9

Assembly of *Retroviridae*

I. Introduction

Retroviruses are widely distributed among vertebrate species, can be transmitted horizontally and vertically, require RNA-directed DNA synthesis for their replication, and are natural agents of oncogenesis in several species (Bishop, 1978). The virion is about 100 nm in size, contains a coiled strand of RNA complexed to proteins (NC), which forms a crescent structure during budding and a dense core after release, and has an envelope bearing clusters of spikes or knobs (Fig. 9-1). The most extensively studied retroviruses are of avian and murine origin, but retroviruses also exist in feline and bovine species, as well as in primates. The prototype avian retrovirus is avian sarcoma virus (ASV), whereas there are two prototypes of murine retroviruses, murine leukemia virus (MuLV), and mouse mammary tumor virus (MMTV). There are also retroviruses in sheep called "lentiviruses"; one of them is visna virus which causes a slow encephalitis in sheep (Haase, 1975). The prototype non-human primate virus is MPMV (Mason-Pfizer monkey virus). More recently, a new group of retroviruses has been isolated from human T cell leukemia (reviewed in Gallo and Wong-Staal, 1982). Since less information is available on the molecular structure and synthesis of primate retrovirus polypeptides, they will not be covered in the first section of this chapter. In most cases, retroviruses are not cytopathic and do not have dramatic effects on cellular metabolism.

We will first briefly review the molecular organization of avian and murine retroviruses, and then the assembly events and mechanisms. The reader is referred to extensive reviews on the subject for more details (Bishop, 1978; Varmus, 1982; Dickson *et al.*, 1982). As in the review of Bolognesi *et al.* (1978), we will attempt to present a unifying view of retrovirus assembly in spite of the well-known differences in the site of NC assembly and virion structure existing between subgroups of this large family.

II. Molecular Organization

Retroviruses have a diploid genome, *i.e.* two identical single-stranded, positive-sense RNA molecules, which are capped and polyadenylated, are found within each
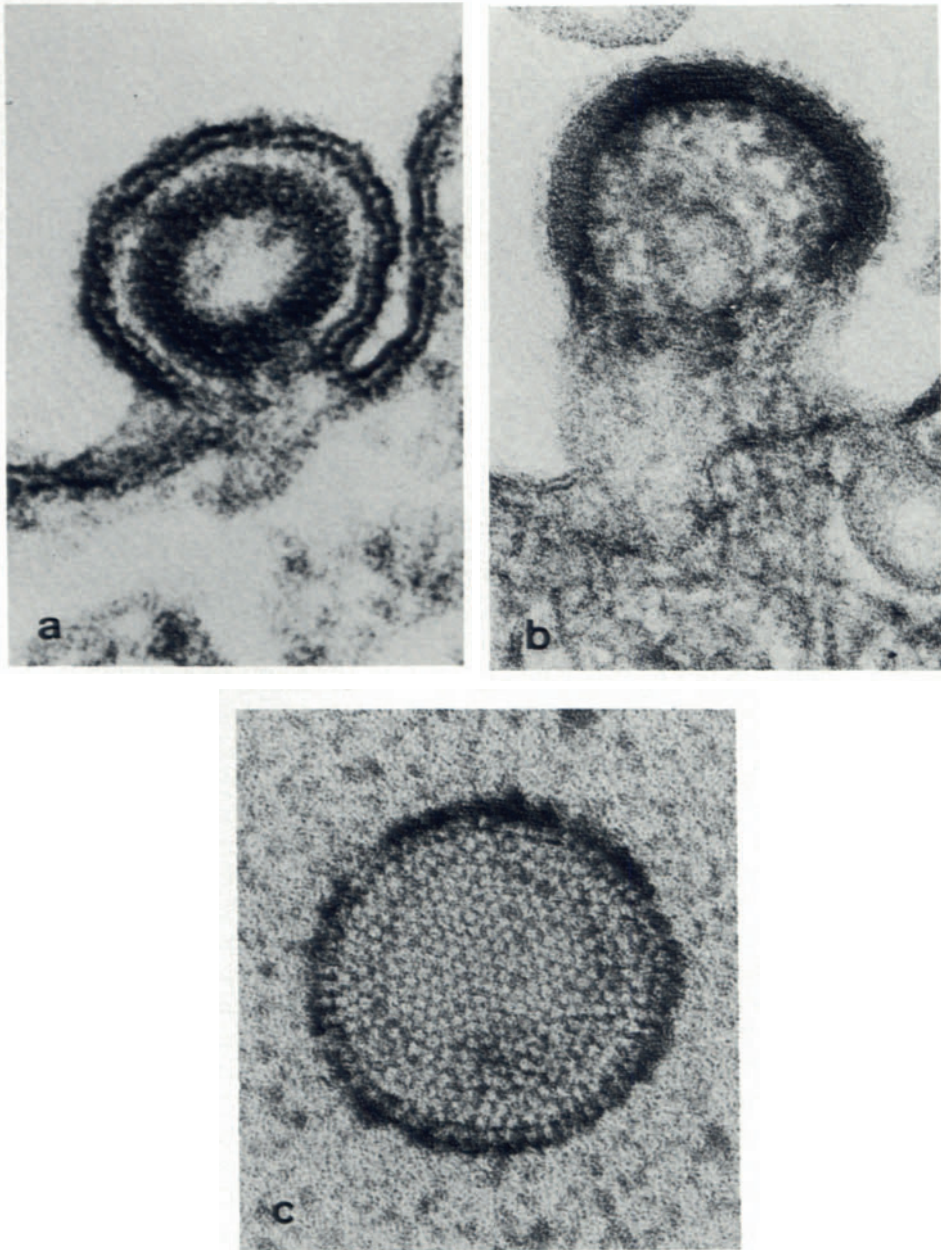
Fig. 9-1. Retrovirus particles budding (a and b) or released from the cell (c). *(a)* Cell infected with Friend leukemia virus. The outer and inner leaflets of the plasma membrane are resolved and in continuity with the leaflets of the virus envelope. The dense core is separated from the virus envelope by an electron-lucent layer. The core is forming a ring, because the virus is almost detached from the cell. *(b)* Visna virus particles budding from a sheep choroid plexus cell. In this case, the dense core is still growing and has the shape of a crescent closely apposed to the unit membrane which displays distinct spikes. *(c)* Mouse mammary tumor virion after negative staining with phosphotungstic acid at neutral pH. Distinct spikes are seen which are sometimes organized hexagonally. Magnifications: (a) ×232,000, (b) ×190,000, (c) ×285,000. [Courtesy of (a) Dr. E. de Harven from De Harven, 1974; reproduced with permission of Academic Press, New York; (b) Dubois-Dalcq *et al.,* 1979 b; reproduced with permission of Academic Press, New York; (c) Dr. C. M. Calberg-Bacq]

virion. This is a unique phenomenon among the known animal viruses, and it facilitates formation of heterozygotes and genetic recombination. Each single-stranded RNA molecule has a short segment at its 5′ end where hydrogen bonds link the two subunits together (Bender and Davidson, 1976). Viral RNA has redundancies (R) at both 5′ and 3′ ends. During the replicative cycle, parental RNA is transcribed by reverse transcriptase *(pol)* into virus-specific DNA (Fig. 9-2) (Varmus, 1982; Hughes, 1983). Synthesis of the first negative DNA strand is initiated by a host tRNA primer which is bound close to the 5′ end of the genome RNA. Reverse transcription produces a linear form of virus DNA which is longer than the haploid subunit of the virus genome. This occurs by the transfer of a nascent DNA strand twice between templates and the fusion of unique sequences (U) at both ends of the genomic RNA (U3 and U5) during reverse transcription (Fig. 9-2). Unintegrated linear virus DNA molecules have long terminal repeats (LTRs) with the sequence U_3-R-U_5 at both ends. LTRs carry regulatory signals, such as a transcription initiation site and a poly(A) addition site. Before virus DNA is integrated into the host cell genome, it exists in the form of circular duplexes, containing either one or two LTRs. Integrated linear virus DNA (provirus) serves as the template for synthesis in the nucleus of virus genomic RNA and of virus messenger RNAs from which virus proteins are synthesized (Fig. 9-2).

