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Quantitation of Endogenous C-type Virion Production in Several Murine Cell Lines

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Quantitation by enumeration of virion particles, measurement of absorbancy at 260 nm, and densitometry on sodium dodecyl sulfate-polyacrylamide gels has shown that mouse L-M cells yielded 7- to 10-fold less endogenous C-type virions than the parental lines, L or L929. The previously noted stimulation of L-M cell virion production by a concomitant 10% increase in fetal calf serum concentration (D. A. Kindig, R. Karp, and W. H. Kirsten, 1968), was not observed.

Endogenous C-type virus particles have been reported in many established cell lines that are commonly used for propagating animal viruses, e.g., mouse L cells (1, 4) or for studies on animal cell hybridization, e.g., Chinese hamster CHO-Kl cells, (6). It has also been reported that certain cell lines, e.g., mouse L-M (5), which are relatively free of externally budding C-type virions, can be converted into a virion-producing line when grown in serum-containing medium. This paper deals with a comparative study of endogenous C-type virion production in such mouse cell lines, and attempts to quantify virion production under several growth conditions (Presented in part at the 73rd annual meeting of the American Society for Microbiology, 6–11 May 1973).

Mouse L-cell fibroblasts (2) in suspension culture were obtained from H. Zweerink, Duke University. L929 cells (10) were provided by M. Wiebe, Duke University, who had initially obtained them from J. Youngner, University of Pittsburgh. The L-M mouse cell line was from the American Type Culture Collection and had a cell repository number of CCL 1.2. The L929 and L-M cell lines were initiated on monolayers, until enough cells could be obtained for subcultivation in suspension flasks. The L and L929 lines used in this study were clearly different; (i) in morphology, viz., the L cells from suspension culture appeared rounder and less crenated than L929; (ii) in duration of growth cycle; and (iii) in degree of interferon induction (M. Wiebe, personal communication). For virion production, all cells were propagated at 37 C in suspension culture in Eagle minimal essential medium (Joklik's modification, Grand Island Biological Co.) containing 5% fetal calf serum. Cultures were initiated at about 3×10^5 cells/ml and grown to densities of 1.0 to 1.5 $\times$ 10^6 cells/ml with a doubling time of 18 to 20 h. Then virions were isolated from supernatant fluids as previously described (9). The isolation involved steps of: (i) ammonium sulfate precipitation, (ii) discontinuous gradient centrifugation, viz., the clarified suspension was layered above 1 ml of 60% sucrose and 2 ml of 38% sucrose before centrifugation for 2 h at 30,000 rpm in an SW41 rotor, and (iii) equilibrium gradient centrifugation through 30 to 60% sucrose density gradients. Virion suspensions were maintained in STE (0.01 M Tris-hydrochloride [pH 7.2], 10^{-3} M EDTA, 0.15 M NaCl).

Our initial observation suggested in a qualitative manner that there was a difference in virion yield between L-M and the parental L or L929 cells. L-cell supernatant fluids exhibited a distinct virion band at the 38%/60% discontinuous sucrose gradient interface, whereas L-M cell supernatants gave a diffuse band throughout most of the sucrose gradient. The entire 38 to 60% region of the L-M gradients, and the interface band from the L gradients were collected for further analysis. These suspensions were then concentrated 30- to 50-fold, by pelleting the virions at 40,000 rpm for 60 min in an SW50 rotor. Based on a protein determination (7), over three experiments for each cell type, 200 to 250 μg of protein was recovered per liter of original L- or L-M cell supernatant. The concentrated discontinuous gradient material was then centrifuged to equilibrium on sucrose density gradients (30 to 60% in STE). For all L strains, a single homogenous band at an average density of 1.16 g/cm^3 was observed (Fig. 1), the peak of absorbancy at 260 nm and virion particles being coincident (Fig. 1, inset). Based on a sum of the absorbancy at 260 nm in the

