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# Infectivity and RNA Patterns as Functions of High- and Low-Dilution Passage of Murine Sarcoma-Leukemia Virus: Evidence for Autointerference Within an Oncornavirus Population

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Heterogeneity of buoyant density and RNA content of virions of Moloney murine leukemia-sarcoma complex [MSV(MLV)] was the result of passage at low dilution. Heterogeneous stocks revealed two major RNA components in the population, with the smaller component, apparent mol wt $4 \times 10^6$ to $5 \times 10^6$, becoming predominant upon serial passage at low dilution. Concomitantly, infectivity titers of both MLV and MSV decreased upon serial passage at low dilution. MSV(MLV) passaged at high dilution retained high titers and a rather homogeneous high-molecular-weight RNA population characteristic of high-buoyant-density virions. Interference of both MLV and MSV replication was demonstrated by employing mixed inocula containing both low- and high-dilution passage stocks of MSV(MLV). In contrast to results with MSV(MLV), MLV freed of MSV by limit dilution did not show heterogeneity of buoyant density or of RNA when propagated at low dilution.

Huang and Baltimore (9) defined defective but biologically active virus particles as those which contain normal structural protein and a portion of the viral genome, replicate in the presence of helper virus, and interfere specifically with intracellular replication of nondefective homologous virus. Such defective particles and the associated phenomenon of autointerference have been demonstrated in many viral systems (2, 4, 10, 11, 16). In this communication we present evidence for autointerference in the Moloney murine sarcoma-leukemia virus [MSV(MLV)] complex. Manning et al. (14) demonstrated virions of a broad density range in populations of MSV(MLV). The RNA prepared from such virus populations was heterogeneous with respect to molecular weight. The reason for this population heterogeneity was not established, but thermal degradation or "nicking" of the RNA during the labeling period was ruled out.

We examined the effects of serial passage, employing high and low multiplicities of infection, on the RNA profile and on the yield of MLV and MSV. The data demonstrate that RNA heterogeneity is not observed with populations of MLV and is only observed for the MSV(MLV) complex after passage at high multiplicity. This increase in RNA heterogeneity and the observed progressive decrease of MLV and MSV titers as a function of passage at high multiplicity are characteristic of autointerference as observed in other systems.

## MATERIALS AND METHODS

The source of Moloney MSV(MLV) and its propagation in cell culture have been described elsewhere (6, 7, 13, 14). Briefly, the procedures are: (i) inoculation of subconfluent cultures of high-passage-level Swiss mouse embryo cells with MSV(MLV) diluted in Liebovitz medium L-15 with 10% fetal calf serum and 4 μg of polybrene per ml (13); (ii) addition of minimal essential medium with 10% fetal calf serum after 1.5 h of adsorption; and (iii) replacement of medium at 48 h, followed by harvest at 72 h postinoculation. For radioactive labeling of virus,

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200 μCi of $^3$H-5-uridine or $^{32}PO_4$ was present in the 10 ml of medium during the 48 to 72 h period.

Assay of MLV on BALB/3T3 cells with XC cells has been described (15). Modifications of the MSV focus assay on BALB/3T3 cells (8) were as previously described (7, 13) except that to insure the presence of sufficient helper virus, $10^6$ plaque-forming units of MLV were added after adsorption of MSV(MLV). Although the bioassays of MLV and MSV were consistent within any one experiment, precise comparisons of titers, and especially of MLV-MSV ratios, could not be made between experiments assayed on different dates.

The MLV employed as a helper in the MSV assay and also used for biophysical studies was derived from MSV(MLV) stock by three serial limit dilution passages; no MSV was detectable in this MLV stock.

Conditions for concentration, isopycnic banding, RNA extraction, and analysis by electrophoresis on 2.4% polyacrylamide gels have been described (6, 14, 18). Apparent molecular weights of RNA were estimated by assuming a linear relation between distance migrated and log molecular weight employing Vero cell rRNA, mol wt 1.67 and 0.70 million, and also, in some gels, Sindbis virus RNA, mol wt 4.0 × $10^6$, as internal markers (17, 18).

