SIMULTANEOUS PURIFICATION OF RNA-DEPENDENT DNA POLYMERASE AND GS-ANTIGEN FROM RAUSCHER LEUKEMIA VIRUS

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SUMMARY: RNA-dependent DNA polymerase and gs-antigen were purified simultaneously from Rauscher leukemia virus by sequential column chromatography on phosphocellulose. The partially purified RNA-dependent DNA polymerase has a molecular weight of 70,000 and is free of cellular DNA polymerase, deoxynucleotidyl terminal transferase, RNase and DNase. The partially purified RNA-dependent DNA polymerase can efficiently copy oligo dT·poly rA and oligo dG·poly rC. The purified gs-antigen shows a single band on SDS-polyacrylamide gel with a molecular weight of 30,000. It is active immunologically and possesses both group and interspecies activity.

Some mammalian oncogenic C-type RNA viruses are vertically transmitted and although the host does not always actively produce virus, the presence of the viral genome can be detected by the synthesis of viral-specific proteins during various stages of development of the host (1, 2). Judicious use of antisera prepared against these proteins, having both species and interspecies specificity, may be used to detect the expression of viral antigens in human tumors.

Three viral antigens have been studied to date: the major internal structural protein (gs-antigen) which possesses both inter- and intraspecies activity, a second polypeptide (possibly from the membrane) which has only interspecies activity, and the viral reverse transcriptase which also has interspecies specificity (3, 4).

Several procedures have been described for purification of these antigens (3-13) but, the methods were designed to obtain either structural or functional proteins, but not both, and few are suitable for large-scale production. The procedure described herein permits the isolation and purification of a

large number of both structural and functional proteins from RLV[1]. Pilot studies show it can be applied to many other C-type oncornaviruses.

MATERIALS AND METHODS: Materials were obtained as follows: radioactive compounds were purchased from New England Nuclear Corporation; unlabelled dNTP, DTT and calf thymus DNA from Calbiochem; phosphocellulose (P-11) from Reeve-Angel; calf thymus DNase and pancreatic RNase from Worthington Biochemical Corp. and synthetic polynucleotides from Collaborative Research. Double isopynically-banded RLV (1000-X concentrate grown in JLS-V9 cells chronically infected with RLV) and rabbit antiserum prepared against purified FeLV were received through the Office of Program Resources and Logistics of the Virus Cancer Program, NCI. RNase H and RDDP were assayed according to the procedure of Bowling et al. (14). Rabbit antisera prepared against purified SSV-1 p30 and partially purified SSV-1 RDDP were gifts from Dr. Wade Parks; rat antiserum prepared against partially purified AMV-RDDP was the gift of Dr. S. Spiegelman; and goat antiserum prepared against pure RLV p30, was a gift from Dr. J. T. August. The complement fixation test for p30 was performed as described by Schmidt (15). Isoelectric focusing was as described by Oroszlan et al. (9).

Simultaneous Purification of RDDP and p30. Fifty ml of RLV were pelleted by centrifugation at 100,000 x g for 90 min. The pellet was resuspended in 5.0 ml of 0.01 M Tris·HCl (pH 7.2), 0.3 M KCl, 0.02 M DTT and 1% Triton X-100. After a 20 min incubation at 37°C the mixture was diluted five-fold with 0.01 M Tris·HCl (pH 7.2), containing 1% Triton X-100 and incubated 15 min at 37°C. The mixture was centrifuged at 27,000 x g for 10 min and the supernatant fluid (S_2) stored at 4°C. The pellet was resuspended in 5.0 ml of 0.01 M Tris·HCl (pH 7.2), 0.02 M DTT and 2% Triton X-100 and incubated at 37°C for 10 min. This mixture was then centrifuged at 27,000 x g for 10 min and the supernatant liquid (S_3) pooled with the S_2 fraction for further purification. The pellet was suspended in 1.0 ml, 0.01 M Tris·HCl (pH 7.2), 0.1 M NaCl, and 0.01 M EDTA for analysis.

The virus lysate (S_2 + S_3) was applied to a phosphocellulose column at a ratio of 1.5 ml phosphocellulose per mg viral protein and eluted with a 10 column-volume linear salt and pH gradient (Fig. 1). When assayed for DNA polymerase activity using oligo dT·poly rA, two peaks of activity were observed, one which eluted at 0.3 M KCl and the second at 0.4 M KCl. When assayed with oligo dG·poly rC, activity was only observed with the peak eluting at 0.3 M KCl. This peak was pooled and used for further studies. Elution of the column with up to 2 M KCl did not yield any additional DNA polymerase activities.