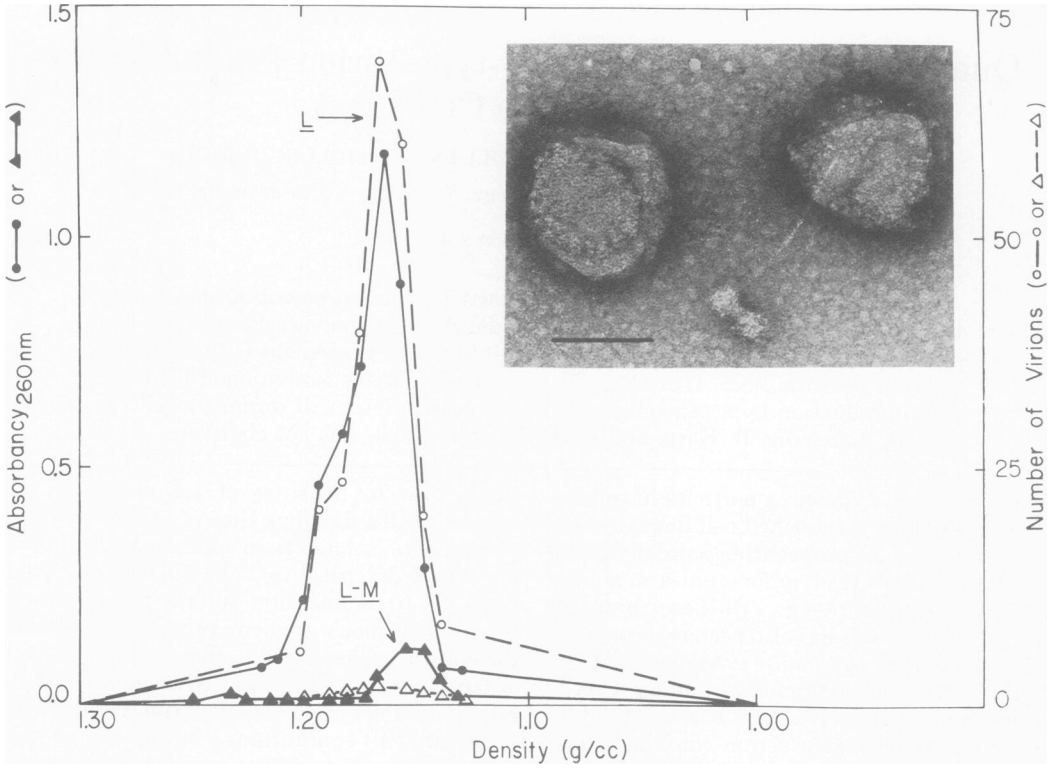

FIG. 1. *Density profile of virions from L (○ and ●) and L-M (△ and ▲) cell supernatant fluids, after sucrose density gradient equilibrium centrifugation. Twenty-drop fractions were collected and absorbancy and virion particle count determinations (see inset for morphology of typical L-cell virions) were made on each fraction. About 1300 μg of protein (7) was layered on each gradient.*

peak fractions around ρ indicating 1.16 g/cc (Fig. 1), we found over two experiments that the average fractional recovery of L-cell virion (LCV) protein after the density gradient step was 57%. This value is based on the assumption that one absorbancy at 260-nm unit of purified RNA-tumor virions corresponds to 158 μg of protein per ml (12). In contrast, the average recovery for L-M was 8% (Table 1). The greater enrichment from concentrated discontinuous gradient materials of L-cell over L-M virions was not unexpected, based on our previous qualitative observation. From the above recoveries, the estimated average yield of LCV was about 140 μg of protein or 2.8×10^{11}-virion particles (12) per liter of cell supernatant. Correspondingly, L-M cells yielded seven- to ten-fold less virions (Table 1). Attempts were made to stimulate the yield of purified L-M virions, both with an increase of 10% fetal calf serum or by growing cells to a higher density. However, only a slight, if any, increase in total virion production was found (Table 1). This result differs from the virion increase implied by the serum activation of [^{3}H]uridine-labeled banded material (ρ = 1.16 g/cm^3) for L-M cells, previously reported (5). However, in the latter study a quantitative estimate of virion yield before and after serum stimulation was not provided. Perhaps, only the virion specific activity was increased due to the stimulation of nucleotide precursor pools or to an increase in the stability of virion nucleic-acid structures. Alternatively, our inability to observe serum stimulation for L-M virions may reflect differences in subcultivation of L-M cells or in the type of growth medium used.

