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Murine leukemia virus morphogenesis: Cleavage of P70 *in vitro* can be accompanied by a shift from a concentrically coiled internal strand ("immature") to a collapsed ("mature") form of the virus core

(virus assembly/proteolytic cleavage/electron microscopy)

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ABSTRACT **Disruption of Rauscher leukemia virus (RLV) with low levels of Nonidet P-40 yielded "immature" cores. These cores have a diameter of about 920 Å, as opposed to the 1300-Å diameter of RLV, possess knob-like protuberances, and contain a concentrically coiled internal strand apposed to the core shell. The two major polypeptide components of immature cores are (*i*) p30, the 30,000-dalton group-specific antigen, and (*ii*) a polypeptide that has the size and antigenic characteristics of P70, the 70,000-dalton precursor protein of the group-specific antigens of murine leukemia virus. Disruption of RLV at high ratios of Nonidet P-40 to virus yielded "mature" cores. These cores have an average diameter of 850 Å, a smooth proteinaceous perimeter, and a collapsed internal strand, and they contain predominantly p30. Treatment of RLV with low levels of Nonidet P-40 for 16 hr at 22° yielded cores that showed (*i*) a 70% decrease in the number of immature forms and concomitant increase in the number of mature forms, (*ii*) a 60–90% decrease of P70, and (*iii*) a 30% increase in a 40,000- to 42,000-dalton protein. These results suggest that maturation of RLV cores is accomplished by cleavage of P70.**

During C-type virus morphogenesis, as studied by thin-section electron microscopy, a crescent is formed directly under the membrane, where the virus buds (1). This crescent, by virtue of its electron-dense staining, has been correlated with the ribonucleoprotein (RNP) component of the virus (1, 2). After budding, the crescent closes into a circle which appears in negative staining as a concentrically coiled double strand tightly apposed to the core shell (3). The particles are then released from the membrane. A question not yet resolved is whether such immature forms are precursors to collapsed, mature forms of the virus, which are the predominant structures seen in purified virus preparations. In mature virus the core has a central, electron-dense region which correlates in negative staining to a collapsed, single-stranded RNP component (3). We have devised a method for separating the immature and mature cores of Rauscher leukemia virus (RLV). We have characterized these cores morphologically and biochemically, and found conditions *in vitro* under which immature cores are converted to mature cores.

MATERIALS AND METHODS

Viruses. Unlabeled RLV was purified from supernatant fluids of chronically infected JLS-V9 cells and supplied to us under the auspices of Jack Gruber of the National Cancer Institute. Virus labeled with [^{35}S]methionine (specific activity 347 Ci/mmol), [^{14}C]glucosamine (specific activity 237 mCi/mmol), ^{3}H-labeled amino acids (average specific activity 27 Ci/mmol), or [^{14}C]acetate (45–60 mCi/mmol) (all from New England Nuclear) was obtained from harvests of Eveline cell (4) supernatant fluids after 20 hr of growth. After the virus was pelleted by differential centrifugation procedures, it was banded on 10–60% (wt/vol) sucrose gradients in 10 mM Tris·HCl/130 mM NaCl/1 mM EDTA at pH 7.3 (TNE). In some cases, ^{3}H- or ^{35}S-labeled purified virus was subjected to sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis, followed by radioautography. The resultant protein pattern was virtually identical to the Coomassie blue-stained bands of RLV.

Nonidet P-40 (NP40) Treatment of Virus and Separation of Viral Cores. Virus [1.8 mg; Lowry assay (5)] in TNE was treated with either 1–4 mg (low concentration) or 7–20 mg (high concentration) of NP40 in 0.3 ml at 4° for 5 min, layered onto 10–40% (wt/vol) sucrose gradients in TNE, and centrifuged at 30,000 rpm for 40 min at 4° in a Spinco SW50-1 rotor. About 20 fractions were then collected, using a tube-piercing device (Hoefer Scientific).