Fractions were assayed for the presence of p30 by both immunodiffusion and SDS gel electrophoresis. Those fractions which showed a strongly stained protein band at 30,000 daltons also showed immunodiffusion activity. These were pooled and dialyzed overnight against two 500 ml changes of 0.01 M BES (pH 6.5) containing 0.001 M EDTA (Buffer B). The dialysate was placed onto a phosphocellulose column (1.0 ml phosphocellulose/mg viral protein) pre-equilibrated with Buffer B and washed with one column-volume of Buffer B. The absorbed protein was eluted with a 200 ml linear gradient of 0.0 to 0.5 M KCl

[1]Abbreviations: RLV for Rauscher leukemia virus, AMV for avian myeloblastosis virus, SSV-1 for simian sarcoma virus, FeLV for feline leukemia virus, RDDP for RNA-dependent DNA polymerase, RNase H for ribonuclease H, p30 for group specific antigen, DTT for dithiothreitol, BSA for bovine serum albumin, SDS for sodium dodecyl sulfate, BES for N,N-bis (2-hydroxyethyl)-2-aminoethane sulfonic acid.

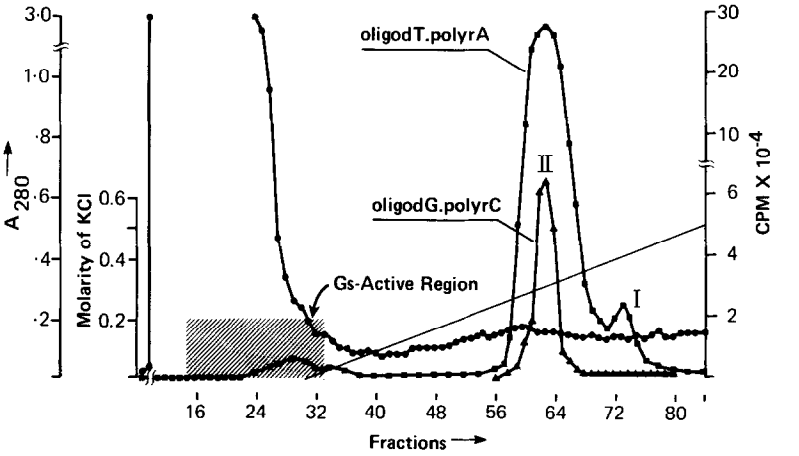

Fig. 1. Phosphocellulose column chromatography of RLV-lysate. The virus lysate was adsorbed on a p11 column (10 x 1.5 cm) pre-equilibrated with 0.01 M Tris·HCl (pH 7.2), 0.002 M DTT and 15% glycerol (Buffer A). The column was washed with two column-volumes of Buffer A and then eluted with a 200 ml linear gradient of Buffer A and 0.01 M Tris·HCl (pH 8.2), containing 0.6 M KCl, 0.002 M DTT and 15% glycerol. Two ml fractions were collected. Aliquots of 25 µl were assayed for polymerase activity (13, 17): (●-●) indicates A_{280}; (■-■) [^{3}H]TMP incorporation with oligo dT·poly rA and (▲-▲) [^{3}H]d-GMP incorporation with oligo dG·poly rC. (II) is the viral RDDP, (I) the cellular DNA polymerase and the shaded area is the p30 region detected by immunodiffusion and SDS gels.

contained in Buffer B. The p30 was detected by immunodiffusion and SDS gel electrophoresis and the positive fractions pooled as the final product.

RESULTS: Purification Procedure. A summary of the purification data for a typical 50 ml virus run is shown in Table 1. Ninety-three percent of the viral protein was solubilized and all of the protein placed on the first phosphocellulose column could be recovered in the various fractions. Recovery of protein from the second phosphocellulose column was close to 100%. An average of 2.3% of the starting viral protein was recovered in the purified RDDP and 11.2% in the purified p30. An average of 2.4% of the RDDP activity was found in the initial supernatant and 1.3% remained associated with the final pellet. The remainder of the activity was placed onto the column and 70% of the starting activity was isolated in the viral enzyme peak yielding an average of 33-fold purified RDDP.

TABLE 1. SIMULTANEOUS PURIFICATION OF RDDP AND p30

RDDP

Step		Total Volume (ml)	Total Activity Units[a]	Total Protein (mg)	Specific Activity (Units/mg)	Fold Purification[b]
I	Virus	50.0	355.1	20.64	17.20	-
II	S_1	48.5	3.66	1.70	2.16	-
III	Final Pellet	1.0	27.8	1.99	13.9	-
IV	Virus Lysate (S_2+S_3)	50.0	321.8	18.80	17.10	-
V	Phospho-cellulose Column (Tris)	17.5	200.1	0.32	625.2	36.3

p30

		Total Volume (ml)	Total Protein (mg)	Gel Immunodiffusion	Electrophoresis
V	Phosphocellu-lose column (Tris)	55	2.75	+	Multiple Bands
VI	Phosphocellu-lose column (BES)	10	1.00	+	Single Band

[a]One unit of activity is the amount of enzyme that incorporates 1 nmole of [^{3}H]TMP into oligo dT-poly rA per 30 min incubation. The assay is linear in the range of 10-80 μg in Step 1 and 250-1000 ng in Step V.

