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Double-stranded DNA Synthesized by RD-114 Virion DNA Polymerase

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INTRODUCTION

Reverse transcriptase, a unique enzyme, found in virions of C-type viruses, possesses two kinds of enzyme activities, namely RNA dependent DNA polymerase which catalyzes the transcription of single-stranded complementary DNA from a viral RNA template, and DNA dependent DNA polymerase as shown by the synthesis of double-stranded DNA[1~3]. Virions of RD-114 virus, an endogenous type-C virus of feline, synthesize double-stranded DNA molecules (dsDNA) as well as single-stranded DNA molecules (ssDNA), and these viral DNAs were used as probes to investigate viral gene sequences in feline and human cells[4]. Normal feline cells contained 100-200 copies of viral gene sequences per haploid DNA quantity, while less than one copy per haploid DNA quantity were detected in human cells in which RD-114 virus can grow efficiently[4]. In this communication will be presented a detailed analysis of RD-114 virus dsDNA population, fractionation of dsDNA by reassociation reaction and further analysis of RD-114 viral DNA sequences in feline and human cells using fractionated dsDNAs as probes.

MATERIALS AND METHODS

Cells and Virus

Preparations of purified RD-114 virions and RD (rhabdomyosarcoma) cells were kindly donated by Dr. Maurice Green, Institute for Molecular Virology, St. Louis University School of Medicine.

P3HR-1 (5): An Epstein-Barr virus producing human lymphoblastoid cell line was provided by Dr. Toyoro Osato, Hokkaido University School of Medicine. Clinical materials were provided by Dr. Ichiro Urushizaki and Dr. Kokichi Kikuchi, Sapporo Medical College.

Preparations of viral [^3H] dsDNA and [^3H] ssDNA probes

Viral [^{3}H] dsDNA and [^{3}H] ssDNA were synthesized by the endogenous DNA polymerase reaction of disrupted virions in the absence and presence

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of actinomycin D (100 μg/ml), respectively, as described (6).

Purification of cellular DNA

Cellular DNAs were purified by the modified procedure of Berns and Thomas as described (4). Cellular DNAs were sonicated to 600–800 nucleotide length by a KUBOTA KMS-250 sonicator at 0°C for 10 min at 250 W.

Reassociation Reaction of [^{3}H] dsDNA

[^{3}H] dsDNA with cellular DNA was denatured at 100°C for 10 min. Reaction mixtures in 0.4 M PB (Phosphate buffer, pH 6.8) were incubated at 67°C. At various incubation periods aliquots were resolved into ssDNA and dsDNA fractions by the batch method of hydroxyapatite chromatography (7). Calf thymus DNA (50–100 μg) was added as a carrier to each fraction. After an addition of trichloroacetic acid to a final concentration of 10% (w/w), precipitates were collected on a nitrocellulose membrane, and counted by a liquid scintillation counter, Beckman LS-230.

Fishing of [^{3}H] ssDNA by RD-114 viral 70 S RNA

[^{3}H] ssDNA, 1.5×10^6 cpm in 800 μl, incubated with 3.4 μg of RD-114 viral 70 S RNA in 2×SSC (SSC : 0.15 M NaCl+0.015 M sodium citrate) and 40% formamide. After 52 hrs incubation at 45°C, [^{3}H] ssDNA hybridized with 70 S RNA was separated from free [^{3}H] ssDNA by the batch procedure of hydroxyapatite chromatography. The hybridized [^{3}H] ssDNA was treated with 0.1 N NaOH at 37°C for 18 hrs and purified [^{3}H] ssDNA was used for the hybridization reaction.

Hybridization reaction of ssDNA with cellular DNA

Purified [^{3}H] ssDNA with cellular DNA was denatured at 100°C for 5 min. Reaction mixtures in 0.4 M PB were incubated at 67°C for 5 days. The hybridized DNA was measured by the batch method of hydroxyapatite chromatography as described above.

RESULTS AND DISCUSSION

Characterization of the DNA product

[^{3}H] DNA product synthesized by the endogenous DNA polymerase reaction of RD-114 virus was purified and strandedness of the product was analyzed by hydroxyapatite chromatography. As shown in Table 1, approximately 80% of [^{3}H] DNA was bound to hydroxyapatite in 0.14 M PB and was eluted with 0.4 M PB, indicating that 80% of the DNA product had a duplex structure. The heterogeneity of dsDNA population was revealed by reassociation kinetics of [^{3}H] dsDNA which showed a marked deviation

from linearity in the Cds/Css versus Cot plot (4, 8). This heterogeneity possibly reflects the decomposition of template genomic RNA during long term incubation and the resulting unequal transcription of each part of genomic RNA into DNA (9-11).

