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Retrovirus Purification: Method That Conserves Envelope Glycoprotein and Maximizes Infectivity

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A Sepharose 4B chromatographic method for purification of retroviruses is described which was less time consuming, increased purified virus yields, conserved viral glycoprotein, and increased recovery of biological infectivity in comparison with conventional sucrose gradient ultracentrifugation techniques.

Several procedures for purification and concentration of retroviruses have been described, including ammonium sulfate precipitation (3, 8), membrane (Amicon) ultrafiltration (7), and high-speed pelleting (6, 13). In all of these procedures, separation of virus particles from cellular contaminants by sucrose density gradient centrifugation is utilized subsequent to the initial concentration. Infectious retrovirus activity, however, is sensitive to both ultracentrifugation and the osmotic shock of suspension in sucrose (6).

We describe here a Sepharose chromatographic technique for purification of murine retroviruses which involves neither ultracentrifugation nor changes in osmotic strength of the suspending fluid. The technique is less time consuming than conventional methods and more conservative of virus biological activity. The viruses studied were a Moloney sarcoma virus-Moloney leukemia virus [MSV(MLV)] complex, grown in rat W/Fu fibroblast line 78A1 (kindly supplied by J. Ferrer and B. Guillemain, University of Pennsylvania Veterinary School, New Bolton Center, Pa.) and the cloned ecotropic AKR virus AKR-L#1, grown in SC-1 cells (kindly supplied by J. W. Hartley and W. P. Rowe). Cells were seeded at 1.5×10^6/75-cm^2 flask and maintained in minimal essential medium with Earle Salts containing 10% fetal calf serum (FCS; GIBCO) and antibiotics. Viruses were radiolabeled with leucine 3 to 4 days after cell passage, using 6 ml of leucine-free minimal essential medium with 10% FCS containing either [^{3}H]leucine (50 μCi/ml) or [^{14}C]leucine (5 μCi/ml). None of the viral polypeptides are leucine deficient, and all are labeled by this procedure (11). Viruses were harvested, centrifuged for 15 min at 10,000 $\times$ g to remove cellular debris, and purified according to the three following procedures.

Method A: pellet/banded virus. Clarified supernatant was pelleted through 25% sucrose for 90 min at 100,000 $\times$ g, the supernatant was decanted, TEN buffer (20 mM Tris-1 mM EDTA-0.1 M NaCl, pH 7.5) was added, and the pellet was resuspended overnight on ice. The resuspended pellet was then spun through a discontinuous 25 to 45% (wt/wt) sucrose gradient in TEN buffer for 2.5 h at 90,000 $\times$ g, and the virus band was collected (6, 11, 13).

Method B: banding. Clarified virus supernatants were concentrated 10- to 50-fold in an Amicon ultrafiltration apparatus with a PM10 membrane at an ultrafiltration rate of 1 ml/min (7). This concentrate was then spun through a discontinuous sucrose gradient, and the virus band was harvested as in Method A.

Method C: Sepharose Cl 4B chromatography. A volume of supernatant concentrated by ultrafiltration (see Method B) which corresponds to 5% of a presterilized Sepharose Cl 4B (Pharmacia) column bed volume was chromatographed at 4°C in TEN buffer; virus appeared in the void volume at a flow rate of 0.5 to 1 ml/min. The fractions containing the virus peak were monitored by both optical density at 260 nm and radioactivity.

Electrophoresis on sodium phosphate-buffered neutral sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE) was performed as previously described (4, 13). Leukemia virus titrations were performed using the standard XC plaque procedure on SC-1 cells (5, 9). Immunoprecipitation of [^{3}H]leucine- and [^{14}C]leucine-labeled virus preparations was performed using a rat anti-MSV polyspecific antiserum followed by SDS-PAGE (12).