## RESULTS

**Heterogeneity in density and RNA content of virions in MSV(MLV) populations.** Labeled MSV(MLV) obtained from cells infected with a low-dilution inoculum (LDI; twofold dilution of a high titer stock) was isopycnically banded. A broad buoyant density range was observed (Fig. 1A). The electrophoretic pattern of RNA from high-density virions from fraction 4 showed a broad distribution with a peak at approximately 6.5 × $10^6$ daltons (Fig. 1B). The same major component appeared to be in the peak fraction (fraction 6), but there was a greater amount of material of lower mobility (Fig. 1C). Low-density virions contained as their major component a species of RNA with a molecular weight of 4 × $10^6$ to 5 × $10^6$ (Fig. 1D). These results are in agreement with the findings of Manning et al. (14) regarding the heterogeneity of certain MSV(MLV) populations. The electrophoretic patterns presented here indicate that there are two distinct major RNA species present in such populations. In

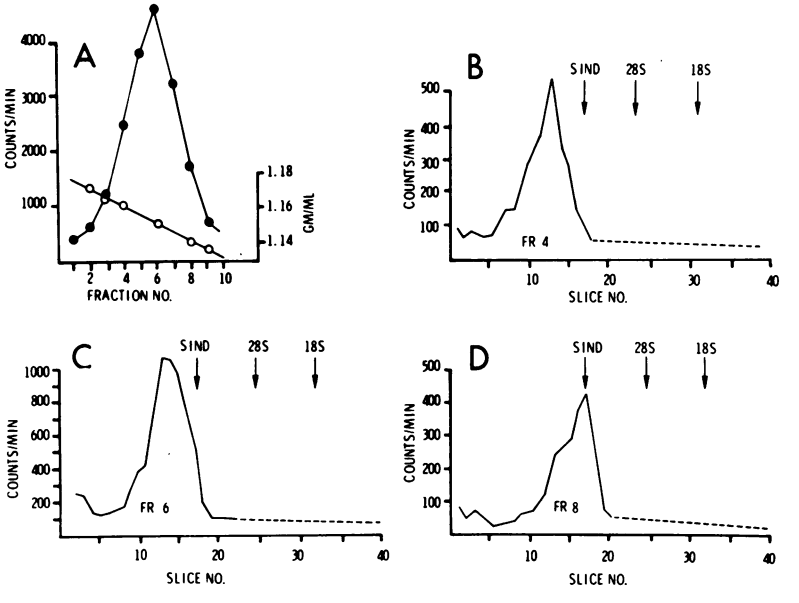


FIG. 1. *Buoyant density distribution of $^3$H-labeled MSV(MLV) virions in sucrose (A), and electrophoretic patterns of RNA from selected fractions (B, C, D). MSV(MLV) was harvested from cells inoculated with a 1:2 dilution (LDI) of a high-titer stock and concentrated by centrifugation (45 min, 204,000 × g, onto a cushion of 50% (wt/vol) sucrose), layered onto a 15 to 50% (wt/vol) sucrose gradient, and centrifuged for 3 h in a Spinco SW50L rotor at 50,000 rpm. Fractions of three drops each were collected; densities were determined by refractive index, and 20-μliter portions were assayed for acid-insoluble radioactivity (A). Only the peak region is shown. Portions of fractions no. 4 (B), 6 (C), and 8 (D) were disrupted by dilution into 1% sodium dodecyl sulfate in dilute electrophoresis buffer (0.1 E, reference 17) and subjected to electrophoresis in 2.4% polyacrylamide gels for 2.75 h at 5.2 V/cm; slices of 1.6 mm each were assayed for total radioactivity. Arrows indicate peak positions of $^{32}P$-labeled Sindbis RNA and $^3$H-labeled Vero rRNA markers; for clarity, radioactivity in marker regions is not shown, but that contributed by MSV(MLV) is approximated by the dashed line (reference 14, and experiments not shown).*

addition to the two major species, there appear to be additional species of RNA present, indicative of the continuous range of apparent mol wt between $4 \times 10^6$ and $9 \times 10^6$.

**Homogeneity in density and RNA of virions of MLV.** In contrast to heterogeneity of MSV(MLV) obtained under LDI conditions, MLV similarly propagated was relatively homogeneous in buoyant density distribution (not shown). The MLV RNA was usually quite homogeneous, as shown in Fig. 2A, where an approximate mol wt of $6.5 \times 10^6$ was estimated. Some heterogeneity of MLV RNA was occasionally observed, as illustrated in Fig. 2B, which also illustrates the much greater heterogeneity of MSV(MLV) RNA. The 4 to 5 million dalton RNA peak from LDI-propagated MSV(MLV) was virtually absent in similarly propagated MLV.

**Serial passage of MSV(MLV) with high- and low-dilution inocula.** The effect of serial passage with LDI and with high dilution inocula (HDI; 200-fold dilution of high-titer stock) on the electrophoretic patterns of RNA extracted from MSV(MLV) virus populations is shown in Fig. 3. RNA from virus populations produced under LDI conditions was quite heterogeneous, with the $4 \times 10^6$ dalton RNA species becoming predominant by the fourth LDI passage. The RNA from populations produced under HDI conditions was relatively homogeneous with a molecular weight of approximately $7 \times 10^6$. There was no observable change in the electrophoretic pattern as a function of HDI passage.