Table 1. *Strandedness of [^3H] DNA Product Synthesized by Endogenous RD-114 Polymerase Reaction*

DNA product* (specific activity)	single-stranded cpm	%	double-stranded cpm	%
Preparation 1 (5.89×10^6 cpm/μg)	2.19×10^5	21.4	8.05×10^5	78.6
Preparation 2 (1.69×10^6 cpm/μg)	0.42×10^5	16.8	2.07×10^5	83.2

* [^{3}H] DNA product synthesized by endogenous RD-114 DNA polymerase reaction for 18 hrs at 37°C.

Isolation of RR, SR-1 and SR-2 fractions

The [^{3}H] dsDNA was fractionated into three fractions, namely rapidly reassociating fraction (RR), slowly reassociating fraction 1 (SR-1) and slowly reassociating fraction 2 (SR-2) according to the reassociation rate. [^{3}H] dsDNA was heat-denatured at 100°C for 10 min and reassociated in 0.4 M PB at 67°C. RR fraction was separated as dsDNA by hydroxyapatite chromatography at a Cot value of 0.63×10^{-3} mole·sec/1. After removal of RR fraction, SR-1 fraction was also separated similarly as a dsDNA fraction at a Cot value of 2.81×10^{-3} mole·sec/1. Sequence complexity of RR and SR-1 fractions were estimated as 1.32×10^6 daltons (13.1% of the viral genome) and 3.34×10^6 daltons (33.1% of the viral genome), respectively (Table 2). Reassociation rate constant of adenovirus type 2 DNA, with a molecular weight of 23×10^6 daltons (13), was used for molecular weight calibration. Sequence complexity of SR-2 fraction could not be determined because of

Table 2. *Complexities of Fractions of RD-114 Virus ds-DNA*

Fraction	Percent of Total ds-DNA	Complexity*1 (daltons)	Fraction of viral genome*2	Viral DNA copies per/Cat kidney Cell
RR	29.2	1.31×10^6	13.1	52
SR-1	19.2	3.34×10^6	33.1	47
SR-2	51.6	—	—	—

*1 Adenovirus type 2 DNA, molecular weight 23×10^6 dalons, was used as a reference.
*2 5.05×10^6 daltons was used as the nucleotide complexity for RD-114 viral RNA (12).

the heterogeneity of this fraction. The addition of poly(A)n (3.5×10^{-3} μg/ml) did not cause any appreciable change in the reassociation rate of RR fraction.

Reassociation of RR fraction in the presence of various cell DNAs

The reassociation reaction of viral dsDNA in the presence of cell DNA is a highly sensitive method for detecting virus-related sequences in cell DNA (4, 8). The sequence of the RR fraction of dsDNA representing about 30% of the dsDNA population was used first to detect the viral DNA sequence in human cell DNA. No appreciable accelerations were observed by the addition of human cell DNA from P3HR-1, a cell line established from Burkitt's lymphoma, or from peripheral blood of patients with acute myelogenous leukemia (AML) as previously described (6). Similarly, DNA from RD cell line established from human rhabdomyosarcoma did not accelerate the reassociation of RR fraction (Table 3). The sensitivity of these measurements showed that less than one copy per diploid quantity of cell DNA was present in these cells.

Table 3. *The RR fraction in cell DNAs*

Cellular DNA (μg/ml)		RD-114 dsDNA (ng/ml)	Increased Rate Factor	Viral DNA copies per cell
E. coli	(190)	0.790	1.00	—
RD cell	(190)	0.790	1.05	<1
Cat kidney + E. coli	(96.4) (93.6)	0.790	3.06	52

The number of viral DNA copies in the cell was calculated from the increased rate factor, using values of 4×10^{12} daltons for mol. wt. of diploid cell DNA and 1.32×10^{6} daltons for the sequence complexity of RR fraction.

Reassociation of SR-1 and SR-2 fraction in the presence of RD cell DNA

The effects of RD cell DNA on the reassociation kinetics of SR-1 and SR-2 fraction were tested. As shown in Fig. 1 and Fig. 2, RD cell DNA accelerate the reassociation of SR-1 and SR-2 fractions slightly. As a result of analysis of the initial reaction (8), approximately 300 copies of 5.8% of SR-1 sequence per diploid cell DNA quantity were estimated to be present in RD cell line. This value of 5.8% of SR-1 sequence corresponds to aproximately 2% of the whole viral genome. These suggest the presence of the viral sequence related to SR fractions and not to RR fraction in RD cell line.