Both viruses studied, MSV(MLV) and AKR, were excluded from the void volume of a Sepharose 4B (exclusion size: 10×10^6 daltons) column. PAGE analysis of virus purified by the

three techniques indicated that both Sepharose C1 4B-purified MSV(MLV) and banded MSV(MLV) produced similar gel profiles. Figure 1 shows, however, that pelleted/banded MSV(MLV) had lower levels of gp69/71 and gp45 than Sepharose-purified MSV(MLV). A higher level of material which does not enter the gel was seen with Sepharose-purified virus in contrast to the other methods; that this material was virion associated was established by immunoprecipitation of disrupted virions with polyspecific syngeneic anti-MSV antiserum, followed by SDS-PAGE analysis (12). The polypeptide pattern of the immunoprecipitate was virtually identical to the Sepharose-excluded virus, demonstrating that all Sepharose 4B-excluded material was recognized as virion antigens and was therefore unlikely to contain cellular contaminants.

To determine whether any unlabeled FCS components copurify with virus in any of the purification techniques, three assays were used; (i) SDS-PAGE followed by Coomassie brilliant blue staining, (ii) optical density absorbance at 260 nm (A_{260}) compared with 280 nm (A_{280}), and (iii) ability of each technique to remove ^{125}I-labeled FCS from a viral preparation. Results of all three show that even at 450 gram-percent FCS, no FCS contamination was noted by SDS-PAGE in pellet/banded or Sepharose-purified preparations. These preparations had consistent A_{260}/A_{280} ratios between 1.23 and 1.24. In contrast, ultrafiltration-concentrated MSV(MLV) banded through sucrose was heavily contaminated by FCS proteins (Fig. 2). These observations were confirmed when ^{125}I-labeled FCS was added as a contaminant to MSV(MLV) prior to purification by Sepharose or pellet/banding. In

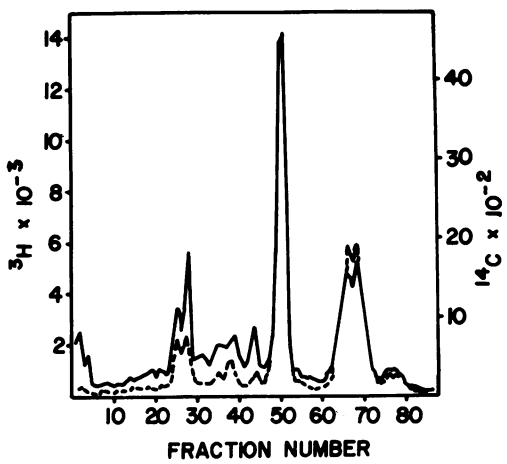

FIG. 1. *SDS-PAGE (7%) illustrating polypeptide patterns of [^{3}H]leucine-labeled Sepharose-purified MSV(MLV)* (——) *cochromatographed with [^{14}C]leucine-labeled pellet/banded MSV(MLV)* (- - -). *Two milliliters of a 20-fold-concentrated [^{3}H]leucine-labeled MSV(MLV) supernatant was chromatographed through a Sepharose C1 4B column (1.6 × 24 cm) in TEN buffer. The void volume of 3 ml was collected and dialyzed against 10^{-2} M phosphate buffer, pH 7.2. A total of 0.02 A_{260} unit of [^{3}H]leucine-labeled Sepharose 4B-purified MSV(MLV) electrophoresed with 0.02 A_{260} unit of [^{14}C]leucine-labeled pellet/banded MSV(MLV). This experiment was repeated five times for MSV(MLV) and four times for AKR virus with similar results.*

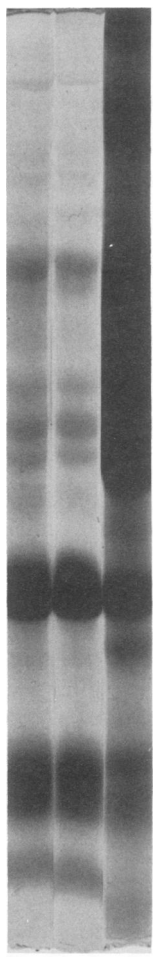

FIG. 2. *PAGE (7%) stained with Coomassie brilliant blue (13). (A) One A_{260} unit of Sepharose-purified MSV(MLV) prepared as above. A_{260}/A_{280} ratio = 1.24. (B) One A_{260} unit of pellet/banded MSV(MLV) prepared as above. A_{260}/A_{280} ratio = 1.24. (C) One A_{260} unit banded MSV(MLV), dialyzed against water, lyophilized, and resuspended for SDS-PAGE. A_{260}/A_{280} ratio = 1.10. (The 260/280 ratio is a comparison between the nucleic acid measurement at 260 nm and protein absorbance at 280 nm.)*

each case, less than 0.05% of input FCS remained after purification and did not represent any specific protein species which co-electrophoresed with MSV(MLV), as analyzed by SDS-PAGE.