The haploid subunit of single-stranded genomic RNA contains three genes necessary for replication of infectious virus. They are, from the 5′ end, *gag,* which
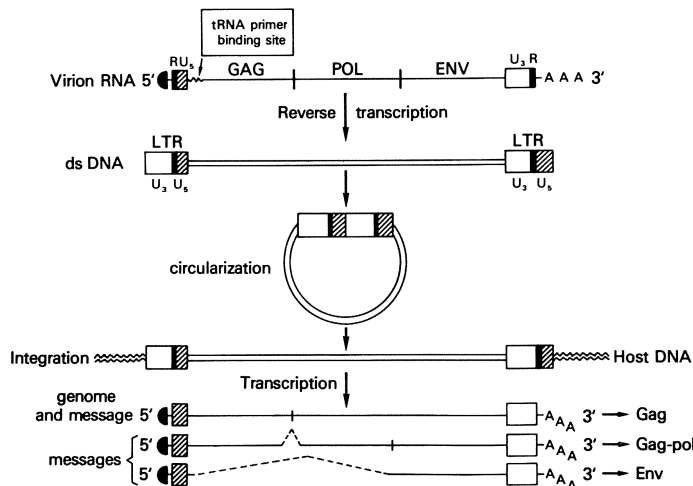

Fig. 9-2. Representation of retrovirus replication and transcription. The virion RNA is capped at its 5′ end and polyadenylated at its 3′ end and contains the three replicative genes, GAG, POL and ENV. There is a short sequence repeated at each terminus called R. A sequence unique to the 5′ terminus is called U5 while a sequence unique to the 3′ terminus is called U3. After reverse transcription, the double stranded DNA has at each end a long terminal repeat or LTR composed of U3, R, and U5. This DNA then becomes a supercoiled circular structure which contains one or two LTR sequences (a circle with two LTRs is represented). The provirus DNA then becomes integrated in the host DNA. Transcription of the provirus DNA results in the formation of new virus genomes and mRNAs. The messages molecules for the structural genes are indicated. The dashed lines in the mRNA diagrams indicate regions spliced out to create subgenomic mRNA molecules. In some cases, the *pol* mRNA is thought to contain a splice site near the *gag-pol* junction. Modified from Dickson *et al.* (1982) and Varmus (1982)

Table 9-1. *Proteins of* Retroviridae: *The gag Molecule and its Products*[1]

	Precursor		*gag 1* Lipid binding, hydrophobic (amino-terminal)	*gag 2* Phosphoprotein, binding to genome	*gag 3* Core shell protein	*gag 4* Basic, RNA- binding	*gag 5* Protease (carboxyl-terminal)
Avian C Prototype, ASV	p76		pp19	p10	p27	p12	p15
Murine C Prototype, MuLV	p65		p15	pp12	p30	p10	?
Murine B Prototype, MMTV	p77		p10	pp21	p28	p14	?

[1] From Dickson *et al.*, 1982.

codes for the core proteins antigens, *pol,* which codes for reverse transcriptase, and *env,* which codes for the envelope glycoproteins (Fig. 9-2). Both *gag* and *env* antigens may be group- or type-specific. The order of these three genes seems invariant among retroviruses. A cellular transforming gene may be added to these genes by a recombination event between transforming and non-transforming strains. More commonly, the acquisition of a host cell transforming gene is done at the expense of virus genetic information.

Virus Proteins

We will now summarize the structure of each protein encoded by the replicative genes and their putative functions. For the purpose of clarity, the *gag* and *env* products, which have similar properties in the prototype viruses ASV, MuLV and MMTV, have been given numbers (Tables 9-1, 9-2, and 9-3).

(1) *gag* (Table 9-1). The primary product of translation of the *gag* gene is a precursor polyprotein (p76^{gag} in ASV, p65^{gag} in MuLV, and p77^{gag} in MMTV), which are cleaved into four or five core proteins. In addition, in MuLV, there is another glycosylated *gag* polyprotein, gp80 *gag* (see section III, p. 158). The cleaved *gag* proteins of ASV, MuLV, and MMTV are listed in Table 9-1 and numbered 1 to 4 or 5 from the amino terminus to the carboxyl terminus. The fifth protein is a virus-encoded protease highly specific for p76 *gag,* which has been found only in avian retroviruses (Pepinski and Vogt, 1983). The *gag* polyprotein probably becomes phosphorylated while still in a precursor form (Bishop, 1978; Bolognesi *et al.,* 1978) and this phosphorylation increases the structural stability of the core (Durbin and Manning, 1982). Within the *gag* polyproteins, one can distinguish four to five domains between the amino- and carboxyl-termini. The first domain corresponds to the hydrophobic membrane protein, the second to the phosphoprotein, the third to the core shell protein, the fourth to the basic RNA-binding protein and the fifth to the virus protease (Barbacid and Aaronson, 1978; reviewed in Dickson *et al.,* 1982). The pp19 of ASV has both lipid-binding and genome-binding properties (*gag* 1).

Table 9-2. *Proteins of* Retroviridae: *The* gag-pol *Molecule and its Products*[1]

	Precursor in cytoplasm	Intermediates at plasma membrane and immature virion	Final product *pol* in mature virions
Avian C (prototype, ASV)	Pr180	Pr130	two subunits $\alpha = 58$ K $\beta = 92$ K*
Murine C (prototype, MuLV)	Pr180	Pr150, 140	p80
Murine B (prototype, MMTV)	Pr160	Pr130	p100

[1] From Dickson *et al.,* 1982.
* Phosphorylated.

(2) *pol* (Table 9-2). MW = 160 to 180. An essential part of all retroviruses is the RNA-dependent DNA polymerase (reverse transcriptase) or *pol* (20 to 70 copies/virus), which is also responsible for the specific incorporation of the primer tRNA necessary for the initiation of DNA synthesis (Fig. 9-2). The reverse transcriptase is apparently expressed by uninterrupted translation from *gag* and *pol* in the 38S mRNA. A splicing mechanism which removes the termination codon at the end of the *gag* gene generates a *gag-pol* mRNA that lacks the *gag* termination signals (Fig. 9-2). p180$^{gag-pol}$ is the product of that read-through translation and contains the antigenic determinants of both *gag* and *pol* proteins. This p180$^{gag-pol}$ cannot be a precursor to any of the mature *gag* gene products but it is the precursor of the active reverse transcriptase found in mature virions. In avian retroviruses, the mature enzyme is a bimolecular complex with two non-identical subunits, α and β, while the murine polymerases consist of only one polypeptide (Table 9-2). Synthesis of the precursor p180$^{gag-pol}$, at low levels probably serves to mediate the incorporation of a small number of polymerase molecules into the virion (Bolognesi, 1978). There are about 50 *gag* molecules for one *pol* molecule in the virion.

(3) *env*. The *env* product is a polyprotein precursor Pr92 in ASV; Pr80 to 90 in MuLV; gPr73 in MMTV, which yields the envelope glycoproteins gp85 and gp37 in ASV, gp70 and p15E in MuLV, and gp52 and gp36 in MMTV (Table 9-3). In both avian and murine retroviruses, the smaller of the two cleavage products is the one anchored in the cell membrane (spike anchor or *env* 2), while the larger one makes the body of the spike and knobs (surface spike or *env* 1) and is attached to *env* 2 by disulfide bonds (Fig. 9-3, Table 9-3). The smaller product is often more highly conserved, although many type-specific variations have been observed in MMTV-gp36 (Calberg-Bacq *et al.*, 1981). In avian retroviruses, gp85 and gp37 can form a disulfide-linked dimer or tetramer (Ewert and Halpern, 1982). In MuLV, gp70 is linked to p15E not only by disulfide bonds but also by noncovalent associations (Montelaro *et al.*, 1978). A model for the membrane orientation of p15E and its interactions with gp70 has been proposed (Fig. 9-3) (Pinter and Honnen, 1983). The
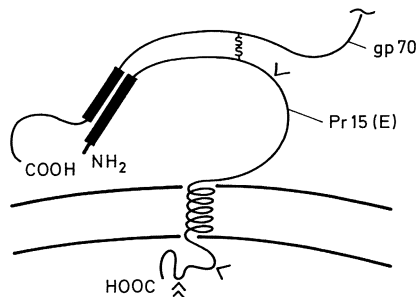

Fig. 9-3. Diagram of structural features of the two envelope proteins of MulV, gp70 and p15E. Arrowheads represent sites sensitive to trypsin cleavage. The double arrowheads represent the natural cleavage sites used in the late processing of pr15E to p15E. The carboxyl-terminal non-polar region of p15E is represented traversing the membrane in a helical conformation. The heavy lines represent non-polar regions at the aminoterminus of p15E and carboxyl terminus of gp70, which may be noncovalently associated. In addition, gp70 and p15E are linked by disulfide bonds. (Courtesy of Dr. A. Pinter, from Pinter and Honnen, 1983; reproduced with permission of ASM Publications, Washington, D.C.)

Table 9-3. *Proteins of* Retroviridae: *The* env *Molecule and its Products*[1]

	Precursor	env 1 Surface spike protein	env 2 Spike-anchoring protein
Avian C (ASV)	Pr92	gp85	gp37
Murine C (MuLV)	Pr80–90	gp70	pl5E*
Murine B (MMTV)	gPr73	gp52	gp36

[1] From Dickson *et al.*, 1982.
* Nonglycosylated.

various domains of MuLV gp70 have been analyzed recently using monoclonal antibodies (Pinter *et al.*, 1982). These studies demonstrate that gp70 contains structurally distinct amino- and carboxy-terminal domains with multiple disulfide bonds within but not between these domains. In MMTV, three gp52 and three gp36 molecules make a trimeric structure and a rosette pattern resembling the prominent spikes of MMTV (Dickson *et al.*, 1982) (Fig. 9-1c). Using monoclonal antibodies to MMTV gp52, it was demonstrated that the three patterns of antigenic reactivity (type-, class, and group-specific) were related to individual determinants on the gp52 molecule (Massey *et al.*, 1980).

III. Intracellular Synthesis of Virus Components

We describe here the site of synthesis of the virus precursors, as well as their transport to the site of assembly and their cleavage or post-translational modification. The precursor, *gag*, is synthesized on free ribosomes from the 28S mRNA and is found diffusely throughout the cytoplasm of MuLV-infected cells when viewed by immunofluorescence (Satake and Luftig, 1983) (Fig. 9-4a). In contrast, the core shell protein, p28, of MMTV is detected in patches and inclusions in infected cells of the mouse mammary gland (Kozma *et al.*, 1979) (Fig. 9-5). However, one should realize that the detection of a *gag* product by immunological methods does not necessarily mean that this product has already been cleaved from its precursor.

