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Takuzo Oda* Sekiko Watanabe†

Shiro Hanakawa‡ Takashi Nakamura**

*Okayama University,

†Okayama University,

‡Okayama University,

**Okayama University,

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Takuzo Oda, Sekiko Watanabe, Shiro Hanakawa, and Takashi Nakamura

Abstract

A permeable cell system has been developed by treatment with digitonin for studying in vitro DNA replication of chromatin. DNA replication of simian virus 40 nucleoprotein complexes (SV40 chromatin) in digitonin-treated permeable cells was analyzed by electrophoresis in agarose-gel. Autoradiography of the agarose-gel revealed that [32P]dCTP was incorporated in SV40 DNA I, II and replicating intermediates. The time course of the incorporation indicated the complete replication of SV40 DNA and chromatin with a full number of nucleosomes. The digitonin-treated permeable cell system will serve as a useful system for studying in vitro DNA replication of chromatin.

KEYWORDS: digitonin, permeable cells, DNA replication in vitro, SV40 chromatin replication, gel -electrophoresis, autoradiography

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BRIEF NOTE

COMPLETE *IN VITRO* DNA REPLICATION OF SV40 CHROMATIN IN DIGITONIN-TREATED PERMEABLE CELLS

Takuzo ODA, Sekiko WATANABE, Shiro HANAKAWA* and Takashi NAKAMURA

*Department of Biochemistry, Cancer Institute, and *Department of Orthopedic Surgery, Okayama University Medical School, Okayama 700, Japan*

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Abstract. A permeable cell system has been developed by treatment with digitonin for studying *in vitro* DNA replication of chromatin. DNA replication of simian virus 40 nucleoprotein complexes (SV40 chromatin) in digitonin-treated permeable cells was analyzed by electrophoresis in agarose-gel. Autoradiography of the agarose-gel revealed that [^{32}P]dCTP was incorporated into SV40 DNA I, II and replicating intermediates. The time course of the incorporation indicated the complete replication of SV40 DNA and chromatin with a full number of nucleosomes. The digitonin-treated permeable cell system will serve as a useful system for studying *in vitro* DNA replication of chromatin.

Key words: digitonin, permeable cells, DNA replication *in vitro*, SV40 chromatin replication, gel-electrophoresis, autoradiography.

Nucleotide-permeable cell systems have been developed for studying *in vitro* DNA replication in eukaryotic cells (1–12). Methods used for permeabilization of cells involve cold treatment with 2-mercaptoethanol (1–3), the use of detergents (4–7), hypotonic (8, 9) and hypertonic conditions (10), diethylaminoethyl-dextran (11), and lysolecithin (12). We have found a permeable cell system induced by treatment with digitonin to be extremely useful for studying both *in vitro* DNA replication of chromatin and oxidative phosphorylation of mitochondria. Digitonin, a steroid glycoside, binds with cholesterol and other β-hydroxysterols, which localize predominantly in the plasma membrane (13). Therefore, in permeable cells treated with low levels of digitonin, intracellular membranes are expected to be preserved relatively intact. Another advantage of this system is that it allows exogenously supplied macromolecules to reach intracellular sites (14).

Simian virus 40 nucleoprotein complexes (SV40 chromatin) have a nucleosome structure similar to that of cellular chromatin and serve as a useful model

system for studying the structure and function of cellular chromatin (15–17). Efficient replication of SV40 DNA in isolated nuclei or nuclear extract requires the addition of cytosol (17). In the present communication, we report the complete *in vitro* DNA replication of SV40 chromatin in digitonin-treated permeable cells without the addition of cytosol.