Additional evidence supporting a decreased virion yield from L-M cells came from both sodium dodecyl sulfate-polyacrylamide gel and thin-section electron microscope studies. For the electrophoresis studies, the technique was as described (9). The 7.5% sodium dodecyl sulfate-polyacrylamide gel electropherograms of concentrated discontinuous gradient materials from L-M cell cultures only showed a slight

TABLE 1. *Amount of material in virion peaks after density gradient centrifugation*[a]

Strain	No. of particles	OD_{260}[b]
L (1 to 1.5 × 10^6)	97 (249)[c]	4.63 (0.84)[d]
L929 (1 to 1.5 × 10^6)	92	4.86
L-M (5% FCS) 1.1 × 10^6	6 (4)	0.77 (0.53)
L-M (5% FCS) 1.7 × 10^6	11	0.93
L-M (15% FCS) 1.2 × 10^6	3	0.29

[a] The summed peak fractions presented here have the gradient background subtracted.

[b] Optical density.

[c] Parentheses represent separate density gradient experiments.

[d] This value may be anomalous, since there was a high background of absorbing material in nonvirion fractions.

Coomassie staining of the major LCV protein (Fig. 2, arrow) regardless of whether the fetal calf serum concentration was 5 or 15%. As quantified on a Helena Industries Quick-Scan device, only about 10% as much staining density of this 27,000-dalton band was found for L-M when compared to L- and L929-gradient materials.

For thin-section electron microscopy, pellets containing up to 3 × 10^8 cells were washed twice in isotonic phosphate buffer (310 mOsm, pH 7.2) by centrifuging the cells at 3,500 × *g* for 10 min in a Sorvall; then fixed for 3 h in 0.15 M sodium cacodylate (pH 7.4) buffered 5.0% glutaraldehyde, and postfixed for 1 h in 0.2 M Veronal acetate buffer (pH 7.4) containing 1.0% O_sO_4; dehydrated through ethanol and propylene oxide; embedded in Epon 812 (Shell Chemical Co., New York, N.Y.); and sectioned by means of a Sorvall-Porter-Blum MT-2B ultramicrotome (about 60.0-nm silver sections). Sections were stained with saturated uranyl acetate and lead citrate, and examined in a Philips EM 300 electron microscope. Only rarely was a C-type particle encountered budding from the L-M membrane (Fig. 3B), whereas many such particles were seen for L cells (Fig. 3A and inset). Occasionally an immature C-type particle was seen apparently trapped within a cytoplasmic vacuole of L-M cells (Fig. 3B inset). Again only 10% as many virions were found associated with L-M compared to L-cells (Table 2). It should also be noted that the lack of any significant numbers of C-type particles in