    Many observations suggest indeed that *gag* cleavage occurs mostly at the assembly site at the plasma membrane. For instance, in ASV infected cells, protease p15 (*gag* 5) is only detected at sites of virus assembly under the plasma membrane, suggesting that *gag* cleavage occurs only at the membrane (Vogt *et al.*, 1979). Avian cells contain a cellular protease which cleaves p76^{gag}, allowing the release of p15, which will then cleave the other products (Vogt *et al.*, 1982). Such a protease is not present in mammalian cells which, therefore, do not support good replication of avian retroviruses. Once the avian virus protease, p15, is activated, it cleaves the resulting *gag* intermediate at two to three sites (Fig. 9-6). It is thought that two cellular proteases might be involved in the cleavage of MuLV *gag* (Dickson *et al.*, 1982). Viral protein kinase phosphorylates p65^{gag} at the site of ppl2 (*gag* 2) and triggers the cleavage into two intermediates (Yoshinaka and Luftig, 1982) (Fig. 9-6). There are at least four protease sites in p65^{gag} and one is very near the carboxyl ter-

minus of p15 (*gag* 1), which may thus be a possible *in vivo* cleavage site (Yoshinaka and Luftig, 1981). There is also a protease in MMTV, but its identity with one of the known virus proteins has not been established (Dickson, 1982). Cleavage of p77^{gag} is sequential, releasing first *gag* 1, then *gag* 2 and finally cleaving *gag* 3 and 4 (Fig. 9-6). An exception to the membrane cleavage of *gag* polyprotein is seen with visna virus: the core shell protein, p30, is detected in large amounts in the cell cytoplasm, suggesting that some cleavage of the *gag* precursor occurs inside the cell (Vigne *et al.*, 1982).
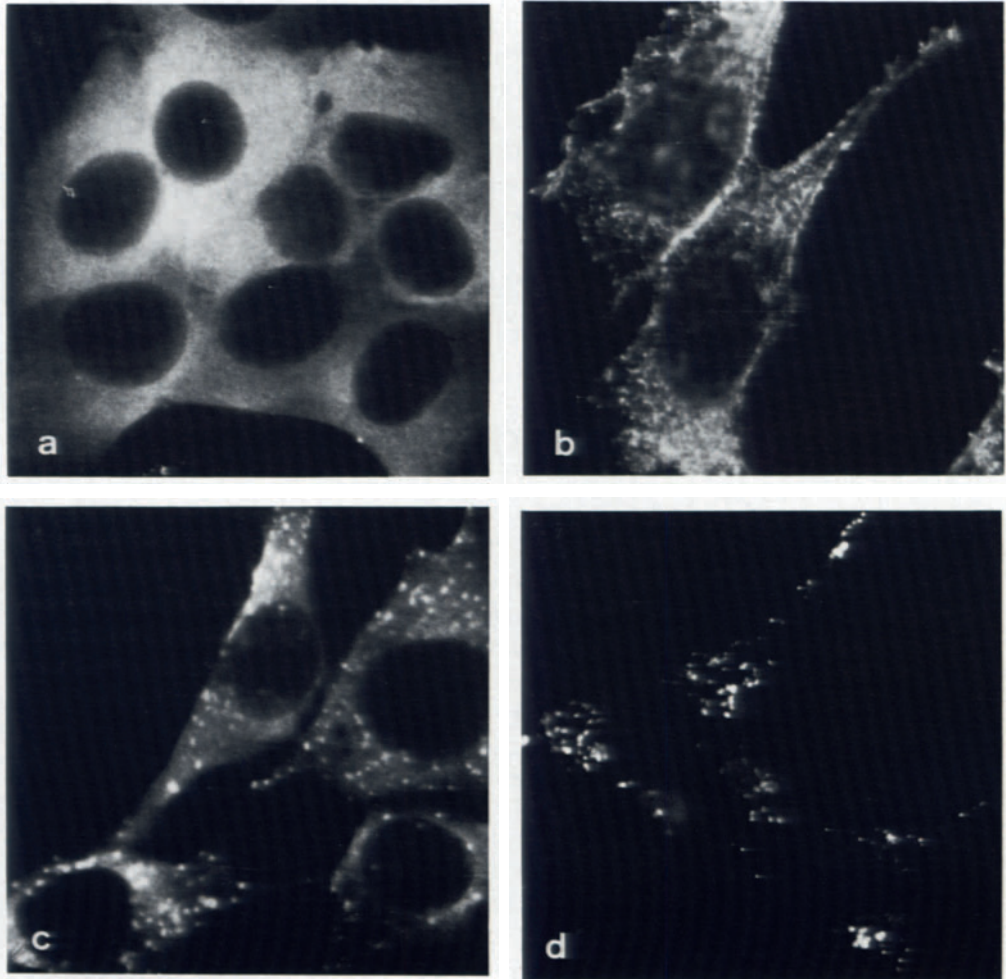

Fig. 9-4. Immunofluorescent staining of four proteins of MuLV after formaldehyde fixation. *(a)* Indirect labeling was performed using as the first antiserum anti-p30. *(b)* Labeling with anti-gp70. *(c)* Labeling with anti-p15. *(d)* Labeling with anti-p15E. The core shell protein, p30, is diffuse throughout the cytoplasm, while all the other proteins are present as fine dots and clusters close to the membrane. Colocalization of p15 and p15E at the membrane level was demonstrated in other experiments. Magnification: ×1290. (Courtesy of Dr. R. B. Luftig, from Satake and Luftig, 1983; reproduced with permission of Academic Press, New York)
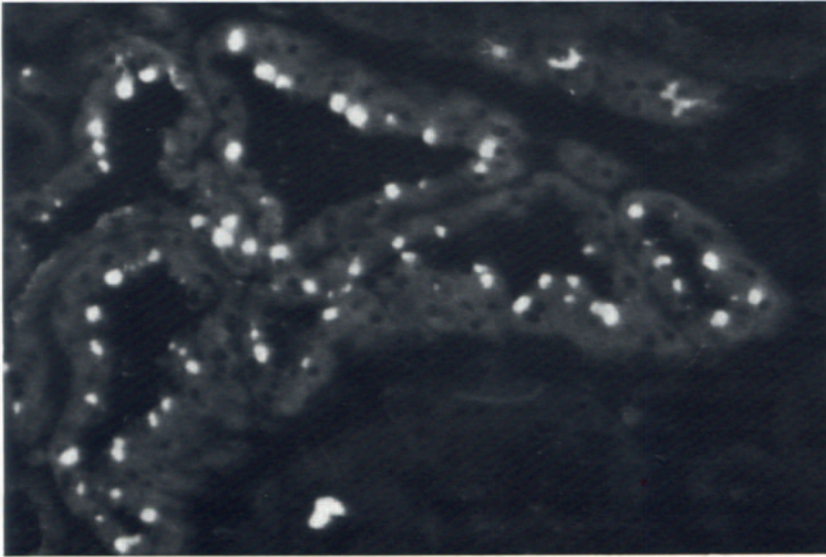
Fig. 9-5. Immunofluorescent localization of the core shell protein, p28, of MMTV in the mouse mammary gland in lactation. This *gag* gene product is clearly located in inclusions inside the epithelium. Magnification: × 360. (Courtesy of Dr. S. Kozma, from Kozma *et al.,* 1979; reproduced with permission of Cambridge University Press, England)
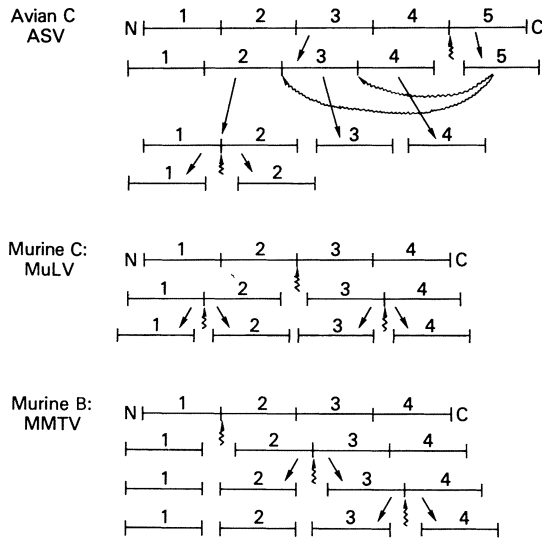

Fig. 9-6. Representation of *gag* cleavage occurring in the three prototype viruses ASV, MuLV, and MMTV. The *gag* products are named 1 to 5 from the amino terminus to the carboxyl terminus, as shown in Table 9-1. ASV has a virus-encoded protease, *gag* 5, which is cleaved by an unknown host cell protease and then activated to cleave the other part of *gag,* at least at two sites. *gag* 1 and 2 are further separated by an unknown protease. Enzyme cleavage sites are indicated by the zig-zag arrows. Cleavage of MuLV occurs in a more symmetrical way and two different proteases, one probably viral, the other probably cellular, appear to be involved. MMTV cleavage occurs in a cascade from the amino terminus toward the carboxyl terminus of the *gag* molecule; no virus-encoded proteases have been identified yet (modified from Dickson *et al.,* 1982)

In MuLV-infected cells, there is a second *gag* protein which is glycosylated (gp80 *gag*) and synthesized independently from p65 *gag*, probably because two different initiation sites in MuLV RNA give rise to the two polyproteins. Virus-specific 28S mRNA associated with free ribosomes might synthesize p65^{gag}, while membrane-bound 38S RNA synthesizes the precursor of gp80 *gag* (Schultz *et al.,* 1981; Dickson *et al.,* 1982). Glycosylated *gag* is not phosphorylated like p65^{gag}, but contains sugar residues and an additional peptide sequence at the amino terminus is not found in p65^{gag}. Such a sequence may function as signal peptide. Additional glycosylation may occur during transport through the Golgi to the cell surface, where the protein might be shed from the cell, probably as cleavage products. Association of gp80 *gag* with the cell surface and with extracellular matrix components has been described (Edwards *et al.,* 1982) and may alter the growth of the cell. Thus, this polyprotein is not present in the virion and is probably not involved in its assembly.

