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The Rapidly Reassociating Fraction in Double-stranded DNA Synthesized by Rauscher Murine Leukemia Virus.

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SUMMARY

Double stranded DNA (dsDNA) product of the endogenous DNA polymerase reaction by the virion of Rauscher murine leukemia virus was analyzed by DNA reassociation kinetics. At least two fractions, rapidly reassociating (RR) and slowly reassociating (SR) fractions were present in dsDNA. The RR fraction represented approximately 65% of the total dsDNA and had a sequence complexity of 8×10^5 daltons, equivalent to 10-15% of the total viral genome. DNAs from human cells including Burkitt's lymphoma cells and a case of acute myelogenous leukemic cells accelerated the reassociation reaction of RR fraction to some extent, suggesting the existence of related sequences to RR fraction in these cells. However, the hybridization experiment using single-stranded viral DNA probe, representing the majority of the genomic RNA sequence, showed that only a little, if any, of the single-stranded DNA probe sequences are present in these cells. Possible explanations and the significance of the above results are discussed.

INTRODUCTION

Single-stranded and double-stranded viral DNA are synthesized by virion endogenous DNA polymerase reactions of avian and mammalian C-type RNA tumor viruses (1-6). In the first step, single-stranded DNA is transcribed from genomic RNA by virion RNA dependent DNA polymerase reaction and then, subsequently, double-stranded DNA is synthesized on the DNA template by virion DNA dependent DNA polymerase reaction (7, 8). Actinomycin D selectively inhibits DNA dependent DNA polymerase reaction (5, 9) and the synthesized DNA in the presence of actinomycin D is single-stranded DNA (ssDNA) complementary to most of the sequences of genomic RNA (10). This ssDNA probe was used for the detection of virus-specific sequences in

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human neoplasia such as human leukemic cells, lymphoma cells and sarcoma cells (11-13). On the other hand, double-stranded DNA (dsDNA) synthesized in the absence of actinomycin D is rather heterogenous in its sequence complexity and most of the populations represents only a part of the genomic RNA (6, 14-16). These characteristics of dsDNA are disadvantageous when used as a probe for detecting viral DNA sequences in cell DNA. However, the homogenous population in dsDNA, if isolated and characterized, would be one of the most sensitive probes which can be used for the detection and quantitation of probe DNA sequences in cell DNA by the reassociation kinetic method (16, 17).

In this communication, the isolation and characterization of homogenous double-stranded DNA population synthesized by Rauscher murine leukemia virus (R-MuLV) DNA polymerase reaction are carried out and trials for the detection of virus-related sequences in human cellular DNA are done using two different probes, dsDNA and ssDNA made by MuLV DNA polymerase reaction.

MATERIALS AND METHODS

Virus

Virus preparations of purified R-MuLV (JLS-V9) and RD-114 virus were kindly supplied by Dr. Maurice green, Institute for Molecular Virology St. Louis University School of Medicine.

Preparation of viral [^3H] dsDNA probe

Viral [^{3}H] DNA was synthesized by the endogenous DNA polymerase reaction of disrupted virions as described (16). The DNA product was purified by the SDS-phenol method (16), followed by precipitation with 2 volumes of ethanol in the presence of *E. coli* tRNA (150 μg/ml) and treatment with RNase A (100 μg/ml) in 0.007 M sodium phosphate buffer, pH 6.8 (PB) for 60 min at 37°C. Then, dsDNA was isolated on hydroxyapatite chromatography by elution with 0.4 M PB after the removal of single-stranded DNA with 0.14 M PB containing 0.4% SDS (16). Alkali treatment of dsDNA was performed by 0.2 N sodium hydroxide for 2 hours at 37°C. After neutralization, dsDNA was dialyzed against 0.1 × SSC (SSC : 0.15 M sodium chloride-0.015 M sodium citrate).

Preparation of [^3H] ssDNA

Viral [^{3}H] ssDNA was synthesized by the endogenous DNA polymerase reaction of disrupted virions in the presence of actinomycin D (100 μg/ml) as described (18). [^{3}H] ssDNA was purified by the SDS-phenol method, followed by ethanol precipitation as described above. Alkali treatment of

ssDNA was performed by 0.1 N sodium hydroxide for 30 min at 80°C and after neutralization, ssDNA was dialyzed against 0.1×SSC (18).