The effect of serial passage under LDI conditions on yields of infectious MLV and MSV is shown in Fig. 4. MSV titers continued to decrease through five successive passages. MLV, on the other hand, decreased only through the third passage, remaining stable for the third to the fifth passage. Decrease in total infectivity upon serial passage was not accompanied by a gross decrease in total RNA, but did result in a shift toward the low-molecular-weight component (Fig. 3A, C). As a control, serial passage of MSV(MLV) under HDI conditions showed essentially no change in titers of MSV or MLV (not shown) or in RNA patterns (Fig. 3B, D) through four passages.

**Reduction in virus yield with mixed inocula.** To demonstrate that autointerference was responsible for the reduction in titer, cells were infected with mixed inocula prepared from two different virus stocks. Stock A consisted of an MSV(MLV) harvest from a culture infected under HDI conditions. Stock B consisted of an MSV(MLV) harvest from a culture

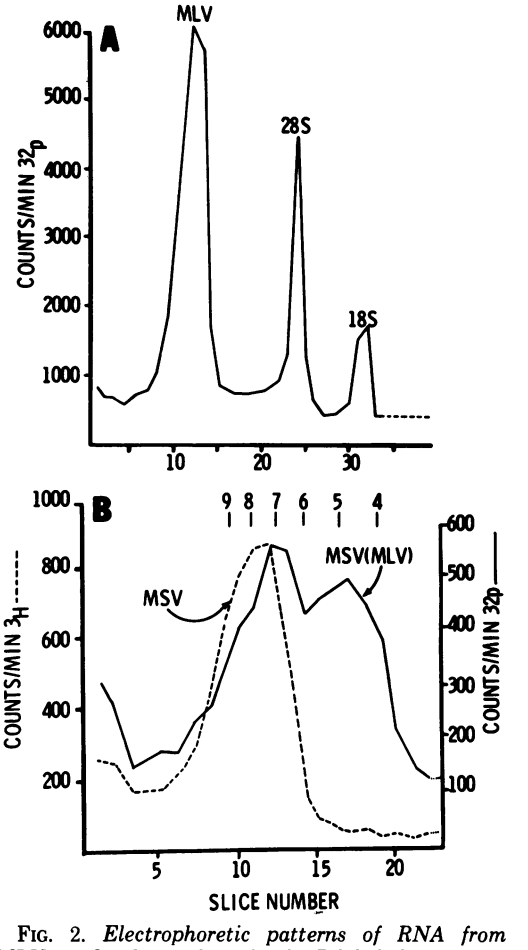


FIG. 2. *Electrophoretic patterns of RNA from MLV. A, Co-electrophoresis of $^{32}P$-labeled MLV and $^{32}P$ RNA from Vero cells. MLV was propagated under LDI conditions (1:2 dilution of high-titer stock) and collected by centrifugation (78,000 × g for 45 min); the virus pellet was disrupted by addition of 1% sodium dodecyl sulfate in 0.1 E buffer and the liberated RNA together with added $^{32}P$ rRNA marker were subjected to electrophoresis as described in Fig. 1. B, Co-electrophoresis of RNA from MLV and MSV(MLV). Cell culture fluids containing $^3H$-labeled MLV and $^{32}P$-labeled MSV(MLV), both propagated under LDI conditions, were mixed and centrifuged. RNA from the mixed pellet was liberated with sodium dodecyl sulfate and subjected to electrophoresis as described above. Only the viral RNA portion of the gel is shown. Apparent molecular weights (× $10^{-6}$) estimated from $^{32}P$ rRNA markers are shown at the top of panel 2B.*

infected under LDI conditions. Yields from cells infected with a constant amount of Stock A (200-fold dilution) and varying amounts of Stock B (2-, 10-, and 100-fold dilutions) were compared with yields from cells infected with Stock A or B alone (Table 1). Reduction in