Hybridization of [^3H] ssDNA with various cell DNAs

To further investigate the DNA sequence possibly related to RD-114 viral genome in human cells, hybridization experiments of human cell DNAs with [^{3}H] ssDNA representing most of the viral genome sequences were carried out. [^{3}H] ssDNA probe was prepared by the endogenous DNA polymerase reaction of disrupted RD-114 virions in the presence of actinomycin D (100 μg/ml), and purified as described (6) after hybridization with RD-114 virus 70 S RNA. Purified [^{3}H] ssDNA complementary to viral 70 S RNA was hybridized with DNAs from RD, P3HR-1 and normal human spleen to a Cot value of 9600. Only a few percent of the input [^{3}H]ssDNA

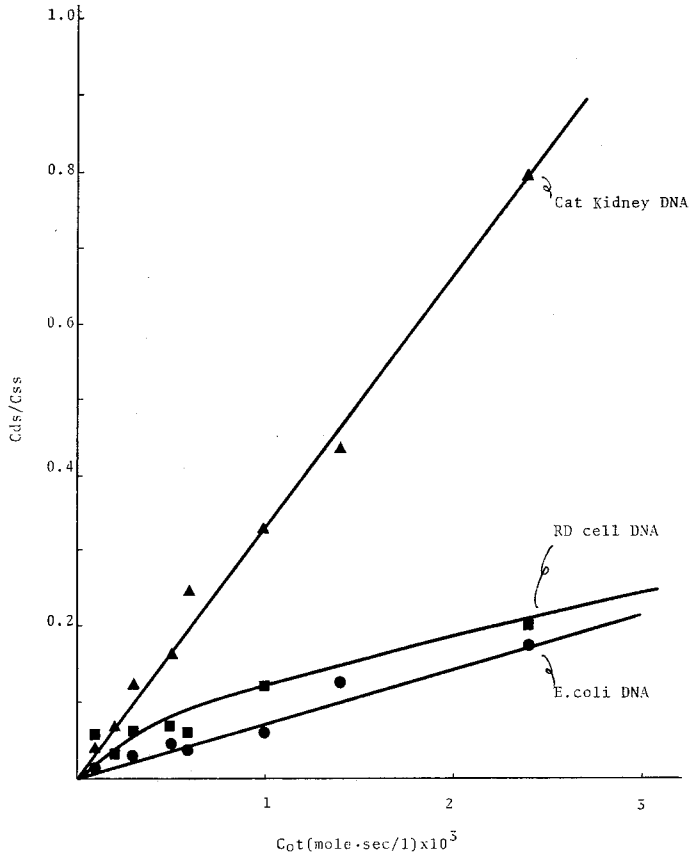

Fig. 1. Reassociation of SR-1 fraction in the presence of cell DNA.
Heat-denatured SR-1 fraction in a concentration of 0.87 ng/ml in 0.4 M PB was incubated with denatured cell DNA. The ratio of dsDNA to ssDNA (Cds/Css) are plotted against Cot of SR-1 fraction. E. coli (181 μg/ml; ●—●), RD (151 μg/ml; ■—■) and Cat kidney (70 μg/ml+E. coli; ▲—▲).

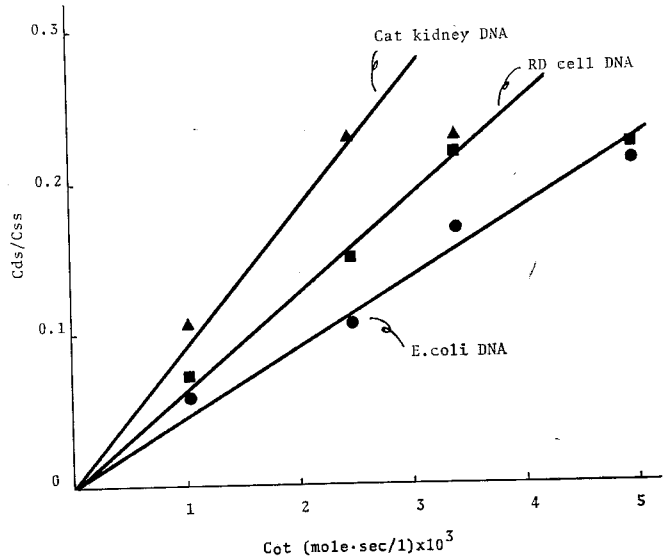

Fig. 2. Reassociation of SR-2 fraction in the presence of cell DNA.
Heat-denatured SR-2 fraction in a concentration of 9.02 ng/ml in 0.4 M PB was incubated with denatured cell DNA at a concentration of 160 μg/ml. The ratio of dsDNA to ssDNA (Cds/Css) are plotted against Cot of SR-2 fraction. E. coli (●—●), RD (■—■), and Cat kidney (▲—▲).