Analysis of Viral Proteins. To 25 μl of each sucrose density gradient fraction an equal amount of two times concentrated electrophoresis sample buffer containing 2% $NaDodSO_4$ and 10% 2-mercaptoethanol was added. The combined sample was boiled for 1 min and analyzed on 12.5% or 7% acrylamide slab gels made by the procedure of Läemmli (6) ($NaDodSO_4$/polyacrylamide gel electrophoresis). The gels were run on a Hoefer SE500 vertical slab gel system for 5 hr at 15 mA. Apparent molecular weights (M_r) of RLV polypeptides were determined relative to a series of known markers, i.e., bovine albumin, egg albumin, chymotrypsinogen, and cytochrome *c*. The numbering of polypeptides (p), precursor polypeptides (P), and glycoproteins (gp) by apparent M_r is according to convention (7).

Partial Purification of P70. Twenty milligrams of RLV and 3×10^5 cpm of [^{35}S]methionine-labeled Friend leukemia virus (FLV) in TNE plus 10 mM EDTA was disrupted with 5% NP40 in a total volume of 2.0 ml, then layered onto a 10–40% sucrose gradient as described before. The P70-rich precipitate (40–70% P70 relative to other bands as determined by $NaDodSO_4$/gel electrophoresis) was collected. In some experiments [^{14}C]glucosamine-labeled FLV (20,000 cpm) was added as a marker for gp69/71 to RLV. After NP40 treatment and gradient centrifugation, >99% of the [^{14}C]glucosamine counts remained at the top of the gradient. The P70-rich precipitates were then applied to a 5% acrylamide preparative slab gel. The P70 protein peak fractions were concentrated with Sephadex G-200 and dialyzed

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; NP40, Nonidet P-40; RNP, ribonucleoprotein; RLV, Rauscher leukemia virus; FLV, Friend leukemia virus; TNE, 10 mM Tris·HCl/130 mM NaCl/1 mM EDTA at pH 7.3; M_r, molecular weight.

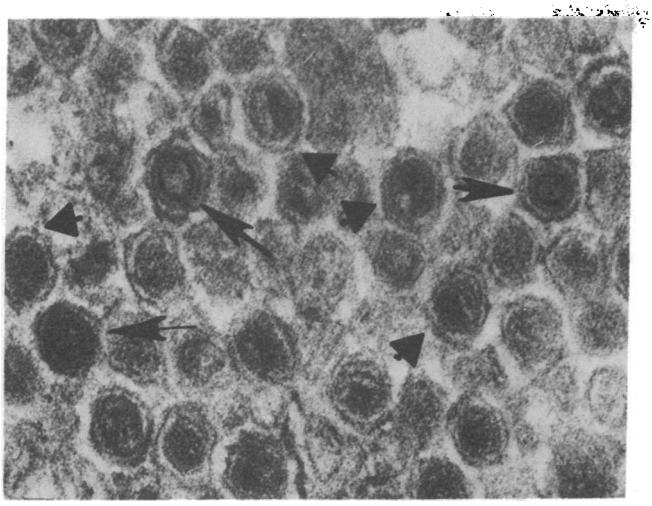

FIG. 1. Thin section of an RLV pellet. About 2 mg of purified RLV was pelleted by centrifugation for 2 hr at 30,000 rpm, fixed, embedded, sectioned, and stained. (×120,000.) Arrows, immature virus particles. Arrowheads, mature particles.

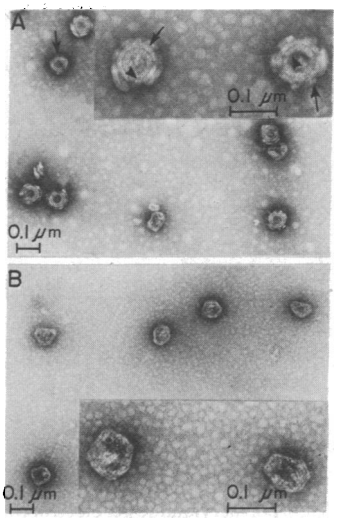

FIG. 2. Morphology of (*A*) "immature" and (*B*) "mature" cores isolated on sucrose gradients after exposure to low and high concentrations of NP40. For (*A*) note the concentrically coiled nature of the RNP (arrowheads) and the appearance of lipid-like protuberances (arrows). The average diameter measured over 25 particles (where the perpendicular dimensions were equal within a 5% error) was 920 Å for coiled cores, as compared to a value of 850 Å for the collapsed cores.

