid was added. After standing for 10 min in an ice-water bath, acid-insoluble precipitates were collected on a Whatman GF/C filter disc, washed with 5% trichloroacetic acid and dried. Radioactivity of the disc was counted as described previously (22).

DNA content was measured by the method of Burton (24).

Results and Discussion

Kinetics of the digestion by DNase I of newly synthesized DNA and bulk DNA are shown in Fig. 1. The word "bulk DNA" was used here to indicate the whole DNA measured by the method of Burton (24). The digestability difference at each time point between newly synthesized DNA and bulk DNA was denoted as the "rapidly digested fraction" of newly synthesized DNA compared to bulk DNA. The rapidly digested fraction of newly synthesized DNA was relatively large when 10 to 20% of bulk DNA became soluble, and then slightly decreased as DNase digestion advanced further (Fig. 1). When the solubility of bulk DNA was lower than 10% or higher than 20%, which was brought about by varying the DNase concentration and /or digestion period, the rapidly digested fraction of newly synthesized DNA was frequently decreased, because of either insufficient or excessive digestion. Therefore, the rapidly digested fraction was measured in the present experiment when 10-20% of bulk DNA became soluble. This condition is similar to the condition at which transcriptionally active genes are preferentially digested by the enzyme (7). The rapidly digested fractions measured when 10-20% of bulk DNA became soluble were almost constant and were independent of the amount of digested DNA, as indicated by little variation in the values of the rapidly digested fraction in repeated experiments. The replicative nature of DNA synthesis in intact cells and also in permeable cells incubated in the replicase substrate mixture was confirmed by autoradiography

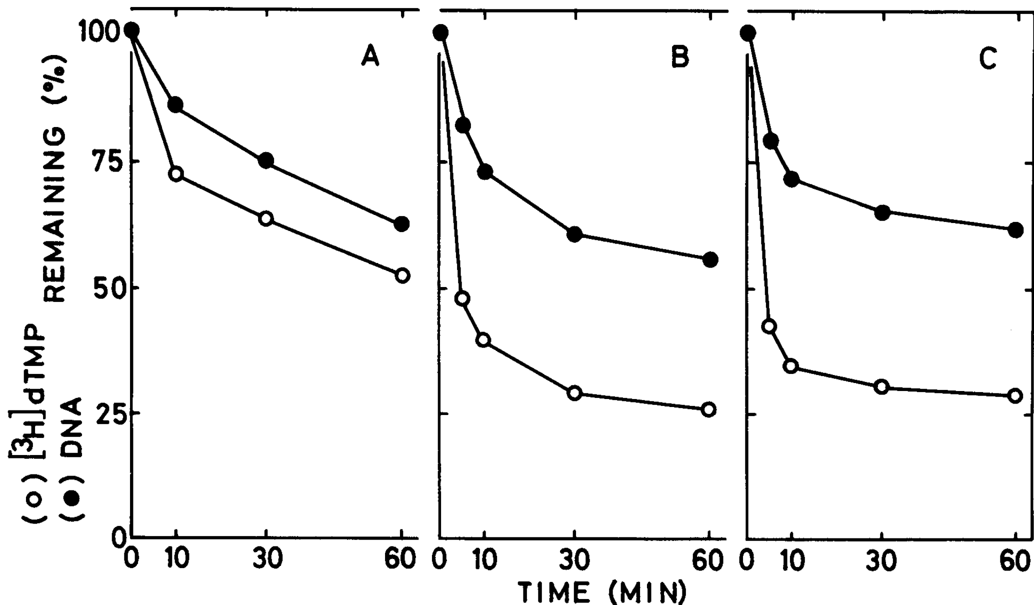

Fig. 1 The percentage of bulk DNA or ^{3}H-labeled, newly synthesized DNA undigested by DNase I after various times of incubation with the enzyme at 37℃. Replicative DNA syntheses in intact cells (A) and in permeable cells (B) were conducted, and DNA was labeled for 10 min with [^{3}H]dThd and [^{3}H]dTTP, respectively. Bleomycin-induced unscheduled (repair) DNA synthesis (C) was conducted, and DNA was labeled for 60 min with [^{3}H]dTTP as described in Materials and Methods. [^{3}H]dTMP-incorporated cells were digested at 37℃ for the indicated intervals with 25 μg/0.5 ml DNase I, and [^{3}H]dTMP and DNA content in the acid-insoluble fraction were measured as described in Materials and Methods.