The amount of infectious leukemia virus recovered with each virus purification technique was determined using the XC plaque assay. Viral supernatants were initially concentrated 20- to 50-fold by an AMICON ultrafiltration-stirred cell apparatus or through hollow fiber ultrafiltration using PM-10 filters. This procedure has been 80 to 100% efficient in concentrating viral biological activity in our hands. Yields from subsequent purification steps were compared with the initial concentrate in XC plaque-forming activity (Table 1). The Sepharose purification technique proved optimal for recovery of infectious viral particles; most striking is the recovery of 100% more MSV(MLV) and AKR virus using Sepharose purification over the widely used pellet/banding technique. AKR virus appears to be most sensitive to inactivation by the pellet/banding technique. Further analysis of preparations shown in Table 1 demonstrated a correlation between XC plaque-forming activity, recovery of p30, and A_{260} units (Table 2); i.e., recovery of virus is greatest in Sepharose preparations, and is decreased by more than 50% in pellet/banded preparations. The level of viral gp69/71 in relation to p30 was measured by SDS-PAGE; banding resulted in a slight decrease of gp69/71, and the gp69/71 to p30 ratio in pellet/banded preparations was decreased by 50% as compared with Sepharose purification. Therefore, it appears that the pellet/banding technique gives less than 50% recovery of viral particles, and these virions have only 50% of the gp69/71 of viral preparations purified by Sepharose chromatography.

Sucrose banding of viral concentrates also preserved biological activity to a high degree (Table 1), although at the cost of significant contamination by FCS proteins (Fig. 2). This contamination of banded virions by serum proteins is reflected in a lowered A_{260}/A_{280} ratio in this preparation compared with the other methods of enrichment (Table 2). Therefore, the recovery of virion proteins was assayed by determining

TABLE 1. *Virus titer in XC plaque assay of purified virus preparations*[a]

Virus	Total no. of PFU in expt: 1	2	3	Avg % of input XC PFU[b]
78A1 MSV-(MLV)				
Supernatant[c]	4.5×10^6	5.8×10^{3}[d]	2.8×10^6	100
Concentrate[e]	4.5×10^6	6.0×10^3	2.4×10^6	96 ± 7[f]
Sepharose[g]	4.0×10^6	5.9×10^3	1.8×10^6	85 ± 15
Banded[h]	3.3×10^6	2.7×10^3	1.7×10^6	60 ± 11
Pellet/banded[i]	2.2×10^6	2.5×10^3	1.0×10^6	43 ± 5
AKR				
Supernatant[c]	1.1×10^5	5.0×10^4	1.3×10^6	100
Concentrate[e]	9.6×10^4	5.5×10^4	1.3×10^6	99 ± 9
Sepharose[g]	7.0×10^4	5.2×10^4	9.5×10^5	80 ± 17
Banded[h]	4.0×10^4	ND[j]	1.3×10^6	68 ± 32
Pellet/banded[i]	8.0×10^3	0	8.3×10^3	3 ± 3

[a] XC plaque titrations were done on purified virus prepared from equal portions of viral supernatant in three different experiments. The XC titer is representative of the total XC PFU in each concentrate. All samples were prepared on the day of titration.

[b] Average percent recovery of input XC units was calculated by defining the viral supernatant to have 100% biological activity.

[c] Supernatant XC PFU are those in 40 ml of unconcentrated 1-day-old MSV(MLV) or AKR virus supernatant.

[d] Samples frozen and thawed 2× before titration.

[e] Concentrate XC PFU are those in 2 ml of a 20-fold concentrate of 1-day-old MSV(MLV) or AKR virus supernatant.

[f] Standard deviation.

[g] Sepharose XC PFU were derived from a 2-ml concentrate of MSV(MLV) or AKR virus supernatant that had been chromatographed through a Sepharose 4B column (1.6 × 25 cm) washed in TEN buffer, and the void volume viral peak of 2.5 ml was pooled.