extensively against TNE. Double immunodiffusion was carried out with P70, using Hyland (Costa Mesa, CA) Immuno-plates. The goat antiserum used was raised against purified p10, p12, p15, or p30. It was obtained from R. Wilsnack, Huntingdon Research Laboratory, and had the following radioimmunoassay titers: 3,200 for gp69/71, 38,000 for p30, 2,000 for p15, 4,000 for p12, and 4,080 for p10. Crossreactivity among components was less than 50.

Incubation of Virus *In Vitro* in the Presence of NP40. A sample containing 1.4 mg of virus was treated with 4.0 mg of NP40, divided into two equal portions, and incubated for 16 hr; one was held at 4° and the other at 22°. After incubation, 90 μg of each sample was analyzed for total protein composition on $NaDodSO_4$/gel electrophoresis. The remainder was subjected to sucrose gradient centrifugation, and fractions were collected. For each fraction the protein composition was determined. The 350S core band was examined by electron microscopy, and the coiled and collapsed forms were enumerated.

Electron Microscopy. Negative staining of virus and virus components using 5% glutaraldehyde (0.15 M Na cacodylate, pH 7.4) and 2% uranyl acetate (pH 4.2) was done as described (3). Thin sections of 5% glutaraldehyde-fixed virus pellets were prepared as described in ref. 8.

RESULTS

Isolation of "Immature" and "Mature" Cores of RLV Using Selective NP40 Exposure. Unlabeled RLV obtained from the National Cancer Institute, when examined by thin-section electron microscopy, shows both immature and mature virus particles (Fig. 1). We quantitated the amount of each type and found that 11% of the particles (n = 659 virions) had the immature morphology, i.e., a dark concentric ring apposed against the core shell. A parallel examination of virions by a negative staining procedure, namely, the 0.01% $NaDodSO_4$ grid exposure method (3), showed, in agreement with the result from thin-section electron microscopy, that 15% of the particles (n = 690 virions) had an immature morphology, i.e., a concentrically coiled internal component. We found that the cores of these immature virions could be isolated by exposing the virus to a relatively "low" (0.5–3 ratio of NP40 to virus wt/wt) concentration of NP40 detergent, followed by sucrose gradient centrifugation. We settled on routinely using detergent ratios of NP40 to virus of 2–3 to isolate the immature cores. The resultant cores sedimented in a sharp, visible band at 350 S, relative to Sindbis virus, s_{20w} = 280 (9). Morphologically (Fig. 2*A*), these cores appeared smaller (average diameter = 920 Å) than virus (average diameter = 1300 Å), had a dark center (indicating penetration by stain) and a concentrically coiled internal strand component pressed against the core shell, and possessed knobbed protuberances.

Perhaps these protuberances are lipid in nature since their amorphous morphology is similar to that of negatively stained viral envelopes that we have seen previously (3). [^{14}C]Acetate labeling for lipid shows that these cores retain about 10% of the total virion radioactivity, which supports this contention.

Mature cores, which also sediment at about 350 S and which were present to a limited extent (20–30%) after treatment with low amounts of NP40, were obtained as the major core structure when RLV was exposed to high (ratio of NP40 to virus of >4 wt/wt) NP40 concentrations. At these concentrations the immature cores were extensively removed from the 350S core band and formed aggregates at the bottom of the gradient with what appear as membrane vesicles. Morphologically, the mature cores that were isolated appear very different from the immature cores (Fig. 2*B*). The particles were smaller (average diameter = 850 Å) and had a smooth polygonal perimeter. They appear identical to cores observed in a previous study (10). Surprisingly, the yield of mature cores [based on Lowry protein determinations (5) and [^{35}S]methionine labeling profiles, using equivalent amounts of virus as starting material] was approximately half that of the immature cores. Based on the electron microscope observations of intact virions, we would have expected about a 9-fold increase in mature forms. The finding that most of the p30 and p15 is at the top of the gradient even after treatment with low concentrations of NP40 (Fig. 3) suggests that most (>70%) of the mature virus in these unlabeled RLV preparations is unstable to NP40 treatment and is easily solubilized.