Materials and Methods. CV-1 monkey kidney cells were infected with plaque purified small plaque type SV40, strain 777, at a multiplicity of 50 to 100 plaque forming units per cell. Thirty-six to forty hours after infection, the CV-1 cells were trypsinized, calf serum was added, and the cells were washed with buffer A [50 mM Tris-HCl, pH 7.4, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 0.15 M sucrose]. They were then treated with buffer A containing digitonin at the concentration indicated for each 10^6 cells in 0.1 ml for 5 min at 25°C. In the standard assay condition, cells treated with 100 μg/ml digitonin in 0.1 ml were mixed with 0.15 ml of the substrate mixture to make final concentrations of 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 10 μM [^{3}H]dTTP (1 Ci/mmol), 5 mM ATP, 100 μM CTP, 100 μM GTP, 100 μM UTP in buffer A, and incubated for 15 min at 37°C. The radioactivity of [^{3}H] dTMP incorporated into the acid-insoluble fraction was measured on the glass filter in the toluene-PPO-POPOP system. The nature of SV40 chromatin DNA synthesized *in vitro* was analyzed by electrophoresis in 1.5% agarose-gel (18) after incubation under the standard assay conditions replacing [^{3}H] dTTP with [^{32}P] dCTP (4 μCi/ml), followed by extraction of DNA according to the method of Hirt (19) and purification of DNA by Marmur's method (20).

Results and Discussion. The effect of digitonin concentration on DNA-synthesizing activity. The incorporation of [^{3}H]dTTP into the DNA of trypsinized SV40-infected CV-1 cells was increased by treatment with digitonin, as shown in Fig. 1. The optimal concentration of digitonin for [^{3}H]dTTP incorporation was 100 μg/ml digitonin per 10^6 cells. The rate of [^{3}H]dTTP incorporation was nearly proportional to cell numbers up to 10^6 at the concentration of 100 μg/ml digitonin (Fig. 2). The optimal digitonin concentration increased to some extent with increase in the number of cells (Fig. 2). Digitonin treatment at lower temperature (4°C) with no incubation at 25°C decreased the rate of [^{3}H]dTTP incorporation. The time course of [^{3}H]dTTP incorporation was almost linear for 5 min and thereafter proceeded at a decreased rate (data not shown).

Requirements and inhibitors of DNA synthesis in digitonin-treated permeable cells. All deoxyridonucleoside triphosphates (dATP, dGTP, dCTP, dTTP), ATP, and Mg^{2+} were required for efficient DNA synthesis in digitonin-treated permeable cells (Table 1). The elimination of ATP reduced the amount of DNA synthesis to 41%. DNA synthesis decreased to 20% in the absence of ATP, GTP, CTP and UTP. Ethylendiamine-tetraacetic acid (EDTA, 10 mM) completely

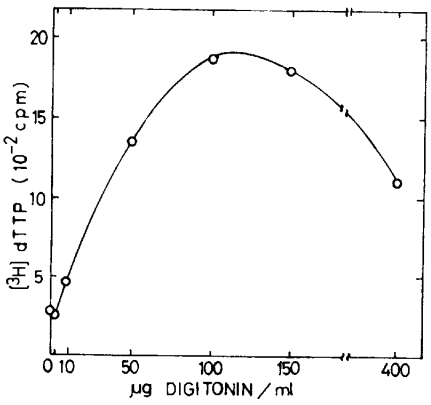

Fig. 1

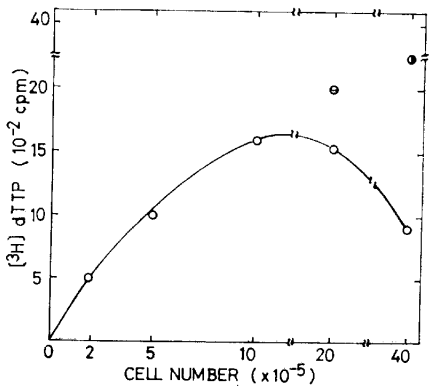

Fig 2

Fig. 1. The effect of digitonin concentration on DNA-synthesizing activity in SV40-infected CV-1 cells.

Fig. 2. The correlation of cell number with DNA-synthesizing activity in digitonin-treated CV-1 cells. ○—○, treated with 100 μg/ml digitonin; ◒ with 200 μg/ml; ◑, with 400 μg/ml.