Cells and tissues

R 17: A R-MuLV producing cell line (19), was provided by Dr. Akinori Ishimoto and Dr. Yōhei Ito, Aichi Cancer Center Research Institute. P3HR-1 (20), Raji (21), NC-37 (22): Human lymphoblastoid cell lines with the genome of Epstein-Barr virus (23) were provided by Dr. Toyoro Osato, Hokkaido University School of Medicine. Clinical materials were provided by Dr. Ichiro Uruschizaki and Dr. Kōkichi Kikuchi, Sapporo Medical College. Leukocytes were separated from the blood of patient by Dextran-Urografin method (24).

Purification of cellular DNA

Cellular DNAs were purified by the modified procedure (16) of Berns and Thomas (25). Cells were lysed in 20 volumes of buffer A (0.01 M Tris-0.1 M EDTA-0.01 M sodium choride, pH 8.1) containing 0.5% SDS, and were digested overnight at 37°C with 100 μg/ml of pronase (Calbiochem, nuclease free, self-digested for 2 hours at 37°C). The sample was treated thrice with equal volumes of phenol and chloroform-isoamylalchol (24 : 1) mixture. Then, 0.02 volumes of 5 M sodium chloride was added, and DNA was spooled out after precipitation with 2 volumes of 95% ethanol. Spooled DNA was dissolved in buffer A, and was treated with 50 μg/ml of RNase A (Sigma, boiled for 5 min. at pH 5.0) for 2 hours at 37°C. Digestion with 100 μg/ml of pronase was performed in the presence of 0.5% SDS. The sample was treated thrice with equal volumes of phenol and chloroform-isoamylalcohol (24 : 1). Then, 0.02 volumes of 5 M sodium chloride was added and DNA was spooled out after an addition of 2 volumes of 95% ethanol. Spooled DNA was dissolved in 0.1×SSC and treated with 0.2 N sodium hydroxide for 2 hours at 37°C, and was neutralized with 0.1 volumes of 3 N sodium phosphate dibasic. DNA was dialyzed against 0.01×SSC. Cellular DNAs were sonicated to 600-800 nucleotide length by KUBOTA KMS-250 sonicator (KUBOTA Tokyo, JAPAN) at 0°C, for 10 min at 250 W.

Reassociation Reaction of [^3H] dsDNA

[^{3}H] dsDNA with cellular DNA was sonicated and denatured at 100°C for 10 min. Reaction mixtures in 0.4 M PB were incubated at 67°C. Aliquots containing 300 to 1000 cpm in 50 to 300 μl were removed at various incubation periods and were resolved into ssDNA and dsDNA fractions by batch method of hydroxyapatite chromatography (26). Calf thymus DNA (100 μg) was added as a carrier to each fraction. After the addition of tri-

chloroacetic acid to a final concentration of 10%, precipitates were collected on nitrocellulose membrane, and counted by a liquid scintillation counter, Beckman LS-230 (Beckman, Palo Alto, Calif., U.S.A.).

Hybridization reaction of ssDNA with cellular DNA

[^{3}H] ssDNA with cellular DNA was denatured at 100°C for 10 min. Reaction mixtures in 0.01 M PIPES buffer containing 0.72 M NaCl and 1.0 mM of EDTA were incubated at 68°C for 5 days. The hybridized DNA was measured by batch method of hydroxyapatite chromatography as described above.

RESULTS

Characterization of DNA product

[^{3}H] DNA product synthesized by the endogenous DNA polymerase reaction of R-MuLV was purified and the 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

Table 1. *Strandedness of [^{3}H] DNA products by the endogenous DNA polymerase reaction of R-MuLV*

	single-stranded		double-stranded	
	cpm	%	cpm	%
Experiment 1*	1.0×10^5	19.2	4.2×10^5	80.8
Experiment 2**	0.7×10^6	17.9	3.2×10^6	82.1

* Specific activity of viral DNA product used: 1.7×10^6 cpm/μg
** Specific activity of viral DNA product used: 6.0×10^6 cpm/μg