[h] Sucrose-banded XC PFU represent biological activity recovered after banding 2 ml of concentrate through 5 ml of 25% sucrose onto a 5-ml 45% sucrose cushion. The viral peak of 2 ml was collected by tapping the gradient from the bottom.

[i] Pellet/banded virus was prepared from 40 ml of viral supernatant (the equivalent of 2 ml of concentrate) as outlined in Materials and Methods.

[j] ND, Not done.

TABLE 2. *Recovery and polypeptide analysis of purified virus preparations*[a]

Virus	[^{3}H]leucine-labeled p30 recovery (^{3}H cpm × 10^{-4}) in expt.			Relative p30 recovery[b]	[^{3}H]leucine-labeled gp69/71 recovery (^{3}H cpm × 10^{-4}) in expt:			Avg gp69/71 to p30 ratio (cpm)[c]	Total A_{260} units	Avg A_{260}/A_{280} ratio
	1	2	3		1	2	3			
MSV(MLV)										
Sepharose	1.74	8.5	15.7	100	0.55	2.6	5.1	0.32 ± 0.01	6.4	1.23 ± 0.01
Banded	1.47	4.6	16.7	81 ± 21	0.41	1.5	4.7	0.29 ± 0.01	5.1	1.12 ± 0.08
Pellet/banded	0.85	1.8	6.8	38 ± 12	0.16	0.34	1.2	0.19 ± 0.01	2.9	1.23
AKR										
Sepharose	0.99	13.2	8.5	100	0.29	3.3	3.0	0.30 ± 0.04	1.3	1.24 ± 0.01
Banded	0.65	10.0	11.8	92 ± 30	0.27	2.6	3.5	0.27 ± 0.02	1.1	1.07 ± 0.10
Pellet/banded	0.27	2.1	0.78	17 ± 7	0.048	0.23	0.19	0.13 ± 0.02	0.35	1.23 ± 0.01

[a] Data was obtained from three separate experiments measuring [^{3}H]leucine-labeled protein recovery as quantitated from SDS-PAGE 7% gels (as in Fig. 1 to 3). Equal portions of labeled virus were purified by each method, and total recovery of each polypeptide was calculated by subtracting background from the sum of peak fractions corresponding to p30 and gp69/71. These experiments correspond to those shown in Table 1.

[b] Mean p30 recovery relative to Sepharose purification of AKR virus and MSV(MLV) ± standard deviation.

[c] Average ratio of [^{3}H]leucine counts per minute recovered in gp69/71 compared with p30 ± standard deviation in the three experiments.

the amount of [^{3}H]leucine-labeled moieties in preparations enriched by these various techniques (Table 2).

Sepharose-purified [^{3}H]leucine-labeled MSV(MLV) was ultracentrifuged through a discontinuous 25 to 45% sucrose gradient to determine whether virus glycoprotein would be removed by this centrifugation step alone. The [^{3}H]leucine-labeled viral polypeptides remaining at the buffer–25% sucrose interface at the top of the gradient were co-electrophoresed with [^{14}C]leucine-labeled pellet/banded virus. Figure 3 shows that, upon centrifugation, whole virions are disrupted at the sucrose interface with an enhanced loss of viral gp69/71. Comparison of p30 levels before and after centrifugation showed that the disrupted virions represented 40% of the initial Sepharose-purified MSV(MLV) p30 and that the resultant banded virions had a 50% decreased level of gp69/71 similar to the pellet/banded virions analyzed in Table 2.

To determine whether the decrease in gp69/71 relative to p30 has any biological significance, AKR virus infectivity was measured by the XC assay with and without DEAE-dextran. The decrease in the absolute infectivity in the absence of DEAE-dextran (Table 3) was most striking in the pellet/banded preparation, which, in this assay, had only 50% of the gp69/71 level of the Sepharose preparation.

