EVIDENCE FOR THE INVOLVEMENT OF SINGLE-STRAND DNA-BINDING PROTEINS IN MAMMALIAN DNA REPLICATION

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1. Introduction

In prokaryotic organisms, single-strand DNA-binding proteins (ssDNA-binding proteins) are required for DNA replication [1]. In mammalian cells, several proteins with affinity to ssDNA have been isolated [2–5], but their roles in mammalian DNA replication were not characterized. In this communication, an experimental system that may contribute to the elucidation of the functions of mammalian ssDNA-binding proteins will be presented. As previously reported [6], cell extract (i.e. a supernatant obtained by high speed centrifugation of homogenized CHO cells) stimulates semiconservative DNA replication in partially lysed CHO cells. It will be shown that ssDNA of low molecular weight inhibits DNA synthesis in this subcellular system, probably by forming complexes with proteins present in the cell extract and required for DNA replication.

2. Materials and methods

2.1. *Materials*

Herring sperm DNA was purchased from Fluka, and [^{14}C]DNA was purified from CHO cells as described [6]. Metrizamide (analytical grade) was obtained from Nyegaard; Co A/S and DNase I from Worthington Biochemical. The source of other compounds has been previously indicated [6].

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2.2. *Partially lysed ("highly lysed") CHO cells and determination of the radioactivity in DNA*

Exponentially growing CHO cells were prelabeled with [^{14}C]dThd (2 nCi/ml; 50 Ci/mol) and detached from petri dishes by trypsin treatment. Partially lysed CHO cells were prepared by treatment with a buffer containing 0.1% Brij-58 and 240 mM KCl as previously described and as referred to as "highly lysed cells" [6]. In this preparation, 20–50% of nuclei were essentially free of cytoplasm and 50–80% of nuclei were surrounded with little cytoplasm. Lysed cells were suspended in buffer A (40 mM HEPES (pH 7.8), 80 mM KCl, 1.5 mM dithiothreitol, 0.5% (w/v) dextran, 225 mM sucrose, 1 mM EGTA, 4 mM $MgCl_2$, 2 mM ATP, 5 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 0.1 mM each of dATP, dCTP, dGTP, CTP, GTP and UTP and 10^{-5} M [^{3}H]dTTP (1 Ci/mmol). In addition, reaction mixtures contained cell extract and heat-denatured (15 min; 95°C) DNA as described in section 3.

After incubation, acid-insoluble DNA was collected on GF/C filters, and radioactivity was determined. To correct for differences in DNA synthesis due to slight variations of cell numbers in the test tubes, ^{3}H/^{14}C ratios were calculated. Further details of this assay system have been described previously [6].

2.3. *Equilibrium density in metrizamide gradients of heat-denatured DNA*

A volume of 100 μl of heat-denatured [^{14}C]CHO DNA (35 μg/ml) was mixed with 100 μl of buffer B (40 mM HEPES, pH adjusted to 7.8 with ~30 mM KOH, 80 mM KCl, 300 mM sucrose, 2 mM dithiothreitol, 1 mM EGTA, 4 mM $MgCl_2$ and 2 mM ATP) or with 100 μl of buffer B containing cell extract at

a concentration equivalent to 4×10^7 cells/ml. To all samples, 4.8 ml of buffer B containing metrizamide was added to give a final metrizamide concentration of 0.4 M. The samples were centrifuged for 70 h at 1°C with 30 000 rev./min in a 50 Ti rotor (Beckman). After centrifugation, fractions were collected from the bottom of the tubes, and the radioactivity and the refractive index in aliquots of each fraction were determined.

3. Results and discussion

3.1. *Inhibition of DNA replication by ssDNA*

To determine the effects of ssDNA on DNA replication, highly lysed cells were incubated at 30°C with cell extract, [^{3}H]dTTP and different concentrations of heat-denatured herring sperm DNA (50% of molecules $<5 \times 10^4$ d). After incubation, radioactivity incorporated into DNA was determined. As shown in Fig.1, heat-denatured herring sperm DNA inhibited DNA replication. Similar results were obtained with purified, heat-denatured CHO DNA of low molecular weight (not shown). Furthermore, little [^{3}H]DNA was synthesized if reaction mixtures containing cell extract and denatured herring sperm DNA were incubated without highly lysed cells, indicating that ssDNA of low molecular weight was a poor template for polymerase(s) in the cell extract (see legend to Fig.1). In contrast, ssCHO DNA with an average molecular weight of 1.5×10^6 daltons exhibited a template activity 20–50 times higher than heat-denatured herring sperm DNA. This template activity was, however, greatly reduced by irradiation of ssDNA with UV light (30 kJ/m^2), whereas this treatment did not detectably affect the capacity of ssDNA to inhibit DNA replication.