and the bromodeoxyuridine monophosphate density shift technique (2, 21, 22). DNA synthesis in bleomycin-treated permeable cells was shown to be unscheduled, reflecting a partial reaction of excision repair type DNA synthesis (2, 21).

The fraction rapidly digested by DNase I of DNA replicated in permeable cells was about 2.5 times that of DNA replicated for 10 min in intact cells (Table 1). The fractions rapidly digested by staphylococcal nuclease of DNA replicated in either intact cells or permeable cells were smaller than those by DNase I. However, the rapidly digested fractions of DNA repaired in bleomycin-treated permeable cells were markedly larger when digested by staphylococcal nuclease than when digested by DNase I (Table 1).

The DNase I hypersensitivity of newly replicated cellular and SV40 chromatin has

Table 1 Fractions of newly synthesized DNA rapidly digested by DNase I

DNA synthesis	Enzyme	Rapidly digested fraction (%)[a] (Mean ± S. D.)
Replicative DNA synthesis		
in: Intact cells	DNase I	13.9 ±4.0(6)[b]
Permeable cells	DNase I	34.7 ±4.8(6)
Unscheduled DNA synthesis		
in: Permeable cells	DNase I	35.5 ±2.1(3)
Replicative DNA synthesis		
in: Intact cells	St. nuclease[c]	10.7 ±2.5(6)
Permeable cells	St. nuclease	22.1 ±3.0(6)
Unscheduled DNA synthesis		
in: Permeable cells	St. nuclease	74.1 ±0.8(4)

a: The rapidly digested fractions were calculated as described in the text.
b: Numbers in parentheses are the numbers of independent experiments.
c: The data (see Ref. 14) obtained by using staphylococcal nuclease (St. nuclease) are shown as a reference.

been reported previously (see Ref. 19). The hypersensitivity was suggested to be due to newly replicated DNA being first incorporated into endonuclease-sensitive "immature" nucleosomes, SV40 (and presumably mammalian) replicating chromosomes containing an endonuclease-sensitive prenucleosomal DNA and Okazaki fragment on each arm of SV40 replication forks, or both prenucleosomal DNA and newly assembled immature chromatin being present on each arm of SV40 replication forks (8, 19). However, the hypersensitive structure has not been clarified yet, especially in mammalian cells.

DNase I sensitivity of newly synthesized DNA in intact cells decreased rapidly by increasing the incubation time for DNA synthesis. When intact cells were incubated with [^{3}H]dThd for 30 min, the [^{3}H]dThd-labeled DNA showed almost the same sensitivity to DNase I as bulk DNA (Table 2). With the same 30 min incubation for DNA

Table 2 Effect of cycloheximide on the production of DNase I-hypersensitive DNA[a]

Time of incubation for DNA synthesis (min)	Cycloheximide (20 μg/0.6 ml)	Rapidly digested fraction of newly synthesized DNA (%) (Mean ± S. D.)
10	−	14.2 ± 4.3 (3)[b]
30	−	0.3 ± 1.2 (3)
10	+	31.0 ± 3.2 (3)
30	+	30.7 ± 2.9 (3)

a: Intact cells were incubated for replicative DNA synthesis in the presence (+) or absence (−) of cycloheximide. The fraction rapidly digested by DNase I was measured as described in Materials and Methods.
b: Numbers in parentheses are the numbers of independent experiments.

synthesis, staphylococcal nuclease sensitivity of newly replicated DNA in intact cells was also reduced to that of bulk DNA (14). The decrease in the rapidly digested fraction with the increase in incubation time is thought to indicate that chromatin organization of newly replicated sites returned to the organization of non-replicating sites (mature chromatin) as reported previously (15). The fraction rapidly digested by DNase I of DNA replicatively labeled with [^{3}H]dTTP for a short time (3 min) was reduced to about half by chasing with cold dTTP for 30 min (Fig. 2). The result indicates that some structural change (possibly toward the maturation) of the newly replicated chromatin occurred after the DNA replication in permeable cells, as shown previously by using SV40 chromosomes in an *in vitro* system (19).