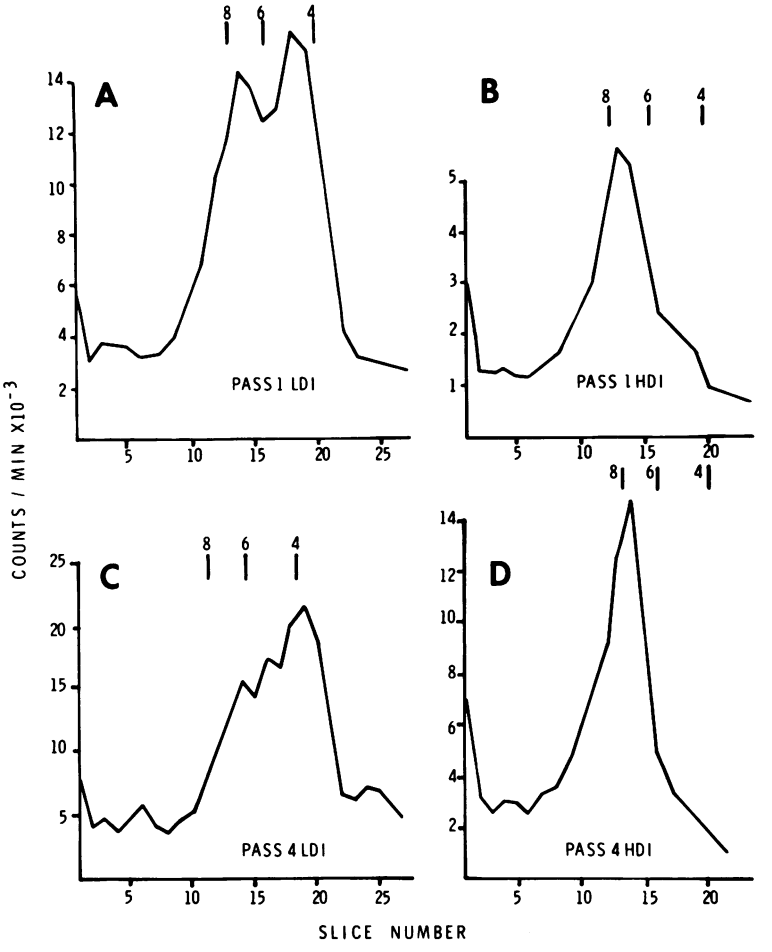


FIG. 3. *Effect of serial pasage with high- (HDI) and low- (LDI) dilution inocula on the electrophoretic patterns of $^3$H-labeled MSV(MLV) RNA. Profiles of RNA from (A) first passage LDI, (B) first passage HDI, (C) fourth passage LDI, and (D) fourth passage HDI. First LDI passage was initiated by inoculation with a 1:2 dilution of a high-titer MSV(MLV) stock; subsequent passages were inoculated with a 1:2 dilution of harvest of previous passage. First HDI passage was initiated by inoculation with a 1:200 dilution of MSV(MLV) stock (same as above); subsequent passages were inoculated with a 1:200 dilution of harvest from previous passage. Preparation of samples and electrophoresis were as described in Fig. 2. Only the viral RNA portion of each gel is shown. Apparent molecular weights (× $10^{-6}$) estimated from $^3$H Vero rRNA markers are shown at the top of each panel.*

yield of both MLV and MSV was found when the inoculum contained 2- and 10-fold dilutions of Stock B; this effect was diluted out at the hundredfold level.

## DISCUSSION

The findings of Manning et al. (14) that populations of MSV(MLV) exhibit a broad buoyant density range correlating with electrophoretic patterns of RNA have been extended in our work. Two major RNA species with apparent mol wt of approximately 4 and 7 million were present in the heterogeneous populations produced under LDI conditions, but only the higher-molecular-weight major species was present in relatively homogeneous HDI populations. Degradation or "nicking" of viral RNA during incubation at 37 C (1, 12) is not a plausible explanation for the distribution of RNA sizes, since the MLV and HDI samples showing homogeneity and the LDI samples showing heterogeneity were all subjected to the same conditions of incubation. Further, Manning et al. (14) observed essentially the same RNA patterns obtained from 3-h and 14-h

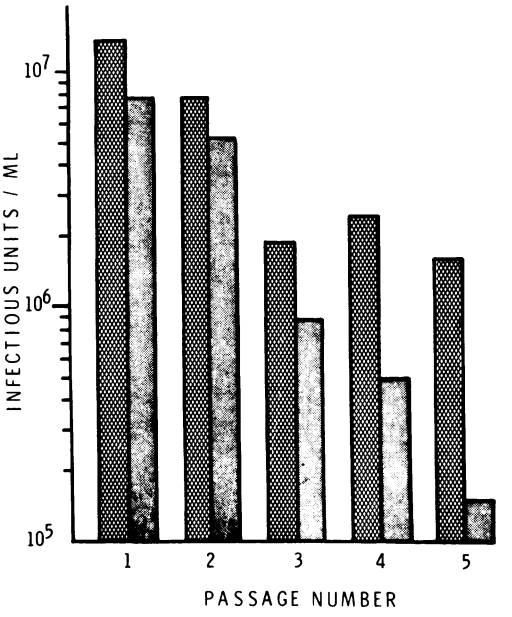


FIG. 4. *Effect of serial passage of MSV(MLV) with low-dilution inocula on MLV and MSV yields from infected cultures. Conditions of passage were described under Fig. 3; culture fluids collected between 48 and 72 h post-inoculation were stored at −70 C, and all were assayed on the same date. MLV, cross-hatched columns; MSV, solid columns.*