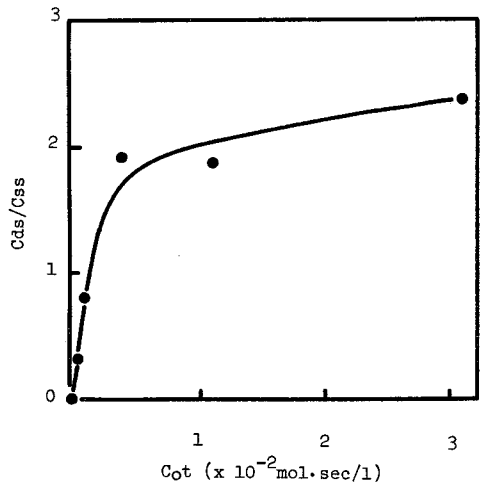

Fig. 1. Reassociation kinetics of MuLV viral [^{3}H] dsDNA.

MuLV viral dsDNA (1.7×10^6 cpm/μg, 1000 cpm/50 μl) was reassociated after sonication and denaturation, and the fraction of reassociated DNA fragments was determined by hydroxyapatite chromatography as described in Materials and Methods. The ratios of dsDNA to ssDNA (Cds/Css) are plotted against the product (Cot) of the initial concentration (Co) and reaction time (t).

was eluted with 0.4 M PB, indicating that 80% of the product had a duplex structure. Reassociation kinetics of [^{3}H] dsDNA was shown in Fig. 1. The presence of at least two fractions, rapidly reassociating (RR) and slowly reassociating (SR) fractions, were clearly shown from the kinetics of the reassociation.

Isolation of RR and SR fractions

For the isolation of RR and SR fractions, heat denatured dsDNA was reassociated. RR fraction was separated as dsDNA by the hydroxyapatite chromatography at the Cot value of 0.4×10^{-3} mol•sec/l. Sequence complexity of RR fraction was estimated from the reassociation rate constant, as approximately 0.8×10^6 daltons (Table 2). Reassociation rate constant of adenovirus type 2 DNA, molecular weight 23×10^6 daltons (27), was used for molecular weight calibration. Reassociation reactions of either RR or SR fractions were not affected by the addition of poly $(A)_n$ in a final concentration of 3.5×10^{-3} μg/ml. Sequence complexity determination of SR fraction was not carried out because of the limitation of the material.

Table 2. *The content and the sequence complexity of RR and SR fraction in [^{3}H] dsDNA product*

Fraction	Content	Sequence complexity*
RR	65%	0.8×10^6 daltons
SR	35%	ND**

* Adenovirus type 2 DNA, molecular weight 23×10^6 daltons, was used as a reference.

** Not Determined.

Reassociation of RR fraction in the presence of various cell DNAs

The effect of various cellular DNAs on the reassociation kinetics of RR fraction was tested. The kinetic curve of RR fraction in the presence of *Escherichia coli* DNA containing no appreciable homologous sequence was shown in Fig. 2 A. Calf thymus DNA did not accelerate the reassociation reaction, while an extreme acceleration effect on the reassociation reaction was observed by the addition of DNA from R-17, MuLV producing cell line, indicating the presence of sequences homologous to RR fraction in cellular DNA of R-17, approximately 300 copies per diploid cell DNA quantity (Table 3). Effects of DNAs from human cell lines on the reassociation reactions were shown in Fig. 2 B. DNAs from P3HR-1 and Raji, cell lines established from Burkitt's lymphoma, and DNA from NC-37, the cell line established from normal peripheral blood, showed acceleration on the reassociation reactions. The addition of leukemic cell DNA from peripheral blood of the