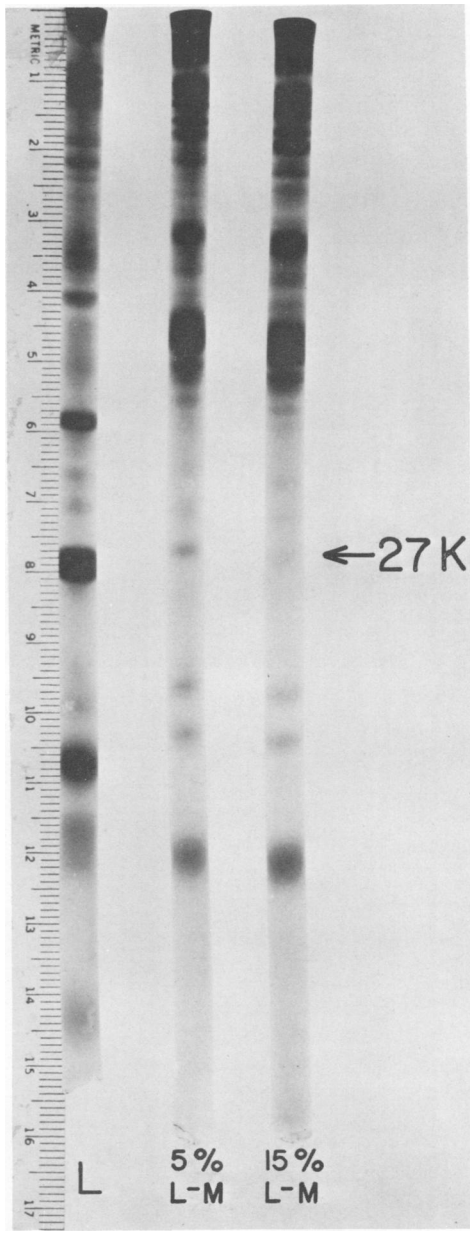

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electropherograms (7.5%) of concentrated shelf material from L or L-M grown with 5% fetal calf serum, and L-M grown with 15% fetal calf serum in the medium. About 125 μg of protein was layered on each gel. Note the marked density only for L, of the 27-K or 27,000-dalton protein, which is the major polypeptide of endogenous C-type virions. Molecular weight for this band was estimated from a marker gel with purified LCV, run simultaneously. The position at 27 K corresponds to band 12, as shown in Fig. 3 of (9).*