Association of P70 Protein with Immature Cores, But Not with Mature Cores. When $NaDodSO_4$/gel electrophoresis of

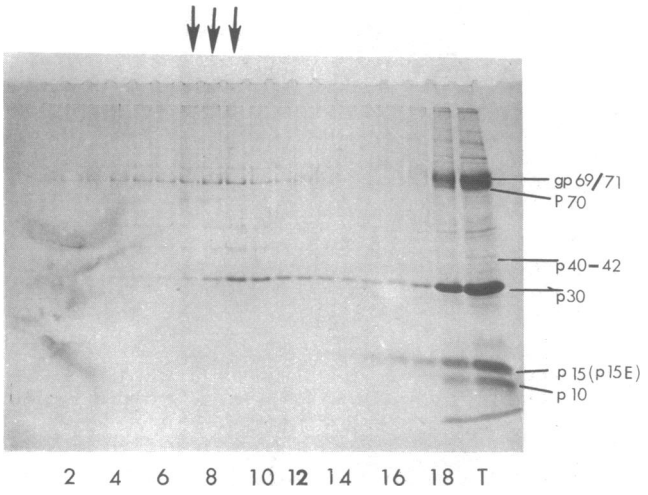

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of gradient fractions after treatment with low amounts of NP40, namely, the NP40/RLV ratio was 3. Arrows point to fractions in which the core band is observed on the gradient. gp69/71, p30, p15, and p10 were identified by comparing the protein bands from the peaks of a guanidine·HCl A 5-m column with the double immunodiffusion profiles obtained with monospecific antisera to the viral proteins. p15E was identified by its position in the void volume of the guanidine column as reported by Ikeda *et al.* (16). The numbers on the bottom refer to gradient fractions from bottom to top (T).

the gradient fractions from treatment with low concentrations of NP40 was performed, we found that the 350S immature core band, which corresponds to fractions 7, 8, and 9 (Fig. 3), was enriched in a 70,000-dalton protein (P70). In total virions, this band is a minor (3% of total staining) component, yet in the immature cores it represents 40–60% of the total stain.

We confirmed that the P70 protein of our RLV preparations apparently corresponds to the *gag* precursor protein by the following experiments. First, the relatively pure P70 antigen (>95% based on the $NaDodSO_4$/gel profile) was tested for immunological crossreactivity by the Öuchterlony double-diffusion method. We observed a common band of precipitation with goat antiserum to p10, p12, p15, and p30 (Fig. 4). There was no crossreactivity with antiserum to gp69/71 nor of P70 with control, unimmunized goat sera. Second, we found that labeled P70 was enriched in immature cores (Fig. 5) that had been obtained from virus purified from [^{35}S]methionine-labeled, chronically infected mouse embryo cells. In this experiment, as in those with unlabeled material, the virus had been treated with "low" NP40 to obtain immature cores. In a third and final experiment, immunoprecipitation of immature cores, labeled with ^{3}H-labeled amino acids, with antiserum to p30 and to p15, each precipitated a P70 component in addition to the respective antigens (unpublished observations).

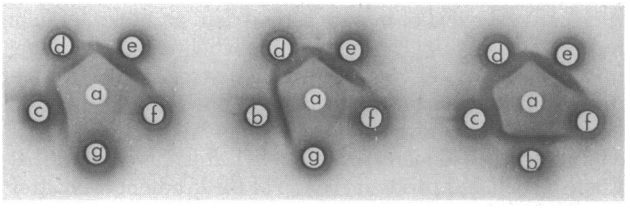

FIG. 4. Double-immunodiffusion pattern of P70 (*a*) against goat antiserum to p10 (*b*), p12 (*c*), p15 (*d*), p30 (*e*), and gp60/71 (*f*) and against control, unimmunized goat sera (*g*). Into the P70 central well we added 50 μg of protein, while 10 μl of serum was added to the other wells. Incubation was for 2 days at 22°. Staining was with Coomassie blue.