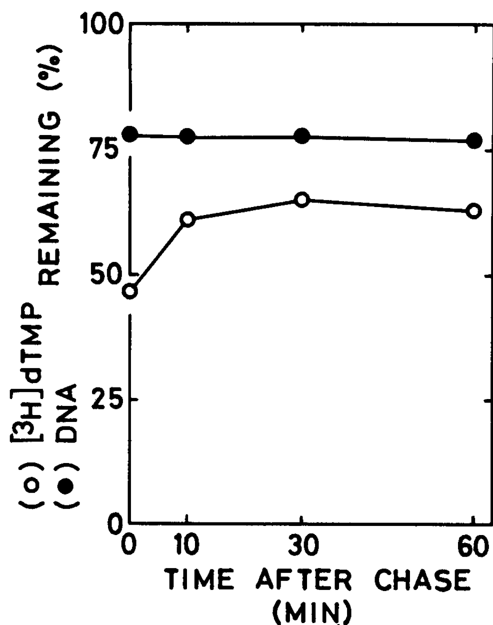

Fig. 2 Effect of chase with cold dTTP after labeling for 3 min with [^{3}H]dTTP in permeable cells on DNase I digestability. Replicative DNA synthesis in permeable cells was conducted for 3 min in the presence of 2.5 μM [^{3}H]dTTP as described in Materials and Methods, and the reaction was chased (continued) by adding 250 μM cold dTTP for the indicated intervals. [^{3}H]dTMP-incorporated cells were digested at 37°C for 10 min with DNase I at 25 μg/0.5 ml, and [^{3}H] dTMP and DNA content in acid-insoluble fraction were measured as described in Materials and Methods.

DNA replication is associated with histone and non-histone protein synthesis in intact cells, but not in permeable cells (14). The difference in the rapidly digested fractions

between replicative DNA synthesis in intact cells and that in permeable cells might be attributed largely to the differences in protein synthesis. To investigate this possibility the effect of protein synthesis inhibition on the DNase I sensitivity of newly replicated DNA was studied. When cycloheximide was added at 20 μg/0.6 ml to the assay mixture for replicative DNA synthesis in intact cells, DNA replication occurred at about half that of the cycloheximide-free control, whereas protein synthesis was almost completely inhibited (14). The fraction rapidly digested by DNase I of newly replicated DNA increased by inhibiting protein synthesis with cycloheximide, and approached the rapidly digested fraction of DNA replicated in permeable cells (Table 2). As reported previously (14), essentially the same result was obtained when staphylococcal nuclease was used as a probe. DNase I and staphylococcal nuclease are different in the specificities for chromatin digestion (1, 3, 4, 7, 8). The former preferentially digests transcriptionally competent chromatin regions, while the latter does not. The digestability difference between the enzymes was also observed in the present experiment (Table 1). The fractions rapidly digested by DNase I were almost the same between DNAs replicated in permeable cells and repaired in bleomycin-treated permeable cells, whereas the fractions rapidly digested by staphylococal nuclease were markedly different between the two DNAs (Table 1). A similarity between the chromatin structure at the sites of DNA replicated in permeable cells and that at the sites of DNA replicated in living cells in the presence of a protein synthesis inhibitor is indicated by the data obtained using these two probes having different specificities. This similarity suggests the possibility that the chromatin maturation process, which can occur in living cells in the absence of protein synthesis (16, 17), takes place in permeable cells.

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