The read-through product *gag-pol* is also synthesized on free ribosomes and cleavage into intermediates occurs at the plasma membrane and in immature virions (Dickson *et al.,* 1982) (Table 9-2). However, the final cleavage necessary for transcriptase activity only occurs during virion maturation after release (see below). This may prevent the enzyme from making transcripts of cytoplasmic RNA inside the infected cells.

In contrast to *gag*, *env* is synthesized on membrane-bound ribosomes inserted in the rough ER membrane and cleaved within the cell. *env* is probably transported to budding sites in vesicles (see Chapter 1). In ASV, unglycosylated pr63^{env}, synthesized on membrane-bound ribosomes, has a hydrophobic signal sequence at the start of the molecule which is removed by proteolytic cleavage. Later, the uncleaved precursor Pr92^{env} (the glycosylated form) is found associated with the rough ER and membrane-bound polysomes but not with newly released virus (Hayman, 1978). The polypeptides gp85 and gp37 are the two cleavage products of avian *env* found in cell lysates (Bosch *et al.,* 1982). Thus, glycosylation and cleavage of *env* probably occur simultaneously in the rough ER and the Golgi apparatus. However, Klemenz and Diggelman (1979) claim that almost all cleavage of the glycoprotein precursor of ASV takes place in freshly budded virus particles. Processing of the particles could be a fast but very late process just before release. In that case, the cleavage point may be on the outer side of the envelope and be accessible only to a host membrane protease. It is possible that the site of cleavage varies with the host cell. In MuLV, only the cleaved mature forms of *env* (gp70 and p15E) are accessible to surface membrane probes (Witte *et al.,* 1977). These two cleavage products of *env* have a patchy membrane localization by immuno-fluorescence, shown in Fig. 9-4b and 9-4d (Satake and Luftig, 1983).

Fig. 9-7. Formation of cytoplasmic NCs (A particles) and budding of MMTV in the mammary gland in lactation. *(a)* Accumulation of numerous A particles with their typical ring-shaped cores in the cytoplasm at 15 days of lactation. *(b)* Production of virions at the cell surface at three days of lactation. One NC is apposed to a cytoplasmic vacuole (arrowhead), while the other is seen inside a budding virion (double arrowhead). Inset shows the mature form of the released virus with an eccentric core. Magnifications: (a) ×22,000, (b) ×60,000. (Courtesy of Dr. C. M. Calberg-Bacq)
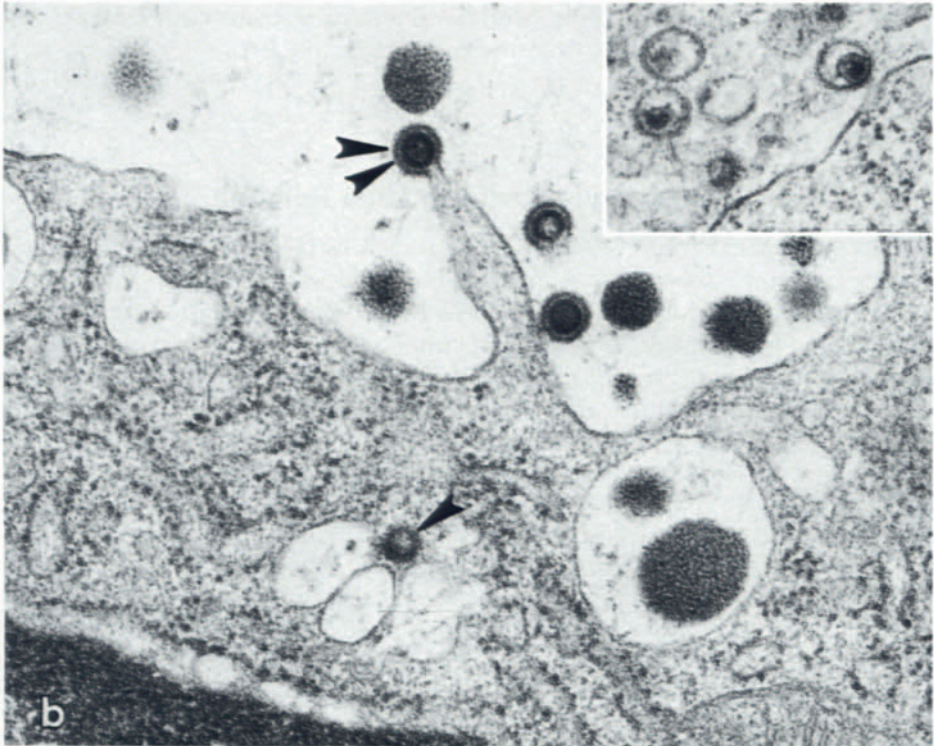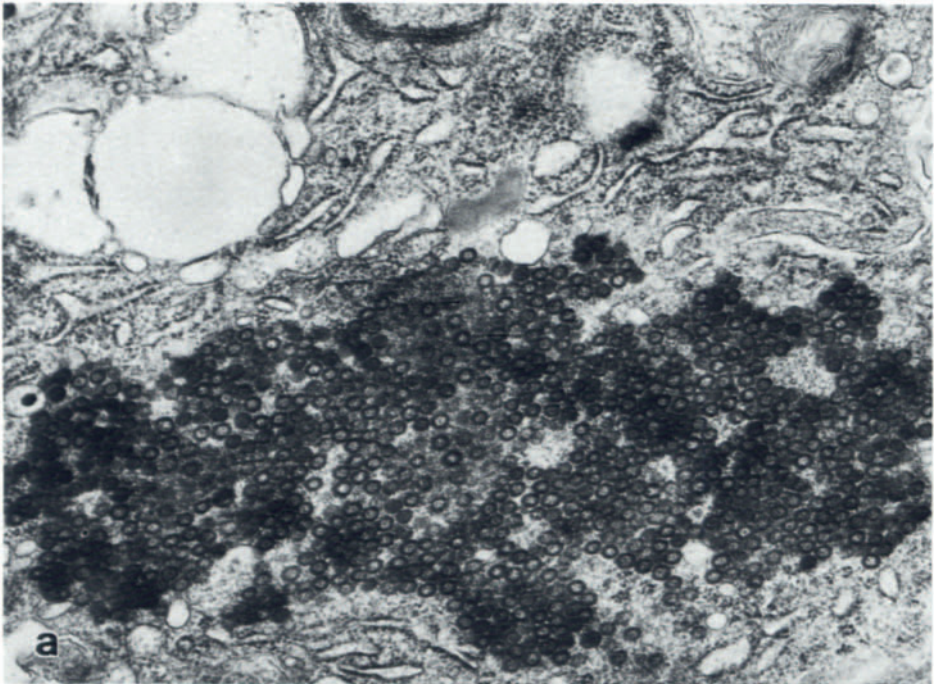
IV. Assembly of Virus Components

Retrovirus assembly has multiple aspects, depending on the types and families. One can distinguish two types: (1) viruses which assemble their NC before interacting with envelope components and budding (Fig. 9-7). These include MMTV (type B retroviruses) and some monkey retroviruses (type D retroviruses, prototype MPMV). (2) viruses which assemble their NC at the site of budding and simultaneously with budding. These are the type C retroviruses such as ASV, MuLV, other murine and avian retroviruses, as well as endogenous primate retroviruses other than MPMV. In these retroviruses, the interaction between *gag* and membrane might be necessary to trigger the final organization of the NC.

The specific case of the intracisternal A particles (IAP) should be mentioned here (reviewed in Schidlovsky, 1977; Kuff *et al.*, 1983). They are found in nonproductive cells of early mouse embryos and many mouse tumors. In contrast to the type C viruses, these particles have their NCs closely apposed to their envelope, and subsequently bud into vacuoles. IAPs are released into cytoplasmic vacuoles but are never released in the extracellular space like type C viruses. They are not known to have biological activity or infectivity, although they contain polyadenylated genomic RNA species and DNA polymerase activity. New observations by Kuff *et al.* (1983) have shown that the DNAs of IAP genetic elements are incorporated into the ϰ light chain gene and thus appear to be movable elements in the mouse genome. Therefore, IAPs are not related to infectious type C virions which only occasionally bud into vacuoles in MuLV and murine sarcoma virus-infected cells (Schidlovsky, 1977) as well as from the cell surface (Fig. 9-8).

NC Assembly Occurs Independently from Budding

MMTV- and MPMV-infected cells show numerous spherical doughnut-shaped structures (70 nm) sometimes organized in inclusions within the cytoplasm (Fig. 9-7a) (Hageman *et al.*, 1981). Assembled NCs have been called intracytoplasmic A particles and form clusters close to cytoplasmic vacuoles. These inclusions can be stained by antibody against the *gag* core protein (see Fig. 9-5). MPMV NCs (or A particles) are similar, except that their outer ring has a more fuzzy appearance than in MMTV.