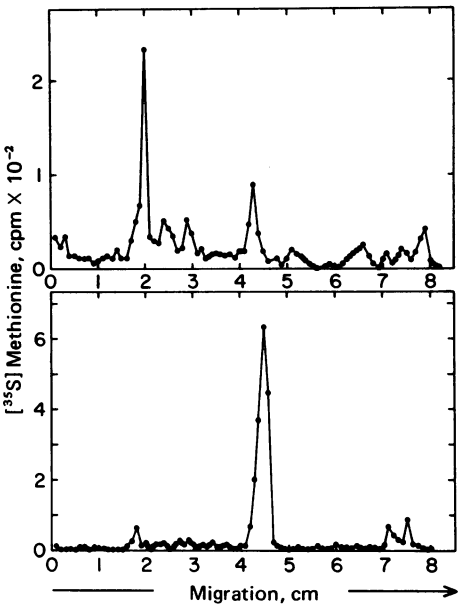

FIG. 5. Isolation of ^{35}S-labeled FLV cores. FLV (50,000 cpm; about 0.01 mg) mixed with 1 mg of RLV was treated with 0.5 or 5.0% NP40/virus (wt/wt). The 350S band obtained in each case was examined by electron microscopy and revealed coiled or collapsed cores, respectively, as described in the legend of Fig. 2. The peak fractions were then pooled, concentrated, and run on $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3). Slices were taken and eluted overnight with 10 mM Tris·HCl (pH 7.3) containing 0.1% $NaDodSO_4$. NCS-Aquasol was used for measuring ^{35}S radioactivity. The major peaks observed for the coiled cores (*Upper*) have M_r corresponding to P70 and p30; for collapsed cores (*Lower*) only p30 is observed. The minor, lower M_r peaks (p15, p15E, and p10) are hard to resolve. The failure to find significant labeling of p15 is consistent with the result of Shapiro *et al.* (14) that this protein lacks methionine.

In contrast to the above results, the corresponding gradient fractions of unlabeled or labeled mature cores did not show any P70. Only a band corresponding to p30 appeared to any extent in the gels (Fig. 6, unlabeled; Fig. 5 *lower*, labeled).

Incubation of Virus *In Vitro* with NP40 at Two Different Temperatures. Now that we had succeeded in isolating a core fraction that was enriched in the immature or concentrically coiled internal strand form of RNP we asked the question, could we observe a conversion of this form to the mature or collapsed form *in vitro*? Jamjoom *et al.* (11) had noted that after incubation of ^{35}S-labeled virus in the presence of NP40 at 37°, there was a cleavage of P70 (Pr4 in their terminology). Using this observation as a starting point, we tried several detergent treatments and incubation regimes. We found that treatment with NP40 at a wt/wt ratio of NP40 to virus of 3:1, followed by incubation at 22° for 16 hr, led to the disappearance of P70 and an increase in a band of M_r 40,000–42,000 (Fig. 7). To determine whether this disappearance was accompanied by a change in core morphology, we incubated NP40 and virus (3:1) at either 4° or 22° and centrifuged the mixture on 10–40% sucrose gradients. In both cases similar gradient profiles were obtained; further, the 350S bands in each case contained the same amount of protein. We then analyzed the 350S core bands by $NaDodSO_4$/gel electrophoresis and electron microscopy. Tracings of the $NaDodSO_4$ gels showed that after 4° incubation of the virus at the low NP40/virus ratio, a large amount of P70 remained, while after exposure at 22° the amount of P70 was negligible. Typical electron micrographs of the cores from the two gradients are seen in Fig. 8. After 4° treatment, immature cores predominated (Fig. 8*A*). In contrast, the cores observed