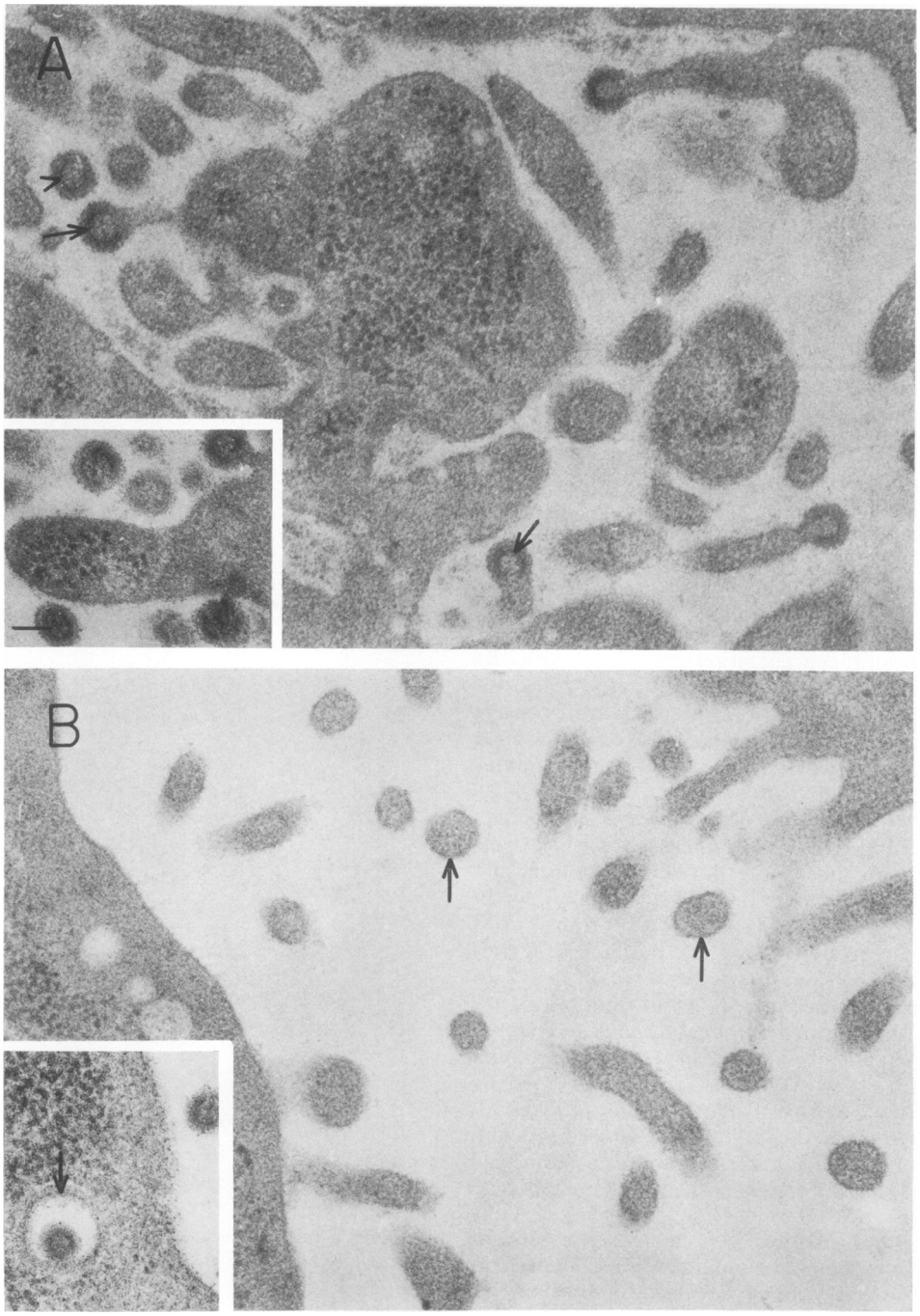

FIG. 3. *Thin-section electron microscopy of pelleted (A) L and (B) L-M cells. Note the many budding (A, arrows) and extracellular (A, arrow-head) C-type virions for L-cells, relative to L-M cells. The predominant feature of L-M cells are the cytoplasmic vesicles (B, arrows). Also, there is an occasional virion-like particle (B, inset arrow), apparently within a cytoplasmic vacuole.*

or near thin sections of cells should not be utilized as sufficient grounds for implying that virions have not been produced. This implication has been made in at least two studies (5, 11). However, although less than 0.1 budding virion per cell per thin section was observed in L-M cells (Table 2), there were 3 to 4 $\times$ 10^{10} virions per liter of cell supernatant fluid that

TABLE 2. *Numbers of virion and nonvirion particles (100 to 200 nm, in size) found associated with L or L-M cells*[a]

Cell Line	Budding particles		Particles in extracellular space		Total % virions	Particles in[b, c] a budding state (%)
	Virion	Nonvirion	Virion	Nonvirion		
L	12	1	25	68	35.0	8
L-M	1 (7)[b]	17	8	222	3.6	7

[a] A nonvirion particle is one that does not have any internal C-type morphology, but instead possesses granular, cytoplasmic-like material (Fig. 3B, arrows). The survey tabulated above was done over 15 cells of each type. However, additional tabulations over more cells showed the same relative percentages.

[b] This refers to intracytoplasmic C-type particles (Fig. 3B) inset arrow.

[c] This per cent is relative to the sum of budding plus extracellular particles. Since the pellets of cells were twice washed, these latter particles were presumed associated with the cells, but released during the processing for thin sections.

could be harvested (Table 1).

As a final point, it is of interest to speculate on the origin of L-M cells. It is possible that by acquisition of the trait whereby cells could be propagated in serum free medium, the L-M line lost or had masked some additional genetic information which was essential for maximal endogenous C-type virion production. Certainly in the light of many recent studies involving observations on endogenous C-type particles (3, 6), as well as stimulation of their production by chemical means (8, 13), it is not at all unusual to expect a piece of genetic information controlling some phase of virion production to become masked or unmasked. The very recent reported induction of abundant numbers of extracellular C-type virus particles from cultures of two nonvirion-producing clones derived from clone NCTC 929 (referred to above as L929) by addition of cytocholasin B (2 or 10 μg/ml) to the growth medium (11), seems to support such a contention.

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