Earlier work had shown that intracytoplasmic A particles of MMTV contain a large polypeptide (MW: 70 K) precursor of the three major polypeptides found in the budding MMTV particles (Tanaka, 1977). It was later demonstrated that these particles contain $p77^{gag}$ precursor, RNA, and $p100^{pol}$ (reviewed by Nusse *et al.*, 1979), all of the components of the virion except *env*. In addition, when the MMTV *gag* precursor is not cleaved or phosphorylated, virus budding at the membrane cannot occur (Nusse *et al.*, 1979). These observations suggest that interactions between NC and membrane trigger *gag* cleavage and allow virus budding. Once NCs are assembled and form A particles, they migrate to the site of budding at the plasma membrane by an unknown mechanism (Fig. 9-7b).
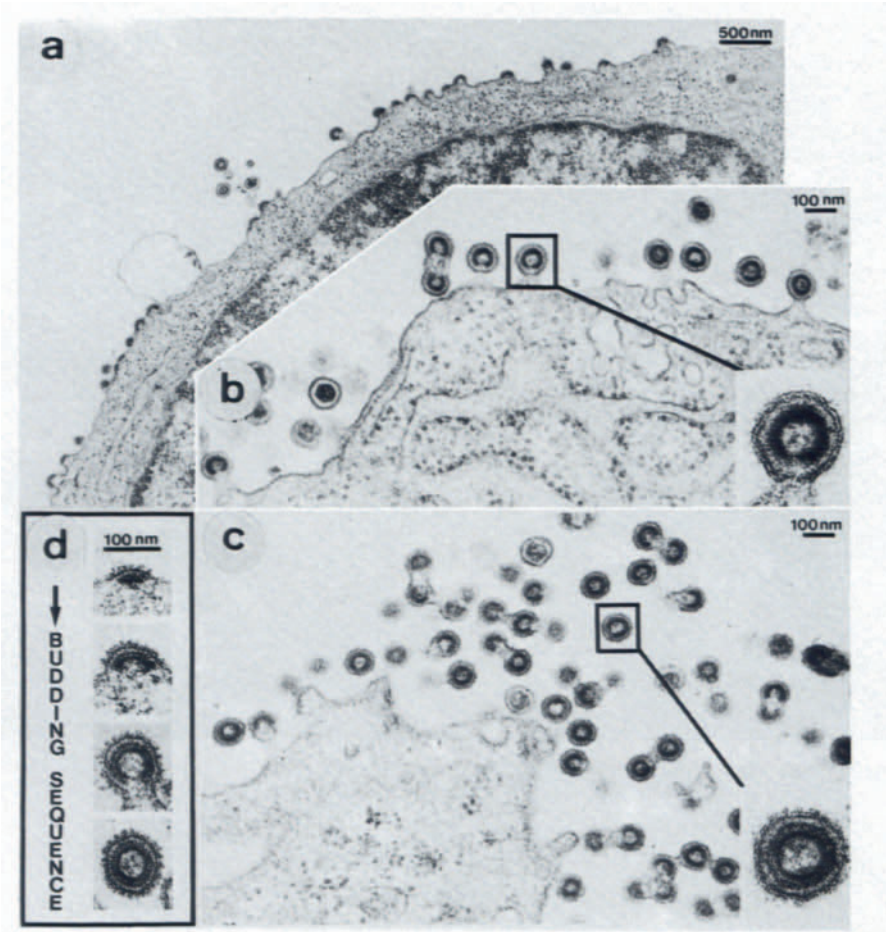
Fig. 9-8. This panel shows a typical leukemia cell producing Friend leukemia virus. *(a)* Numerous particles are seen budding from the cell surface. *(b)* Virions showing no surface projections, a result of treatment with propylene oxyde during embedding. *(c)* and *(d)* Surface projections are readily visible when no propylene oxide is used during embedding. *(d)* The budding sequence, resulting in a immature particle with a circular capsid. (Courtesy of Dr. H. Frank, 1982)

NC Assembly Is Coordinated with Budding

As mentioned earlier, retroviruses which assemble their cores directly at the budding sites are called type C. The budding site and the detailed morphology of the bud vary with the different types of viruses. Type C viruses usually bud at the plasma membrane (Fig. 9-8), but occasionally bud into ER vacuoles or cisterns. The NC then forms a half hollow sphere or crescent during maturation, and this crescent is closely apposed to the bud membrane in visna virus, while it is more distant from the envelope in ASV and MuLV (compare Figs. 9-8 and 9-9c).

The extent of budding, as well as the structural changes in the surface membrane of budding virions, are best seen by scanning electron microscopy and
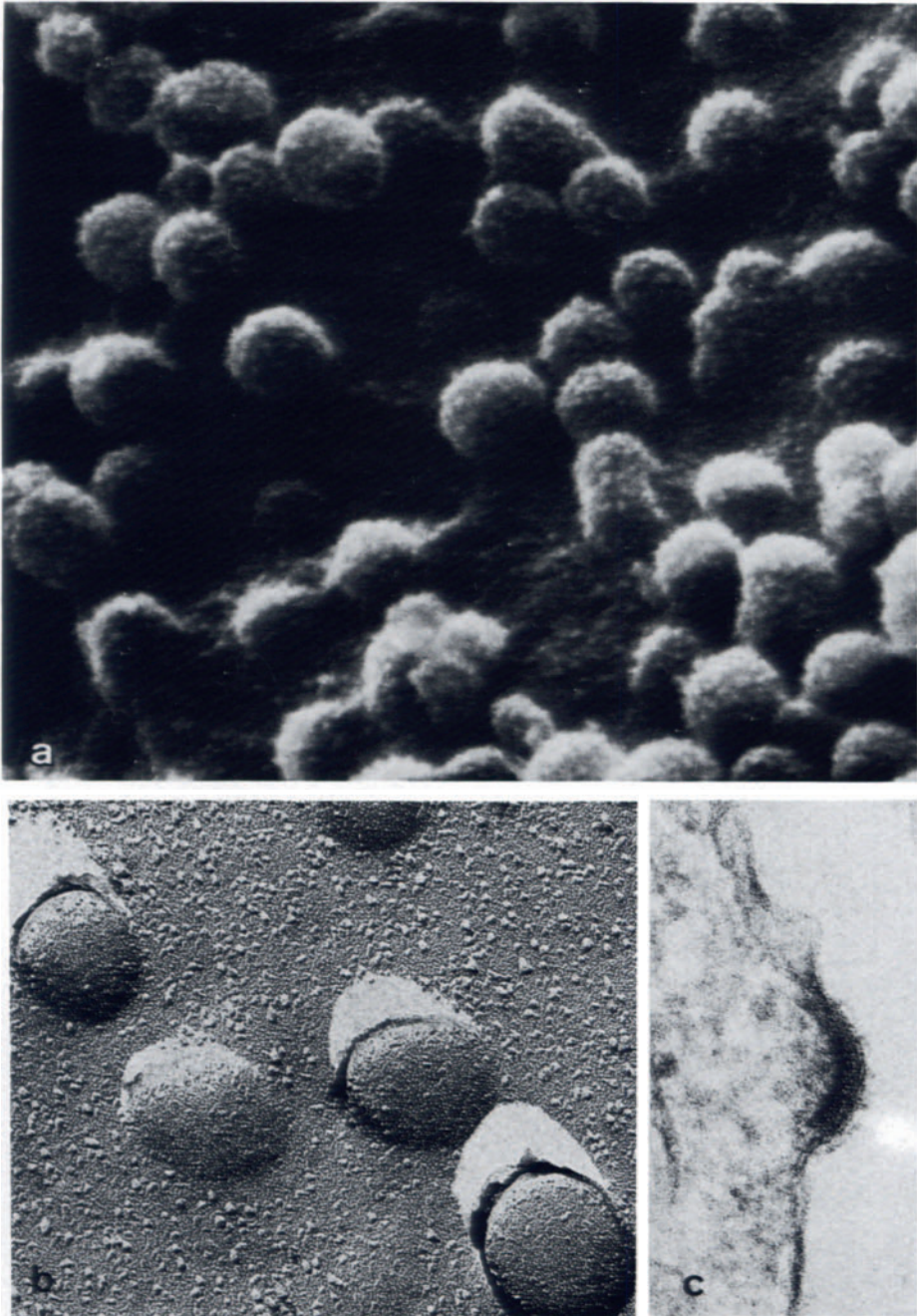
Fig. 9-9. Budding events in sheep choroid plexus cells infected with visna virus. *(a)* High resolution scanning electron microscopy reveals numerous virus buds with distinct knobs, probably corresponding to groups of spikes. *(b)* Early budding sites are detected after freeze-fracture of the membrane of an infected cell. The protoplasmic face is devoid of intramembrane particles (8–13 nm) at the site of budding. *(c)* Thin section of an early bud with its typical crescent-shaped NC directly under the envelope, which is covered with delicate spikes. Magnifications: (a) ×100,000, (b) ×120,000, (c) ×170,000. [(a) From Dubois-Dalcq *et al.,* 1979 a; reproduced with permission of Academic Press, New York]

freeze-fracture techniques (Demsey *et al.*, 1977), as seen in cells infected with visna virus (Fig. 9-9a, b) (Dubois-Dalcq *et al.*, 1976 b). The exclusion of the usual intra-membrane particles from the virus bud may correspond to the exclusion of host membrane proteins. The surface knobs likely correspond to groups of spikes which are visible only where the NC is apposed to the membrane (Fig. 9-9c).