TABLE 1. REQUIREMENTS AND INHIBITORS OF DNA SYNTHESIS IN DIGITONIN-TREATED PERMEABLE CELLS[a]

Conditions	Activity (% of control)
Complete	100
−dATP	91
−dGTP	63
−dCTP	56
−dATP, dCTP & dGTP	55
−ATP	41
−ATP, GTP, CTP & UTP	20
−dATP, dCTP, dGTP, −ATP, CTP, GTP & UTP	4
$-Mg^{2+}$ + EDTA (10mM)	0
−DTT + NEM (10mM)	0
+araCTP (0.6mM)	21

a The complete system for DNA synthesis measured the incorporation of [^{3}H]dTTP into DNA under the standard assay conditions. The amount of DNA synthesized was 1,600 cpm (3.6 pmoles) in the complete system. All reactions are expressed as a percentage of the control complete reaction system.

inhibited DNA synthesis in the absence of $MgCl_2$. N-ethylmaleimide (NEM, 10 mM) also completely inhibited DNA synthesis in the absence of dithiothreitol.

Nature of DNA synthesized in digitonin-treated permeable cells. More than 80% of [^{3}H]dTMP incorporated into DNA was recovered in the Hirt supernatant. Electrophoretic analysis in 1.4% agarose-gel of DNA purified from the Hirt supernatant showed clear separation of forms I, II, and III of SV 40 DNA by staining with ethidium bromide (Fig. 3A). The majority of the SV40 DNA was form I DNA. Autoradiography of this agarose-gel revealed that [^{32}P]dCTP was incorporated into forms I, II and replicating intermediates of SV40 DNA (Fig. 3B). The amount of [^{32}P]dCTP incorporation increased with the advance

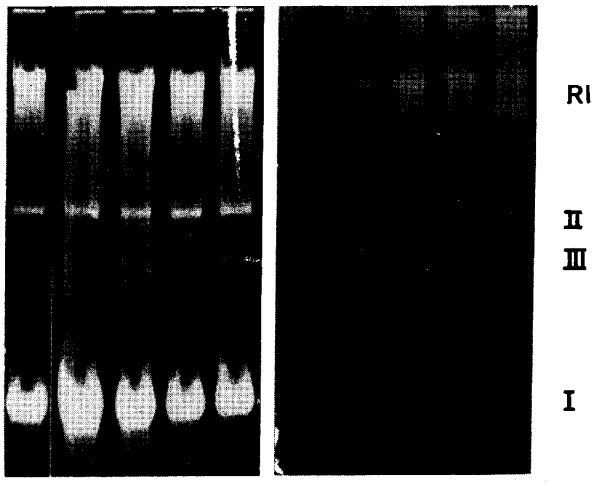

Fig. 3. Gel electrophoretic analysis of SV40 DNA synthesized in digitonin-treated permeable cells under the standard assay conditions. A, ethidium bromide staining; B, autoradiography of [^{32}P]dCMP incorporated into DNA, exposed for 7 days at −80°C; reaction times for DNA synthesis of the samples in a, b, c, d and e were 0, 5, 15, 30 and 60 min, respectively. I, II, III and RI indicate forms I, II, III and replicating intermediates of SV40 DNA.

of reaction time; being very small in form I DNA after 5 min incubation, increased in both form I and II DNA after 15 min, and further increased in form I DNA after 30 and 60 min. These results indicate that the major part of replicating SV40 DNA completed replication in digitonin-permeable cells. Since closed circular molecules of SV40 DNA with insufficient nucleosome formation give multiple bands corresponding to the numbers of superhelical turns between the bands of form I and II DNA in the agarose gel (15), the formation of form I DNA indicates the complete replication of SV40 chromatin with a full number of nucleosomes. The digitonin-treated permeable cell system will therefore serve as a useful system for studying *in vitro* DNA replication of chromatin.

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