These data indicate that Sepharose 4B purification of MSV(MLV) and AKR virions allows for greater recovery of infectious retrovirus particles, is less time consuming, alleviates the need for ultracentrifugation, does not require dialysis of a viral sample to remove sucrose (in our hands, dialysis decreased biological activity 1,000-fold), conserves viral glycoproteins, and allows the removal of very high concentrations of serum from a viral sample in one step. Although gel filtration has been used in purification procedures for other virus systems (14), it has not been reported as the principal method of separation in the retrovirus system. The procedure described allows concentration of virus

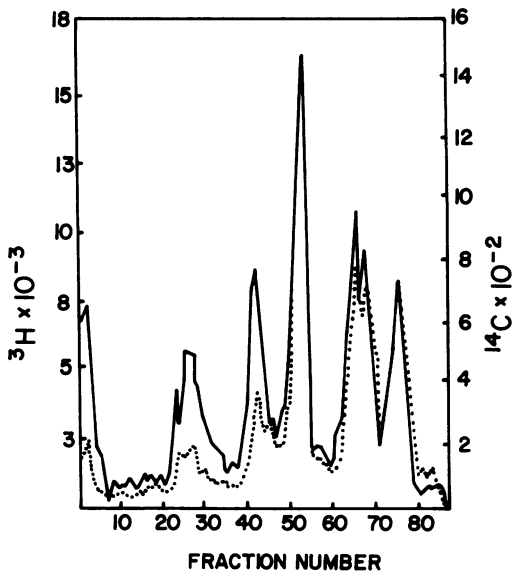

FIG. 3. *SDS-PAGE (7%) illustrating polypeptide patterns of [^{3}H]leucine-labeled virion polypeptides which remained at the top of a 25 to 45% discontinuous sucrose gradient after ultracentrifugation of [^{3}H]leucine-labeled Sepharose-purified MSV(MLV)* (——) *co-electrophoresed with [^{14}C]leucine-labeled pellet/banded MSV(MLV)* (- - -). *The [^{3}H]leucine-labeled polypeptides represent 40% of the input Sepharose-purified MSV(MLV) p30 level.*

TABLE 3. *Biological activity of purified virus with and without DEAE-dextran*[a]

Virus	XC PFU/ml without DEAE-dextran[b] (a)	XC PFU/ml with DEAE-dextran[c] (b)	b/a[d]	gp70/p30[e]
Stock AKR supernatant	6.8×10^3	1.5×10^4	2.1	
Sepharose-purified AKR	3.0×10^4	1.25×10^5	4.2	0.29
Pellet/banded AKR	5.0×10^2	1.0×10^4	20	0.14

[a] Two equal volumes of [^{3}H]leucine-labeled AKR virus supernatant were purified by pellet/banding or Sepharose chromatography. Equal portions of each purified virus preparation were assayed for biological activity to comparison with a standard AKR virus stock.

[b] Standard XC plaque assay without pretreatment of mouse embryo fibroblast monolayer with DEAE-dextran.

[c] Same as footnote *a* above except with DEAE-dextran pretreatment (9).

[d] Ratio of b to a; relative XC plaque-forming enhancement due to DEAE-dextran pretreatment.

[e] gp70:p30 ratio as determined by SDS-PAGE analysis as in Table 2.

and change to a desired buffer in one step while conserving maximal virus biological activity and integrity of the envelope glycoprotein.

The conventional pellet/banding and banding techniques yield a virus population with reduced infectivity and gp69/71 levels compared with Sepharose purification (Tables 1 and 2). Conservation of the envelope glycoprotein is desirable, as this protein binds to the surface of lymphoid targets (1) and susceptible but not resistant fibroblasts (2). The effects due to conservation of gp69/71 on Sepharose-purified virus was seen using the XC plaque assay without DEAE-dextran prior to inoculation (10). No apparent difference between the relative infectivities of the Sepharose and pellet/banded purified virus were seen with the use of DEAE-dextran in all XC assays initially (Table 1). However, a decreased surface glycoprotein content was correlated with a significantly impaired infectivity in the absence of DEAE-dextran pretreatment of target cells (Table 3). The method presented should be particularly helpful in the study of initial virus-cell interactions requiring a viral purification technique that is conservative of envelope glycoprotein.

It should be noted that the method described allows purification of retroviruses to a high degree when retroviruses represent the only particles released by cells into the fluid phase (medium, serum, ascites). If, however, one suspects contamination with other particles (such as mycoplasma) which may be separable on the basis of size and/or density, other techniques of purification subsequent to the above described method will be required.

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