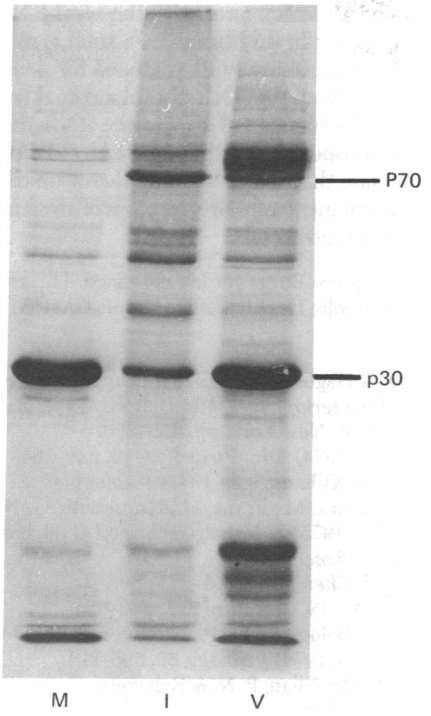

FIG. 6. Protein composition of mature and immature cores. RLV (1 mg) was treated with either low (3 mg) or high (5 mg) concentrations of NP40, the visible core band fractions were pooled, and 25-μl samples were removed for analysis by $NaDodSO_4$/gel electrophoresis. M, Collapsed core fraction from treatment with high concentration of NP40; I, immature core fraction from the sample treated with low concentration of NP40; V, virus.

after 22° treatment were either partially or completely collapsed (Fig. 8*B*). A control to show that treatment alone at 22° in low concentration of NP40 was not sufficient to convert coiled to collapsed cores is presented in Table 1. Here N^{α}-tosyl-L-lysylchloromethyl ketone, which inhibits the P70 specific protease activity of RLV (12), also prevents the morphological conversion.

Quantitation of the $NaDodSO_4$ gel patterns and enumeration of the different types of cores seen in the 350S band after treatment at 4° and 22°, is presented in Tables 1 and 2. From Table 2, the relative change in protein composition for the core bands exposed at 22° compared to that at 4° showed (average over two experiments) about a 90% decrease in P70. There was a concomitant increase for p40–42 going from 15 to 45% of the

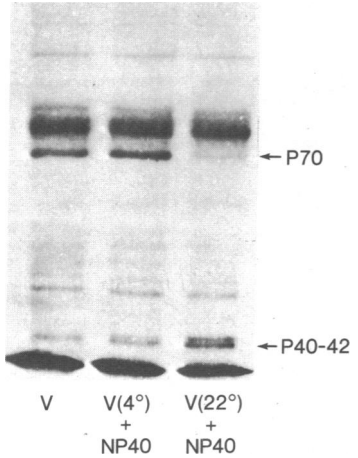

FIG. 7. $NaDodSO_4$/gel electrophoresis (7% gels) of total virus (V) after exposure to NP40 (3:1) for 16 hr at 4° or 22°.

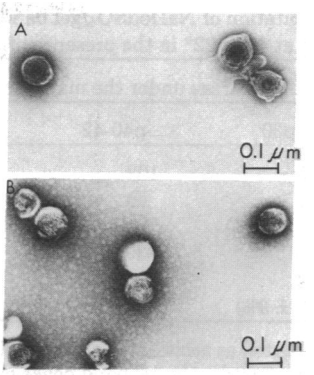

FIG. 8. Electron micrographs of core band fractions from NP40-treated RLV incubated at 4°(*A*) or 22°(*B*).

total staining over all bands measured. This p40–42 band has not been extensively described before and it may be an intermediate cleavage product in morphogenesis of murine leukemia virus. We have found in support of this contention that P70 is cleaved to both p40–42 and p30, when a partially purified P70 proteolytic factor fraction (12) is added to immature cores labeled with ^3H-labeled amino acids. Barbacid *et al.* (13) have observed p40–42 in RLV-infected cells, so that it may be a specific cleavage product *in vivo* as well as *in vitro*. The decrease in coiled and concomitant increase in collapsed forms is shown in Table 1.