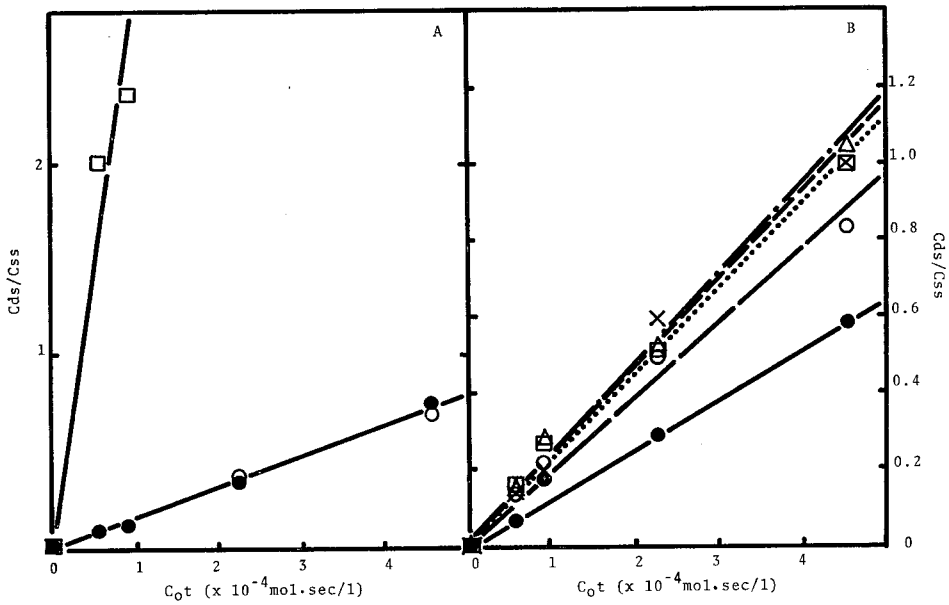

Fig. 2. Reassociation of RR fractien in the presence of various cellular DNAs.

Heat denatured RR fraction in a concentration of 0.9 μg/ml was incubated with denatured cellular DNA at a concentration of 238 μg/ml. The ratios of ssDNA to dsDNA (Cds/Css) are plotted against Cot of RR fraction.
A. *E. coli* (●——●), Calf thymus (○——○), R-17 (□——□), B. *E. coli* (● —●), P3HR-1 (○— —○), NC37(△— — —△), Raji (□·····□), and AML (×—·—×),

Table 3. *Reassociation of RR fraction in the presence of various cellular DNAs*

Cell	Cell DNA (μg/ml)	Viral DNA (ng/ml)	Increased rate factor	Copy numbers per cell*
Experiment 1				
R-17	238	0.9	16.5	290
Calf thymus	238	0.9	1.00	0
E. coli	238	0.9	1.00	0
Experiment 2				
P3HR-1	238	0.9	1.48	9
NC 37	238	0.9	1.76	14
Raji	238	0.9	1.73	14
AML	238	0.9	1.74	14
E. coli	238	0.9	1.00	0

* The copy number of RR sequence in diploid cell DNA quantity was calculated from the increased rate factor, using values of 4×10^{12} daltons for the molecular weight of diploid cell DNA and 0.8×10^{6} daltons for the sequence complexity of MuLV RR fraction.

Table 4. *The absence of RD-114 viral dsDNA segment in human cells**

Cellular DNA (μg/ml)		RD-114 dsDNA (ng/ml)	Increased Rate Factor	Viral DNA copies per cell
E. coli	(190)	0.790	1.00	—
P3HR-1	(190)	0.790	1.05	<1
AML	(190)	0.790	1.05	<1
Cat kidney + *E. coli*	(96.4) (93.6)	0.790	4.03	119

* The number of viral DNA copies in diploid cell DNA quantity was calculated from the increased rate factor, using values of 4×10^{12} daltons for the molecular weight of diploid cell DNA and 0.8×10^{6} daltons for the sequence complexity of rapidly reassociating RD-114 dsDNA segments.

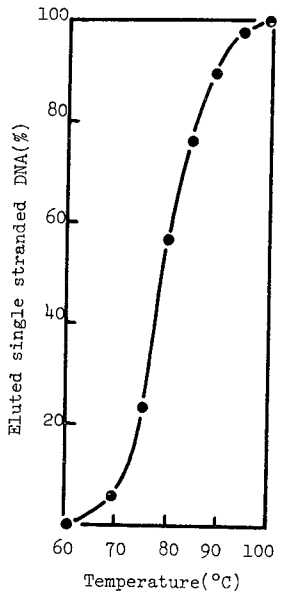

Fig. 3. Melting characteristics of DNA-DNA hybrid formed between RR fraction and AML DNA.