The molecular events underlying virus assembly and budding are not quite elucidated yet. Of the three genes common to all non-defective retroviruses, only the *gag* gene must be functional to allow encapsidation of genomic RNA and packaging and is sometimes called the "particle-making machine" (Vogt *et al.*, 1982; Dickson *et al.*, 1982). The *gag* molecule appears to contain "all the components to achieve a three-dimensional configuration that facilitates assembly" (Dickson *et al.*, 1982).

How is the virus genomic RNA chosen by gag polyprotein for packaging? Most likely the amino-terminal end of *gag* has some of the RNA-binding properties of its cleavage product. These proteins may find more binding sites on virus genomic RNA than on cellular or virus mRNA. There may be a specific site on the genome that interacts with a virion protein to direct RNA packaging specifically (Mann *et al.*, 1983). Retroviruses package full length RNA but not the spliced *env* mRNA (see Fig. 9-2), indicating that an essential signal might be in the region spliced out to form *env* mRNA. A mutant of Moloney MuLV with a deletion of 350 bases between the left long terminal repeat (LTR) and the start of the *gag* codon is also defective in packaging (Shank and Linial, 1980; Watanabe and Tenim, 1982; Mann *et al.*, 1983). So in this case, the key to packaging appears to be a site on the RNA rather than a region encoding a protein needed for packaging.

Interactions Between NC and Envelope Proteins

It has been suggested that *gag* molecules migrate to the cell membrane, bind to the virus genome, and cluster together at virus assembly sites before cleavage occurs (Naso *et al.*, 1982). This would lead to the formation of an electron-dense crescent under the membrane and a partially mature bud prior to cleavage of the *gag* proteins (Witte and Baltimore, 1978). However, membrane association of *gag* has not yet been demonstrated by immunofluorescence (Fig. 9-4a). Close interaction between *gag* molecules may trigger a change in *gag* conformation under the membrane, which may enhance binding to the genome of the phosphoproteins and small basic proteins of *gag*. Clustering of virus components is apparently inhibited in inter-feron-treated cells, because interferon alters membrane structure and function (see Chapter 1) and therefore might hamper the lateral mobility of *gag* and *env* molecules and their cleavage (Naso *et al.*, 1982; Sen and Pinter, 1983).

Virus Budding

As mentioned earlier, cleavage of *gag* appears to be a major event during budding. Cleavage of MuLV *gag* is slightly less efficient than cleavage of avian *gag*. Some, if not all, of the *gag* products may self-associate under the membrane and yield

11*

proteins organized in concentric shells (Pepinski *et al.,* 1980). The core shell protein (*gag* 3) has been localized in concentric dots in the NCs of budding visna virus by electron microscopic immunocytochemistry (Fig. 9-10a). In MuLV, the core shell protein may form higher-order homotypic multimers and the hexon subunits seen in isolated cores (Fig. 9-10b) (Nermut, 1972; Langue *et al.,* 1973). Probably, the core shell protein molecules have some specific associations with the NC. In addition, it is not rare to see radial strands between the outer shell and the envelope, which may correspond to binding sites between core and envelope (Yuen and Wong, 1977).

Generally, the less *gag* is cleaved, the more immature the particle. Observations on murine sarcoma virus (Gazdar MSV) suggest that budding and release may occur with an uncleaved *gag,* but the released virions have a dense crescent close to the membrane in contrast to the normal mature virions which have a central core (see below) (Pinter and de Harven, 1979). Cleavage of *gag* can be triggered *in vitro* and is associated with virion maturation (Yoshinaka and Luftig, 1977). A *ts* mutant of MuLV in which *gag* is not cleaved at the non-permissive temperature yields large amounts of this precursor in the cell and produces submembraneous electron-dense crescents which do not mature to budding particles (Yeger *et al.,* 1978). When these cells are switched to permissive temperature, synchronized budding begins in 2 to 4 min (Yeger *et al.,* 1978). Similarly, a *ts* mutant of ASV yields only atypical viruses with abnormal cores (Rohrschneider *et al.,* 1976). No core shell protein and hydrophobic phosphoprotein were found in these cores, suggesting that the first protein is necessary for core assembly, whereas the second is required for interaction with the membrane.

Once cleavage of *gag* has occurred, the hydrophobic proteins of the amino-terminal end of *gag* associate noncovalently with the spike anchoring protein of *env.* For instance, a fraction of pp19 molecules (ASV) associate with gp37, while p15 (MuLV) associates with p15E (Fig. 9-4c and d). Co-localization of these two proteins at the membrane level has been recently demonstrated by immunofluorescent staining with monoclonal antibodies in MuLV-infected cells (Sataka and Luftig, 1983). The polypeptide pp19 (*gag* 1) in avian retroviruses has been proposed as the mediator of packaging, since it has both lipid- and RNA-binding regions. These two functions are performed, respectively, by *gag* 1 and 2 in MuLV and MMTV. Thus the amino-terminal end product of *gag* may play a role analogous to the M protein of paramyxo-, myxo-, and rhabdoviruses (Simons and Garoff, 1980; Weiss and Bennett, 1980; Weiss, 1980). Studies on pseudotypes between the retrovirus avian leucosis virus (ALV) and the rhabdovirus VSV further support this concept. Indeed, a VSV mutant (*ts* 045), defective in G, the envelope glycoprotein, can acquire retrovirus glycoproteins, suggesting that the M protein of a rhabdovirus can interact efficiently with *env* products. Similarly, a VSV mutant in M (*ts* 31) allows functional assembly of rhabdovirus G into ALV, as if the amino-terminal product of *gag* (pp19) can interact with VSV rhabdovirus G, as well as with retrovirus glycoproteins. However, in this situation, only the retrovirus genome can be rescued, not the rhabdovirus one, probably because hydrophobic *gag* pp19, a phosphoprotein, cannot specifically interact with the VSV genome.

What exactly is the role of *env* glycoproteins in virus budding? Nondefective retroviruses have, in their genomes, information leading to infectious particles.
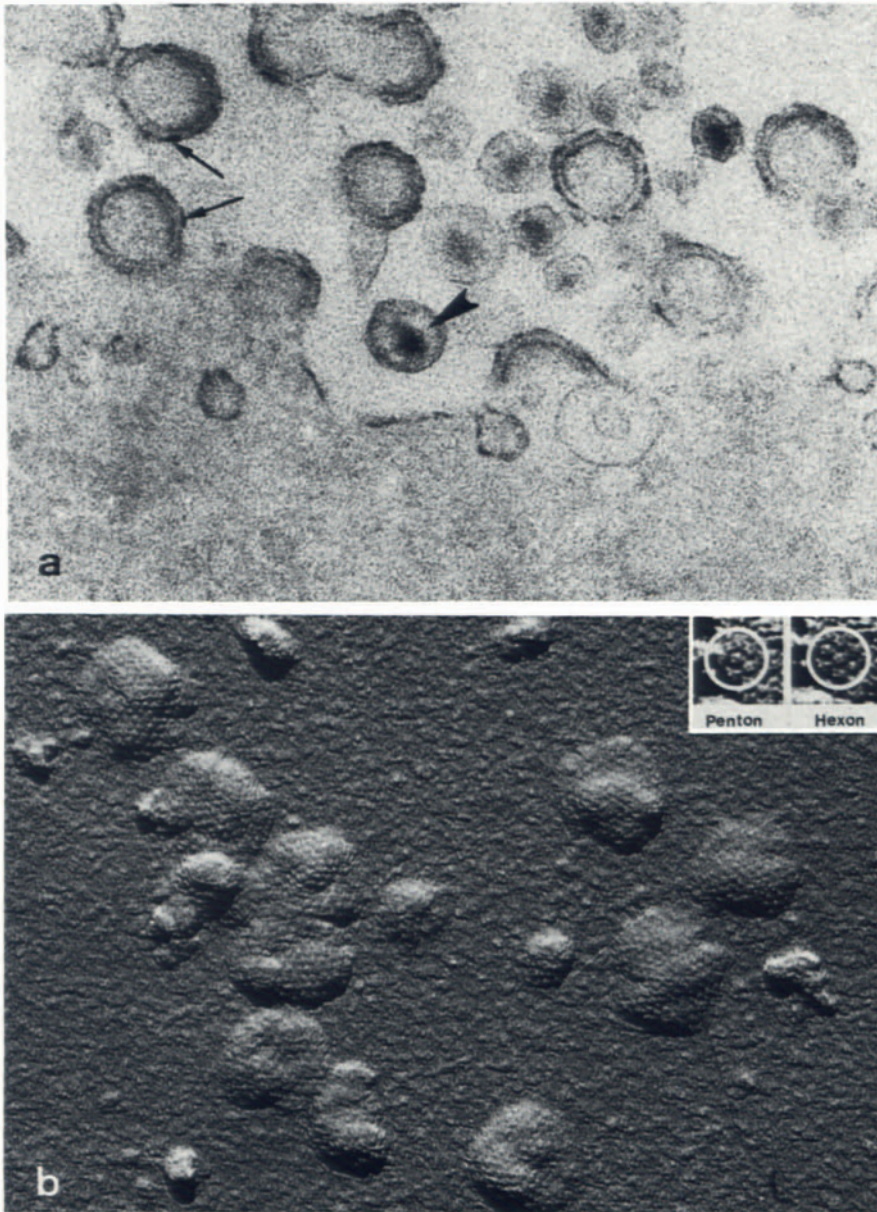
Fig. 9-10. Visualization of the core shell protein of retroviruses. *(a)* Immunoperoxidase labeling of the core shell protein (p30) of visna virus. The section is unstained and a black electron-opaque product corresponds to the localization of the protein. p30 is organized in dots, mostly in the outer part of the virus capsid in virus buds (small arrows). The detached mature viruses are smaller and have a central core, sometimes labeled by the antibody (arrowhead) (no counterstain). *(b)* Isolated cores of Friend leukemia virus after freeze-drying, shadowed with platinum carbon. The cores are covered with hexagonal structures, probably composed of core shell protein, organized in pentons and hexons as shown in the inset. Magnifications: (a) 80,000, (b) 125,000. [(a) from Dubois-Dalcq *et al.,* 1979 b; reproduced with permission of Academic Press, New York; (b) Courtesy of Dr. H. Frank]