[b]Sp activity of any step ÷ sp activity of Step 1.

Properties of Phosphocellulose Purified RDDP. RNase, DNase, RNase H and RNA polymerase activities were determined at each step during purification and the purified enzyme was devoid of RNase, DNase or RNA polymerase activity. However, it does contain RNase H, when tested with [^{3}H]RNA-calf thymus DNA. Sedimentation analysis through glycerol gradients revealed that the RDDP activity migrated as a single peak (using oligo dT·poly rA as primer-template),

TABLE II. TEMPLATE SPECIFICITIES OF RLV-RDDP

Template	[^{3}H]d-NMP Incorporated	[^{3}H]NMP incorp. (pmoles/μg/30 min)
oligo dT	TMP	0.70
poly rA	TMP	0.02
oligo $(dT)_{12}$poly rA	TMP	871.0
oligo $(dG)_{12}$poly rC	d-GMP	292.0
oligo $(dT)_{12}$poly dA	TMP	0.5
oligo $(dC)_{12}$poly rI	d-CMP	41.0
70S RNA	TMP	0.3
70S RNA + oligo dT	TMP	3.2

The assays (0.1 ml) were performed under optimum conditions using 250 ng of phosphocellulose purified RDDP and 2 μg of primer-template.

with an apparent molecular weight of 70,000. Chromatography on a Sephadex G-100 column standardized with molecular weight markers (BSA, ovalbumin, pancreatic DNase and pancreatic RNase) yielded a single peak of activity with the same elution volume as BSA.

Various synthetic primer-templates were tested for activity with RDDP and the results are shown in Table II. No activity was observed with either single-stranded poly rA or oligo dT indicating the absence of deoxyribonucleotidyl terminal transferase activity. Good activity was observed with oligo dG·poly rC as primer-template, which had been shown previously to be a viral RDDP-specific template (16). The enzyme was completely neutralized by rabbit antibody. It was partially neutralized (20-25%) by rabbit antibody prepared against partially purified SSV-1 RDDP. The RLV-RDDP was not inhibited by the rat antisera prepared against partially purified AMV-RDDP.

Properties of the Purified p30. SDS polyacrylamide gel electrophoresis was used extensively to detect the p30 (Fig. 2). A minimum value of 16 ng

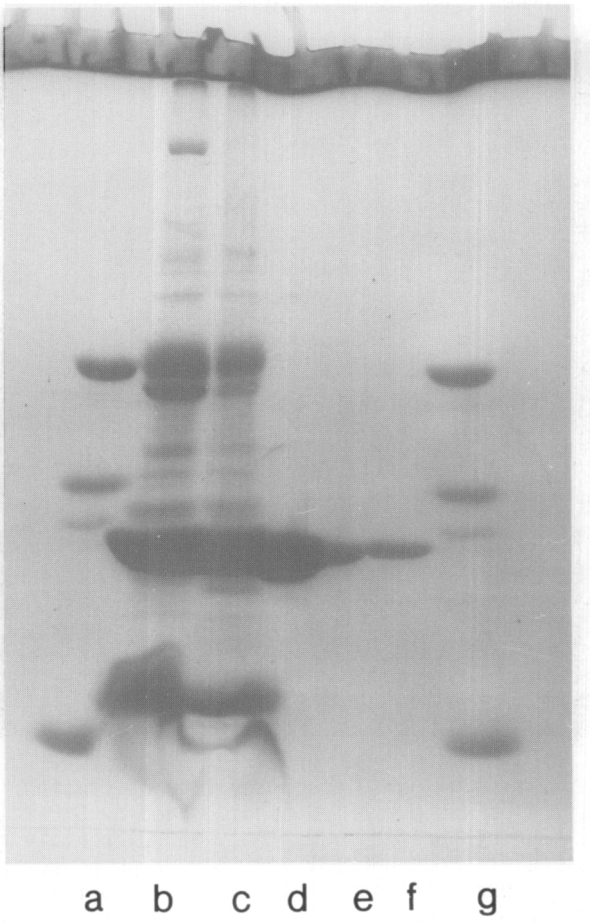

Fig. 2. Polyacrylamide gel electrophoresis. SDS-treated proteins (10-50 μg) were run as previously described (15). (a) and (g) are standards containing 10 μg of each molecular weight marker, BSA (MW ∿ 69,000), ovalbumin (MW ∿ 44,000) pancreatic DNase (MW ∿ 30,000) and sperm whale myoglobin (MW ∿ 17,800); (b) indicates 50 μg of virus; (c) 50 μg virus lysate; (d) 30 μg of protein obtained from first phosphocellulose column; (e) 20 μg of protein after BES dialysis and (f) 16 μg of purified p30.

protein could be visualized on the gel and when 16 μg of the purified p30 was placed upon the gel, only a single protein band corresponding to a molecular weight of 30,000 was observed. This indicates that the material is greater than 99.9% homogenous.