DISCUSSION

By the selective exposures of RLV to low [NP40/virus = (0.5–3)] or high (NP40/virus >4) detergent concentrations, we have been able to isolate either immature or mature cores. The immature cores contain P70, which probably corresponds to the

Table 1. Coiled and collapsed cores appearing in core bands after incubation at 4° or 22° in the presence of NP40*

Exp.	% of cores with coiled or collapsed morphology: Coiled	Collapsed	Total no. of cores
1 (4°)	68 ± 6†	22 ± 4	443
2 (4°)	81 ± 3	9 ± 3	233
1 (22°)	17 ± 5	74 ± 2	466
2‡ (22°)	11 ± 5	77 ± 0	285
3§ (22°; TLCK⁻)	21 ± 9	77 ± 11	761
(22°; TLCK⁺)	63 ± 3	36 ± 3	350

* Cores were examined at an electron microscope magnification of ×21,000 or 27,000. A determination was made from prints or on the electron microscope as to whether the internal RNP was concentrically coiled, collapsed, or of indeterminate morphology. By indeterminate forms we mean those particles where the lipid has so covered the core that the stain does not penetrate into the central region. Such forms make up at most about 10% of the particles in any one experiment.

† The averages are for particles counted over two identically prepared grids. A minimum of six nets per grid was counted per experiment.

‡ In this experiment about 33% of the collapsed cores were partially collapsed (see Fig. 8*B*).

§ In Exp. 3, 5 mM N^{α}-tosyl-L-lysylchloromethyl ketone (TLCK) was not$^{(-)}$ or was$^{(+)}$ added before incubation of RLV at 22° in 1% NP40 (low); the cores were anlayzed by $NaDodSO_4$/gel electrophoresis. The samples showed P70 in a cleaved (TLCK⁻), or uncleaved state (TLCK⁺), similar to the amounts seen in Table 2.

Table 2. Quantitation of $NaDodSO_4$/gel bands from cores incubated at 4° or 22° in the presence of NP40*

Exp.	Relative areas under the major protein bands†		
	p30	p40-42	P70
1 (4°)	125	103	258
2 (4°)	377	45	148
	(46 ± 20%)	(15 ± 7%)	(40 ± 14%)
1 (22°)	283	339	25
2 (22°)	534	315	12
	(53 ± 9%)	(45 ± 8%)	(3 ± 1%)

* The units are presented as arbitrary Coomassie blue-staining units calculated from areas under the gel band peaks made from Helena Quick Scan tracings. Each point is the sum of areas taken from core peak fractions 7, 8, and 9.

† The percents are the average ±SEM over the two experiments. The sum of p30 + (p40-42) + P70 is taken as 100%.

major core precursor polyprotein described by other laboratories (11–14), a concentrically coiled RNP interior component, and lipid-like protuberances on the perimeter of the shell. Mature cores lack P70, have a collapsed interior component, are about 50–100 Å smaller, and lack protuberances on the outer shell. After incubation of virus for 16 hr at 22° in the presence of NP40, a proteolytic activity is expressed which leads to the cleavage of P70 and apparently causes the conversion of coiled to collapsed cores. The partially collapsed cores observed in Fig. 8*B* probably represent an intermediate stage in the conversion, since lipid is still retained on the exterior of the core shell.

The protuberances on coiled cores may represent an association of a hydrophobic end of P70 with a fragment of the viral envelope, since [^{14}C]acetate labeling shows the presence of residual lipid in these cores. If p15 is also hydrophobic, then, based on the recent data of Barbacid *et al.* (13), which indicates that P70 (Pr65 in their terminology) is arranged in the sequence NH_2-p15-p12-p30-p10-COOH, we suggest that P70 is also localized from the outside to the inside of coiled cores in this sequence. Cleavage of P70 would then result in p15 remaining in the hydrophobic layer, while p30 takes part in formation of the core shell, and p10 associates with the viral RNA to form the RNP. Since p10 is highly basic (10), such a role for it in forming the RNP is consistent with the need for neutralization of negative charges from the RNA phosphate backbone during morphogenesis. The binding of P70 to the RNA may initiate P70 cleavage, as proposed by Jamjoom *et al.* (11). It would be interesting to isolate the P70 proteolytic factor, so that it could be compared to the intracellular proteases of murine (11) and avian (15) virus-infected cells.

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