They thus code for specific molecular interactions between *gag* and *env* components. The *gag* proteins are sufficient to produce a physical particle and *env* confers infectivity to the virion. Mutants deficient in glycoprotein assembly yield non-infectious particles at non-permissive temperatures (Pinter and de Harven, 1979). It appears that *env* proteins are not essential to virus budding and other markers in the membrane may direct the assembly of *gag* molecules in the *env* mutants (Dickson *et al.*, 1982). Reduced cleavage of the *env* precursor have been observed in ASV mutants with a defect in the glycoprotein (Hardwick and Hunter, 1981). The precursor is found in the virions and some anomalous cleavage occurs in the extracellular space; as a result, the surface spike protein gp85 falls off the envelope. Thus, correct cleavage is essential to make an infectious particle. Similarly, monensin treatment of MPMV-infected cells results in non-infectious budding viruses. Again, the *env* precursor is migrating, but not cleaved, in the monensin-treated cells, and therefore its cleavage products are not inserted into the virion (Chatterjee *et al.*, 1982). Mooren *et al.* (1981) showed that antibody cross-linking of the spike-anchoring protein p15 E inhibits budding of a MuLV *ts* mutant (*gag* cleavage lesion) at permissive temperatures, as if this protein was essential in initiation of budding. In contrast, most molecules of the surface spike glycoprotein gp70 can be cross-linked without inhibiting budding. Thus, although the integrity of the surface spike glycoprotein is not required for budding, that of the spike-anchoring protein may be necessary to this process.

The location of *env* proteins definitely determines the budding site of various retroviruses at the basolateral domains of polarized epithelial cells (Roth *et al.*, 1983 a). The positioning of the cleaved products of *env* in the plasma membrane may help *gag* to find its site of interaction with the spike-anchoring protein. Monensin treatment of MuLV-infected cells blocks entry of the glycoprotein gp70 into the Golgi apparatus (Srinivas *et al.*, 1982). In this case the glycoprotein does not migrate further to the cell surface and the virus buds into vacuoles instead. Thus the site of budding depends at least partly on the presence of *env* in a specific membrane domain.

Virus Release and Post-Release Maturation

Very little is known about the mechanism of virus release. A molecular event, however, has been correlated with release of MuLV (Green *et al.*, 1981 b). Removal of the carboxyl-terminal tail of the spike-anchoring protein p15E at the time of budding may trigger association of the *env* proteins, causing localized pinching of the membrane and virus budding.

Retrovirions undergo further maturation after release (Fig. 9-11c and d). This consists of a shift from a virus with a concentrically coiled internal NC and an electrolucent center, to a collapsed core situated in the middle of the virion (type C and type D, such as MPMV) or eccentrically (type B, such as MMTV). The central core of MMPV is sometimes triangular or tubular in shape. The reasons why mature virions have an eccentric core in some families is not clear; perhaps this may indicate a loss of binding between *gag* proteins in the core and *env* components so
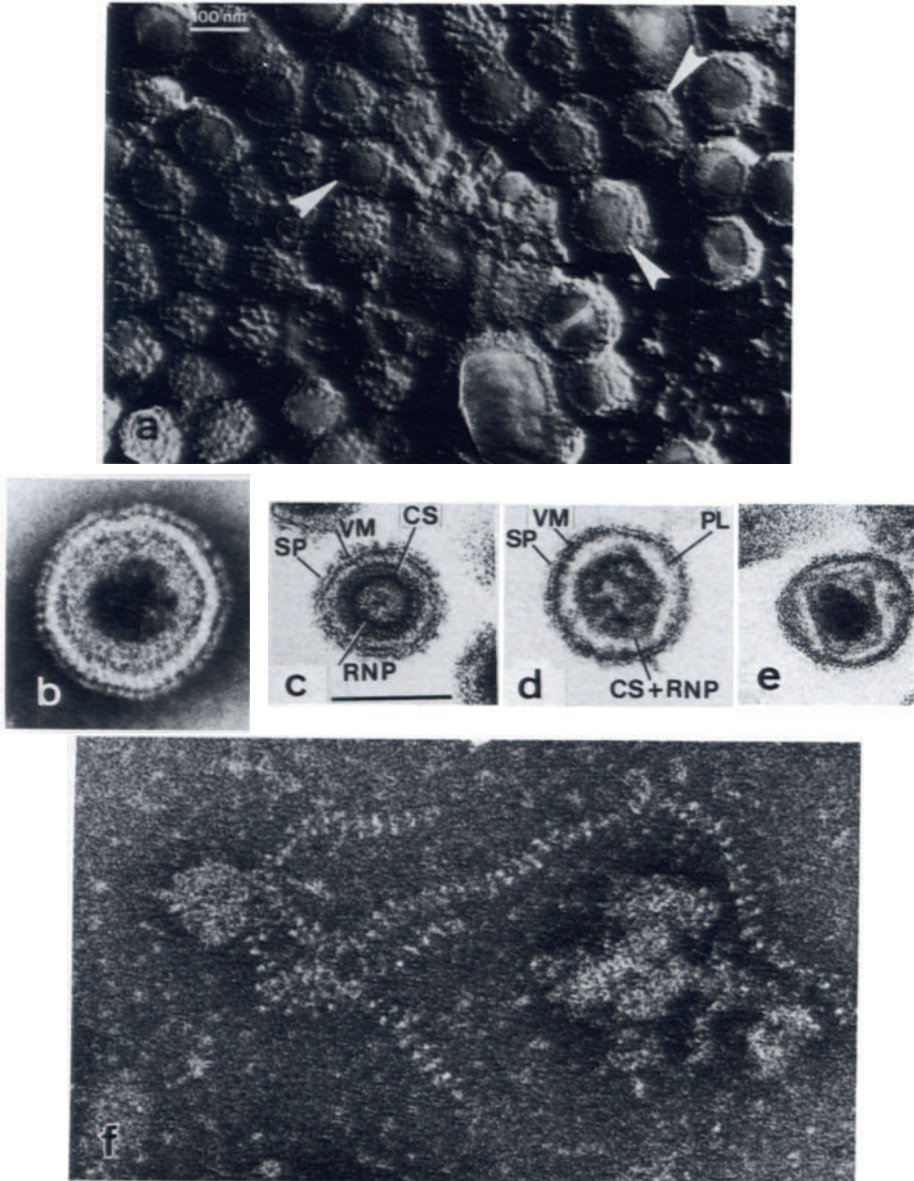
Fig. 9-11. Structure of immature and mature released retrovirions. *(a)* Clusters of Friend leukemia virus particles which have been freeze-dried. The knobs on their surface are very clear and, in some cases, part of the envelope has been lifted away, revealing the core shell under the envelope (white arrowheads). *(b)* Negatively stained immature MMTV after fixation with glutaraldehyde. Note the prominent spikes which appear to be radially connected with the capsid ring. *(c)* and *(d)* Thin sections of immature and mature Friend leukemia virus respectively. *SP* surface projections; *VM* virus membrane layer; *CS* core shell; *RNP* ribonucleoprotein (NC); *PL* protein layer. *(e)* Mature visna virion with a layer between the core and envelope. *(f)* Negatively stained NC with helical symmetry released from Friend leukemia virus after treatment at pH 3.5. Uranyl acetate staining. Magnifications: (b) × 227,000, (c) and (d): bar = 100 nm, (e) × 120,000, (f) × 220,000. [(a), (c), (d) and (f) courtesy of Dr. H. Frank, and (b) of Dr. C. M. Calberg-Bacq]