The duplex DNA formed in the presence of a large excess AML DNA was isolated by eluting with 0.4 M PB from hydroxyapatite, and the thermal stability of the duplex DNA was measured by hydroxyapatite column chromatography as described (28).

patient with acute myelogenous leukemia (AML) also accelerated the reassociation reaction. From the degree of acceleration, it can be calculated that around 10 copies of the sequences of RR fraction are present in these cells (Table 3). However, DNAs of P3HR-1 and AML did not accerate the reassociation of [^{3}H] dsDNA of RD-114 virus (Table 4). These evidences suggest the presence of the related sequences to MuLV in DNAs from at least some of the human cell lines and tissues. Melting characteristics of duplex DNA made by the hybridization between a large excess of DNA from AML cells and RR fraction were shown in Fig. 3. The meting curve

was sharp and had Tm at 79°C.

Hybridization of ssDNA with various cell DNAs

[^{3}H] ssDNA probe was prepared by the endogenous DNA polymerase reaction of disrupted MuLV virions in the presence of actinomycin D (100 μg/ml) and hybridized with DNAs from P3HR-1, AML, and normal human spleen to a Cot value of 7000. Only a few per-cent of the input [^{3}H] ssDNA was hybridized with DNAs from these cells as shown Table 5. Similar results were obtained by using [^{3}H] ssDNA probe after purifying by the hybridization with MuLV 60-70S RNA.

Table 5. *Hybridization of MuLV [^{3}H] ssDNA with various cell DNAs*

cell DNA	input (cpm)	[^{3}H] DNA in DNA-DNA (cpm)	hybridized* (%)
P3HR-1	976	67	2.9
AML	1005	67	2.7
human spleen	999	85	4.6
R-17	882	294	29.4

* The background (the mixturte of [^{3}H] ssDNA and *E. coli* DNA at 0 time; 3.9%) was subtracted.

DISCUSSION

Our results showed that at least two populations, rapidly reassociating (RR) and slowly reassociating (SR) fractions were present in the dsDNA product of the endogenous DNA polymerase reaction by R-MuLV virions. The heterogeneous characteristics of dsDNA product by MuLV DNA polymerase reaction was quite similar to that of dsDNA product by Rous sarcoma virus DNA polymerase reaction (15, 17). This possibly reflects the instability of template genomic RNA (29, 30). The RR fraction represented approximately 65% of dsDNA product and its sequence complexity was estimated as 0.8×10^6 daltons by reassociation kinetics. Both RR and SR fractions did not contain poly$(A)_n$ sequence. Sequence complexity of genomic RNA of murine leukemia virus, which corresponds to 35S subuit of 60-70S RNA, has $3\text{-}3.5\times10^6$ daltons : $6\text{-}7\times10^6$ daltons as dsDNA (31). Therefore, the complexity of RR fraction, 0.8×10^6 daltons, corresponds to 10-15% of the viral genome.

DNA which contains the sequence homologous to dsDNA probe accelreates quantitatively the reassociation reaction of the probe and the copy numbers of the sequence in DNA can be estimated from the degree of ac-

celeration (14, 16). Approximately 300 copies of "RR" sequence per diploid cell DNA quantity were detected in cellular DNA of R 17, MuLV producing cell line. DNAs from Burkitt's lymphoma cell lines (P3HR-1 and Raji), from cell of a case of AML, and from NC 37, a lymphoblastoid cell line of normal human blood origin accelerated the reassociation reaction of RR fraction. The duplex DNA formed by the reassociation of RR fraction in the presence of a large excess of AML DNA had a sharp melting profile. On the other hand, no significant acceleration effect of DNAs from P3HR-1 and from AML on the reassociation reaction of RD-114 dsDNA was observed. These suggest the presence of the sequence related to the MuLV RR fraction in the above human cell lines.

However, only a limited amount of ssDNA probe complementary to most of the genomic RNA sequences were hybridized to P3HR-1 and AML DNA. Possibly, the RR fraction may reflect sequences of some particular region of genomic RNA most of which is not present in ssDNA probe. Alternatively, the hybridization of ssDNA with cellular DNA is rather insensitive, and it would be very difficult to detect a small region of the viral sequences in cell DNA by this method. Also, a careful investigation should be performed to rule out completely the effect of some murine cellular sequences other than that of viral genomic RNA possibly present in RR fraction before concluding the presence of viral genomic sequences in these cells. As shown above, the reassociation kinetic method using RR fraction is highly sensitive for the detection and the quantitation of the probe DNA sequence in tumors or transformed cells, and would be of great use and importance as one of the viral genome detection methods for primary survey experiments if further detailed analysis is followed.

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