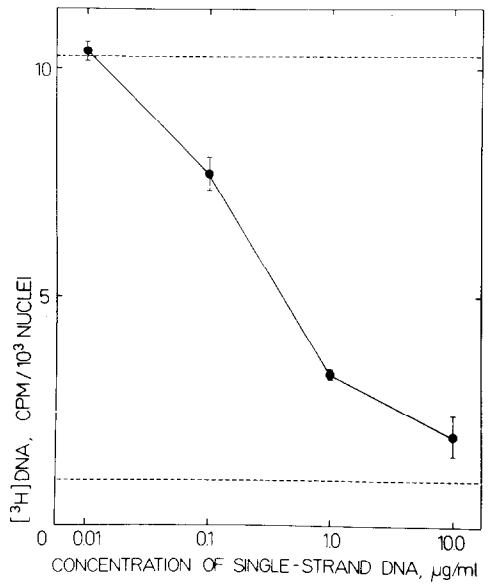

Fig.1. Inhibition of DNA replication by heat-denatured herring sperm DNA. Highly lysed cells were suspended in buffer A containing [^{3}H]dTTP, cell extract at a concentration equivalent to 10^7 cells/ml and heat-denatured DNA at the concentrations indicated in the figure. The incubation was carried out in test tubes containing ~10^5 nuclei in a volume of 20 μl. After incubation for 60 min at 30°C, radioactivity in DNA was measured. To determine template activity of heat-denatured DNA, [^{3}H]DNA synthesized in 20 μl of the reaction mixture without highly lysed cells was measured, and these values (25 cpm, 44 cpm, 32 cpm and 80 cpm in incubation mixtures containing 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml and 10 μg/ml of denatured DNA, respectively) were subtracted to obtain net incorporation of [^{3}H]dTTP by 10^5 highly lysed cells. Vertical bars indicate the range of duplicates. Dotted lines: [^{3}H]DNA synthesized by highly lysed cells incubated in buffer A (lower line) or in buffer A containing cell extract without ssDNA (upper line).

3.2. *Interaction of ssDNA with cell extract*

To determine the effect of different cell extract concentrations on the incorporation of [^{3}H]dTTP, highly lysed cells were incubated in the reaction mixture containing cell extract with or without 1 μg/ml of heat-denatured herring sperm DNA. As shown in Fig.2, no inhibitory effect of denatured DNA was observed if cell extract was present at a concentration equivalent to 10^8 cells/ml. A 5-fold dilution of cell extract had little effect on highly lysed cells incubated without denatured DNA, while in the reaction mixture containing denatured DNA, [^{3}H]dTTP incorporation was decreased. Thus, ssDNA interacted with factor(s) in the cell extract that stimulated DNA replication in highly lysed cells. In the absence of cell extract, the incorporation activity observed in reaction mixtures with or without denatured DNA was indistinguishable, suggesting that DNA replication not stimulated by cell extract was not affected by ssDNA.

To demonstrate that ssDNA of low molecular weight does not serve as a template if incubated with highly lysed cells and without cell extract, CHO cultures were density-labeled for 30 min with BrdUrd prior to preparation of highly lysed cells. These were incubated for 60 min at 30°C with 10^{-5} M [^{3}H]dTTP (15 Ci/mmol) and with or without 1 μg/ml of heat-

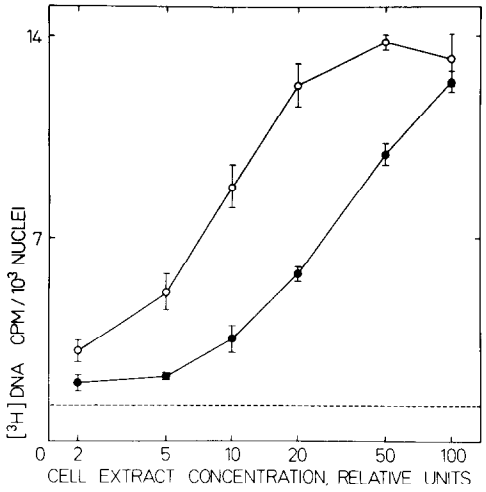

Fig.2. Effect of cell extract on DNA replication by highly lysed cells incubated with and without heat-denatured DNA. Highly lysed cells were incubated for 60 min at 30°C in buffer A containing [^{3}H]dTTP, with (•) or without (○) 1 μg/ml of heat-denatured herring sperm DNA. Cell extract concentrations in the reaction mixtures are indicated in the figure, and 100 units correspond to a concentration equivalent to 10^8 cells/ml. After incubation, [^{3}H]DNA was determined. [^{3}H]DNA synthesized due to template activity of heat-denatured DNA was less than 5% of that in corresponding test tubes containing highly lysed cells. Values shown in the figure are corrected for this template activity. Vertical bars indicate the range of duplicates. The dotted line indicates [^{3}H]DNA synthesized in highly lysed cells incubated with buffer A in the absence of cell extract, with or without 1 μg/ml of heat-denatured herring sperm DNA.