that the core is free to move in various positions inside the envelope. Alternatively, eccentric cores may have a point of attachment to the envelope (type B). On the other hand, viruses with a central core must have some way to regulate the central position of the NC (type C and type D). It is thought that immature particles are more ordered and stable structures than mature particles, since immature particles have a fuzzy skeleton material extending between core and envelope (Hageman *et al.*, 1981). It is only in the released particle that the polymerase becomes active after cleavage from the *gag-pol* precursor. This is why free virus shows an increase in reverse transcriptase activity and infectivity (Lu *et al.*, 1979).
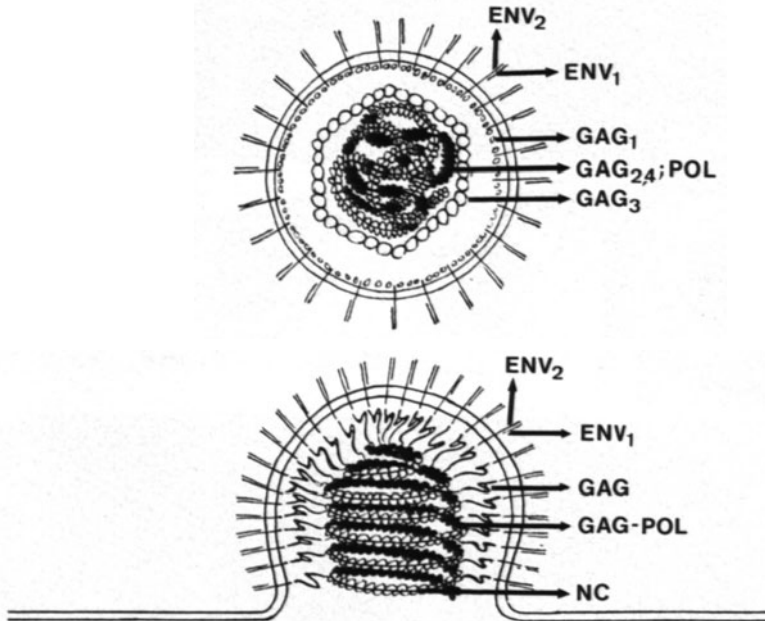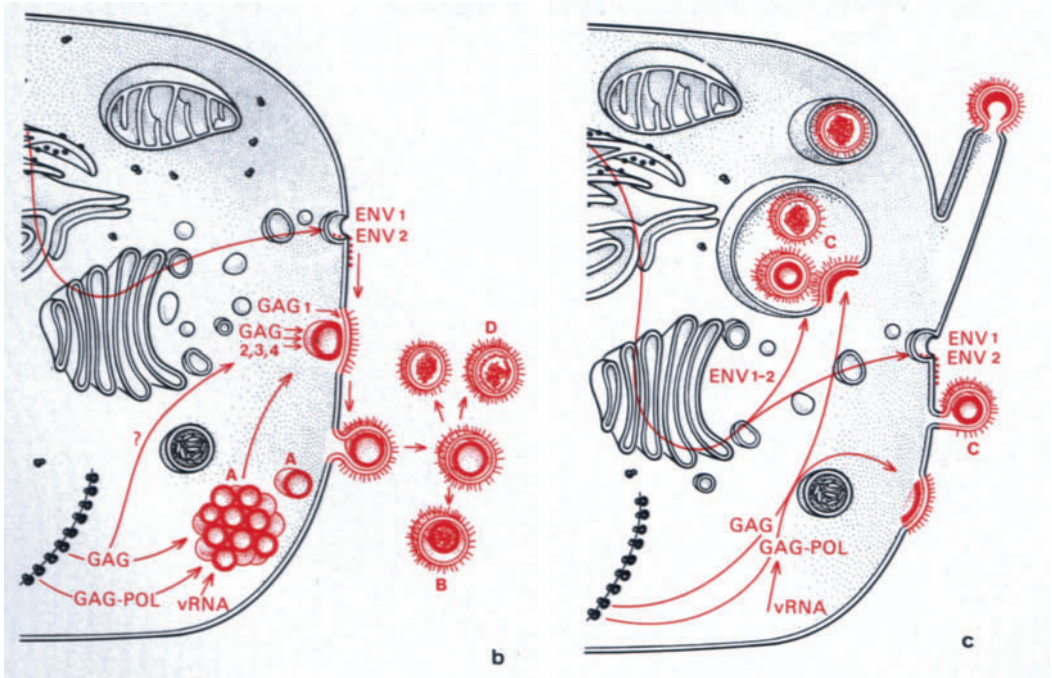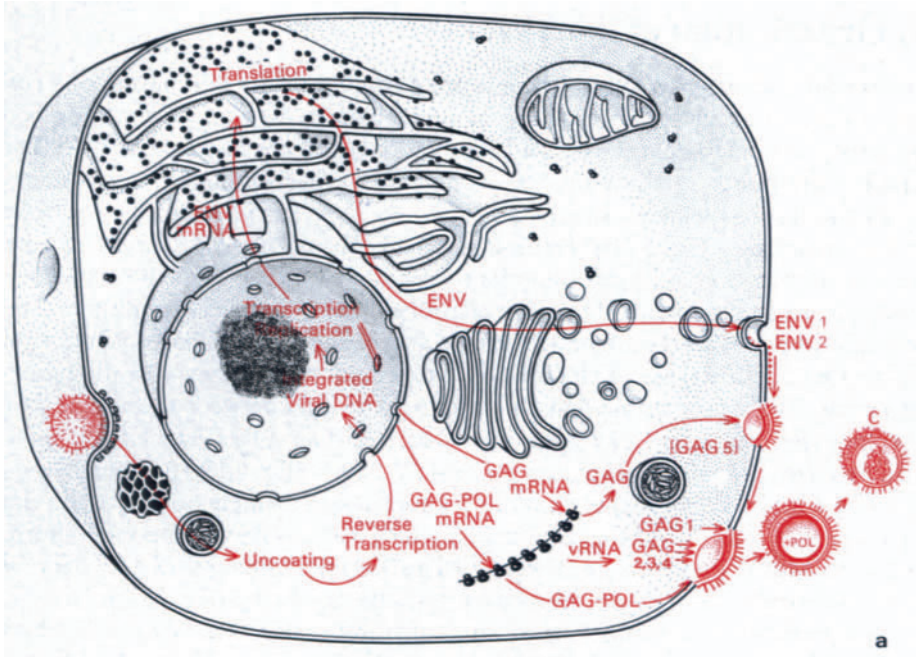

Fig. 9-12. Representation of a budding retrovirus C-type particle and the mature form released from the cells. The locations of the proteins are indicated. Numbers on *gag* and *env* proteins correspond to those given in Table 9-1 and 9-3. NC, nucleocapsid. The *gag* molecule in the budding particle may span the space between the NC and the inner envelope, but its shape is hypothetical. One possible organization of the coiling of the NC in the budding virus is shown. It takes into consideration that the center of the budding particle is clear. It is also possible that retrovirus NC forms a helix similar to that shown in the coronavirus scheme (see Chapter 7, Fig. 7-12)

Fig. 9-13. Representations of the events of retrovirus replication and assembly. *(a)* Maturation of a retrovirus C particle at the membrane. The processes of reverse transcription, integration and replication have been shown in Fig. 9-2. The three mRNAs synthesized in the nucleus are translated on the rough ER (ENV) or on polysomes (GAG and GAG-POL). ENV glycosylation is completed in the Golgi apparatus and cleavage occurs close to or at the cell surface. GAG is cleaved in its five products at the budding site, while POL is cleaved only in the released virion, which acquires a central core. *(b)* Typical maturation events occurring in retroviruses which assemble their NCs in the cytoplasm before budding, such as MMTV and MPMV. Differences in structure between the released virions in these two strains are indicated. B virions have an eccentric core, while D virions appear to have an intermediate layer between the envelope and the irregularly shaped NC. *(c)* Typical murine leukemic cell which can produce virus both at the tips of villi and inside vacuoles

Translation

ENV mRNA

ENV

Transcription Replication

Integrated Viral DNA

Reverse Transcription

Uncoating

GAG-POL mRNA

GAG mRNA

GAG

[GAG 5]

vRNA

GAG-POL

GAG 1

GAG 2,3,4

+POL

ENV 1 ENV 2

C

a

ENV 1 ENV 2

GAG 1

GAG 2,3,4

?

A

A

D

B

GAG

GAG-POL

vRNA

b

ENV 1 ENV 2

C

ENV 1-2

GAG

GAG-POL

vRNA

C

c

V. Organization of the Virion

The virion consists of a 100 nm enveloped particle, usually containing one core (Fig. 9-11d and e), although multiple or tubular cores may also occur. The core consists of a spiral filamentous strand, as proposed earlier by Sarkar *et al.* (1971). This strand corresponds to the virus NC which can be measured after uncoiling by detergent. It is 7–10 nm in diameter and about 1 μ long. Partial coiling can be maintained in isolation (Fig. 9-11f) (Frank *et al.*, 1978). Around the NC core is an outer shell of hexagonally arranged subunits (Nermut *et al.*, 1972) (Fig. 9-10b), probably made of core shell proteins. The exact relationship between these subunits and the coiled RNA is not known. Around the core and its shell is a perinucleoid electron-lucent space. The degree of electron opacity in that zone varies with the type of retrovirus. Virions sometimes have an intermediate layer between the core and the envelope (Fig. 9-11e). The envelope usually bears visible spikes. The longest spikes seen on retrovirus envelopes pertain to MMTV (100 Å) (Fig. 9-11b) (Calberg-Bacq *et al.*, 1976). One spike consists of a spherical knob 55 Å in diameter joined to the viral envelope by a stalk (40 Å long, 20 Å in diameter), whereas ASV spikes seem shorter (50 Å). In MuLV, one spike contains 4 to 6 gp70-p15E complexes (Schneider *et al.*, 1980) and knobs are seen on the surface after freeze-drying (Fig. 9-11a).

A model of the organization of immature and mature retroviruses has been proposed by Bolognesi *et al.* (1978) and later by Dickson *et al.* (1982). Our scheme, modified from these authors, is shown in Fig. 9-12. Fig. 9-13a, b, and c summarizes the events of replication and assembly of retroviruses in the cell.