Table 4. *Hybridization of [³H] ssDNA with various human cell DNAs*

Cell DNA		RD-114 c·DNA*1	% of hybrid formed*2
Cat liver	(1.34 mg/ml)	5.62 ng/ml	37.8%
RD cell	(8.00 mg/ml)	5.62 ng/ml	5.1%
P3HR-1	(8.00 mg/ml)	5.62 ng/ml	5.0%
Normal human spleen	(8.00 mg/ml)	5.62 ng/ml	5.3%
S. Sperm	(8.00 mg/ml)	5.62 ng/ml	3.7%
E. coli	(8.00 mg/ml)	5.62 ng/ml	2.5%

*1 RD-114 ssDNA was made by virion DNA polymerase in the presence of Act. D as described in the text. (Specific activity: 1.78×10^7 cpm/μg)
*2 Hybridization reaction was carried out in 0.4 M PB at 67°C for 120 hrs.

over back-ground levels was hybridized with a large excess of DNAs from these cells as shown in Table 4. The melting characteristics of resulting duplex DNA are shown in Fig. 3. The melting curves were broad and Tm was lower than that of duplex DNA formed by the hybridization between DNA from Cat liver and purified [³H] ssDNA. These showed the

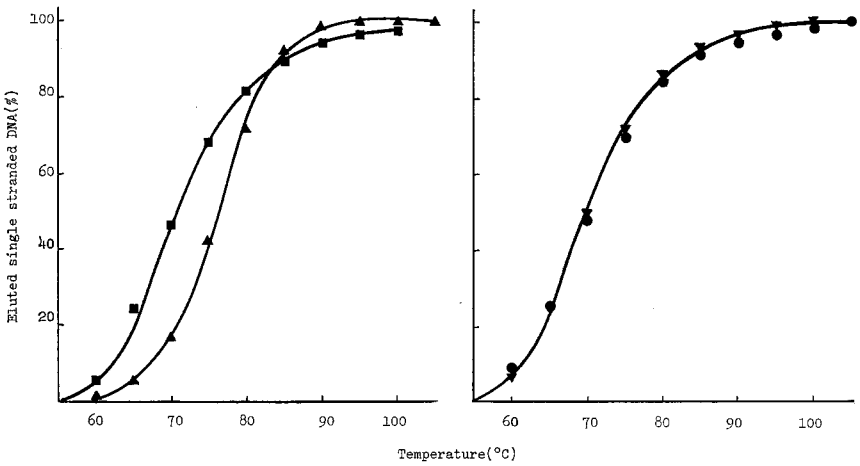

Fig. 3. Melting characteristics of DNA-DNA hybrids formed between purified ssDNA and various cell DNAs.

Thermal stability of the duplex DNA formed in the presence of a large excess cell DNAs were measured by hydroxyapatite column chromatography as described (14). Cat liver (▲—▲), RD (■—■), P3HR1 (▼—▼) and normal human spleen (●—●).

presence of little sequences strictly related to RD-114 viral genome in human cells.

SUMMARY

Double-stranded DNA (dsDNA) product was synthesized by the endogenous DNA polymerase reaction of RD-114 virions disrupted with a detergent. Kinetic analysis of the reassociation of purified dsDNA fraction revealed the heterogeneity of dsDNA population. DNA-DNA reassociation reaction was employed to fractionate the dsDNA population into three fractions, namely the rapidly reassociating fraction (RR), the slowly reassociating fraction 1 (SR-1), and the more slowly reassociating fraction 2 (SR-2). Nucleotide complexities of the RR fraction and the SR-1 fraction were 1.32×10^6 daltons (13.1% of the viral genome) and 3.34×10^6 daltons (33.1% of the viral genome) respectively.

Fractionated dsDNAs, RR, SR-1 and SR-2, were used as probes to investigate RD-114 virus-related DNA sequences in human cell DNA. Human cell DNAs including RD (rhabdomyosarcoma) cell DNA did not significantly accelerate the reassociation reaction of the RR fraction. However, appreciable accelerations by an addition of RD cell DNA were observed for the reassociation reactions of the SR-1 and SR-2, suggesting the presence of RD-114 virus-related sequences in human RD cells. Hybridizations of

DNAs from human cells including RD cells with purified ssDNA representing the majority of the genomic RNA and thermal denaturation experiments of resulting hybrids showed the presence of little sequences strictly related to RD-114 viral genome in human cells.

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