incubation intervals. The virus-cell system used in this work was the same as that used by Manning et al.; whether similar results will be obtained with other oncornaviruses is unknown at the present time. In the more extensively studied Rous sarcoma virus system, one recent study investigated differences in RNA and virion structure in relation to maturation (3) and another studied differences in 36*s* RNA species among virus strains (19). Most Rous sarcoma virus studies employ transformed cells obtained under conditions of high multiplicity of infection (comparable to our LDI), but to our knowledge the possible role of autointerference is not generally considered.

We conclude that the RNA heterogeneity and the reduction of MLV and MSV titers as a function of passage level in LDI experiments are manifestations of an interference phenomenon. We hypothesize that the low-density virions, which contain RNA of $4 \times 10^6$ to $5 \times 10^6$ mol wt, are autointerfering or defective particles as defined by Huang and Baltimore (9). An alternative hypothesis, that interference is related to multiplicity of infection, independent of the presence of any specific type of particle, has not been ruled out; further experiments are required to clarify the role of the low-density virions. The possibility that the interference is due to extracellular interferon is ruled out by the failure of LDI culture fluids to reduce titers of vesicular stomatitis virus in mouse cells (Hackett, unpublished data). The possible role of the MLV component of the MSV(MLV) complex in autointerference remains to be resolved. Under first-passage LDI conditions, MLV failed to show the type of heterogeneity expressed by the complex.

We have considered that MLV and MSV may have different mol wt of 60 to 70*s* or 36*s* RNA, or slightly different buoyant densities, or both. Because of the inability to obtain MSV free of MLV, resolution of these questions is not readily obtained.

A possible difference in molecular weights of the 60 to 70*s* RNA species is suggested in experiments such as that in Fig. 2B; however, heterogeneity, especially of the MSV(MLV) complex, obviates definitive conclusions. Differences in the 36*s* RNA species may exist, but earlier experiments (Fig. 4 of reference 14) showed no detectable difference in the major component from high- and low-density virions.

The apparent mol wt for RNA of MLV and of HDI passage MSV(MLV) estimated by gel electrophoresis in this study, both approximately 7 million, are somewhat lower than the generally accepted mol wt of 10 to 12 million for the genome (5). This discrepancy may be due to uncertainties in the assumption of a linear relation between distance migrated and log molecular weight when extended to high molecular weights, in the true molecular weights of the markers (17, 18), or in the long extrapolation from the markers.

TABLE 1. *Yields from cultures inoculated with mixed high- and low-dilution MSV(MLV) inoculum*

| Inoculum (dilution) | | Yield[a] | |
|---|---|---|---|
| Stock A[b] | Stock B[c] | MLV (PFU/ml) | MSV (FFU/ml)[d] |
| 1:200 | 1:2 | $8.8 \times 10^6$ | $8.6 \times 10^5$ |
| 1:200 | 1:10 | $3.0 \times 10^7$ | $2.8 \times 10^6$ |
| 1:200 | 1:100 | $2.7 \times 10^8$ | $2.4 \times 10^7$ |
| 1:200 | | $1.8 \times 10^8$ | $3.2 \times 10^7$ |
| | 1:2 | $2.0 \times 10^7$ | $2.6 \times 10^6$ |

[a] Culture fluids (48–72 h).

[b] Stock A, virus harvest from a culture infected with high-dilution inoculum (duplicate harvest of that used for RNA profile, Fig. 3D).

[c] Stock B, virus harvest from culture infected with low-dilution inoculum (duplicate harvest of that used for RNA profile, Fig. 3A).

[d] FFU, Focus-forming units.

If autointerference, correlated with heterogeneity of virions and their RNA, is a general phenomenon among the oncornaviruses, studies employing these viruses must be interpreted in the light of this phenomenon. For example, the use of defective virion RNA as a template for DNA synthesis and hybridization may lead to results differing from those obtained with homogeneous RNA from complete virus. There are, of course, broad implications not only in the molecular biology of the virus but also in the whole area of our understanding of the role of oncornaviruses in nature.

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