The purified p30 contains both group and interspecies activity as shown by immunodiffusion (Fig. 3). An average complement fixation titer of 1:128

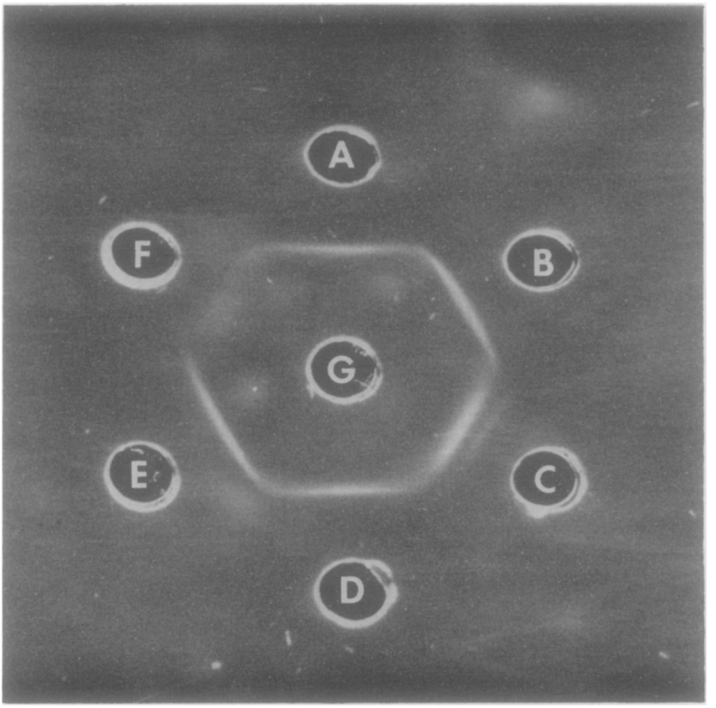

Fig. 3. Characterization of purified p30 by immunodiffusion. Immunodiffusion was performed as described by Strand and August (6). (G) 3 µg of purified p30. (A) and (D) contain antibody prepared against FeLV p30 (B) antibody prepared against RLV; (C) antibody prepared against RLV p30; (E) antibody prepared against Moloney leukemia virus and (F) antibody prepared against SSV-1 p30.

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was obtained with the purified p30 and four units of homologous antibody. Ninety percent of the p30 protein can be iodinated with ^{125}I. Radioimmune assay can detect as little as 0.5 ng of p30 and shows that about 10% of the RLV protein is p30. The p30 has an isoelectric point of 6.5. It is an excellent immunogen in both rabbits and goats (unpublished data).

DISCUSSION: The purification procedure described has been successfully applied to the purification of p30 and RDDP from RLV in amounts ranging from 5 ml to 200 ml virus (10^{12}- 10^{13} particles/ml) per batch.

The starting RLV preparation appears to contain cellular polymerase which elutes from the column at 0.4 M KCl (18). This enzyme appears as a shoulder on the peak of viral enzyme. Since it is active with oligo dT·poly rA

but not oligo dG·poly rC, assaying with both allows one to separate these two activities and pool the viral enzyme to prevent any contamination by the cellular enzyme. The major peaks of RNase, DNase and deoxyribonucleotidyl terminal transferase activity elute from the column prior to the RDDP active fraction. Therefore, contamination of the viral RDDP with these enzymes does not occur.

The p30 sometimes elutes from the first phosphocellulose column in the wash and sometimes at 0.2 M KCl and, in two instances, activity was detected at both regions. If the p30 eluted from the first column in the buffer wash, it eluted from the second column at 0.24 M KCl. If the p30 eluted from the first column at 0.2 M KCl, it eluted from the second column at 0.4 M KCl. However, both peaks showed only a single protein band having an apparent molecular weight of 30,000 on SDS gels and identical group and interspecies specific antigen titers per milligram protein. We are unable to explain this observation at the present time.

The elution pattern of the viral protein from the phosphocellulose column appears to be a function of the age of the infected JLS-V10 cells (17). Consistent profiles were observed using early harvested virus (19 - 3rd to 6th day of culture) whereas, late harvested virus (7th to 10th day of culture) required higher salt concentrations for elution and the effluents contained an 18,000 molecular weight contaminant.

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