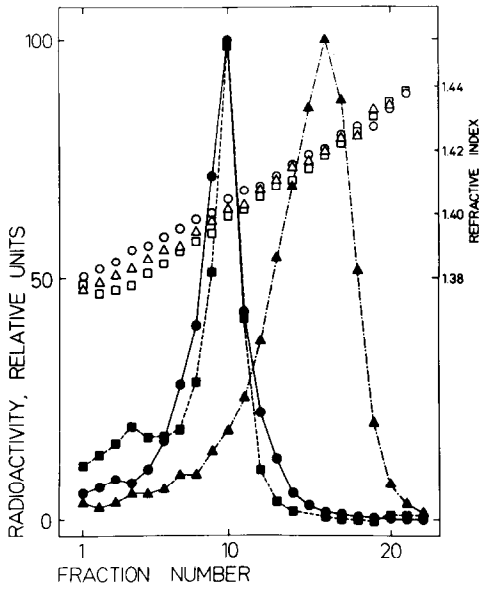

Fig.3. Equilibrium density in metrizamide gradients of heat-denatured [^{14}C]DNA (•, ▲, ■) incubated with (▲, ■) or without (•) cell extract. ■, cell extract in buffer B had been preincubated for 2 h at 30°C with 0.5 mg/ml or pronase E; ○, △, □, Refractive indices. Symbols correspond to those of the radioactivity profiles.

denatured herring sperm DNA. After incubation, the DNA of both reaction mixtures was purified and analyzed in alkaline equilibrium density gradients [6]. Most of the [^{3}H]DNA was density-labeled, indicating covalent linkage between newly synthesized DNA and that preexisting in highly lysed cells, and the density distribution of [^{3}H]DNA was not affected by the addition of heat-denatured herring sperm DNA to the reaction mixture. Thus, [^{3}H]dTTP incorporation attributable to the template activity of ssDNA was negligible.

3.3. *Proteins of cell extract bind to ssDNA and protect it from digestion by DNase I*

To demonstrate that factor(s) stimulating DNA replication form stable complexes with ssDNA and are proteins, native cell extract or cell extract preincubated with pronase E was mixed with heat-denatured [^{14}C]DNA. A metrizamide solution was added, and the samples were centrifuged to equilibrium. As shown in Fig.3, [^{14}C]DNA that had not been mixed with cell extract had a density similar to that of [^{14}C]DNA mixed with pronase-digested cell extract, while [^{14}C]DNA mixed with native cell extract banded at a higher density. These results indicate that factors bound to heat-denatured DNA were proteins forming stable complexes with DNA at relatively high salt concentrations.

To further characterize interaction of these proteins with DNA, cell extract at different concentrations was mixed with purified non-denatured or heat-denatured [^{14}C]DNA and incubated at 0°C with DNase I. After 60 min of incubation, acid-insoluble [^{14}C]DNA was determined. As shown in Table 1, a considerable proportion of heat-denatured DNA remained acid-insoluble if incubated with cell extract at sufficiently high concentrations. On the other hand, most double-strand DNA was digested under these conditions. Thus, cell extract did not inactivate DNase I, but contained proteins binding to ssDNA and protecting it against DNase I digestion, as reported

Table 1
Digestion at 0°C of purified CHO DNA by DNase I in reaction mixtures containing different concentrations of cell extract

DNA in the reaction mixture	Cell extract concentration equivalent to cells/ml	Remaining acid-insoluble DNA after digestion with DNase I %
Heat-denatured	2×10^7	74
	1×10^7	40
	5×10^6	15
	–	2
Non-denatured	2×10^7	13

^{14}C-Labeled DNA (3 μg/ml) was incubated for 15 min at 30°C with cell extract in buffer B. The incubation mixture was then cooled to 0°C. To start digestion of DNA, the reaction mixtures were supplied with 0.11 mg/ml DNase I, 2 mM $MgCl_2$ and 3 mM $CaCl_2$ and incubated for 60 min at 0°C

for purified binding proteins from *E. coli* [7] or KB cells [8].

In conclusion, the results in this report indicate that proteins required for mammalian DNA replication form stable complexes with ssDNA. It has been previously shown [6] that highly lysed cells incubated with cell extract exhibit DNA replication similar to that of intact cells. The experimental approach described in this communication may, therefore, be useful in attempts to elucidate the functions of binding proteins in mammalian DNA replication.

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References

[1] Meyer, R. R., Glassberg, J. and Kornberg, A. (1979) Proc. Natl. Acad. Sci. USA 76, 1702–1705.
[2] Fox, T. O. and Pardee, A. B. (1971) J. Biol. Chem. 246, 6159–6165.
[3] Herrick, G. and Alberts, B. (1976) J. Biol. Chem. 251, 2124–2132.
[4] Otto, B., Baynes, M. and Knippers, R. (1977) Eur. J. Biochem. 73, 17–24.
[5] Novak, B. and Baril, E. F. (1978) Nucl. Acids Res. 5, 221–239.
[6] Reinhard, P., Maillart, P., Schluchter, M., Gautschi, J. R. and Schindler, R. (1979) Biochim. Biophys. Acta 564, 141–153.
[7] Molineux, I. J. and Gefter, M. L. (1975) J. Mol. Biol. 98, 811–825.
[8] Nass, K. and Frenkel, G. D. (1979) J. Biol. Chem. 254, 3407–3410.