The EMBO Journal vol.8 no.9 pp.2653 – 2660, 1989

The GAG precursor of simian immunodeficiency virus assembles into virus-like particles

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Communicated by A.Burny

To examine the potential role of the GAG precursor polyprotein in morphogenesis and assembly of the simian immunodeficiency virus (SIV), we have expressed the *gag* gene of SIV_{Mac} using a baculovirus expression vector. Infection of insect cells with recombinant virus containing the entire *gag* gene results in high expression of the GAG precursor protein, $Pr57^{gag}$. The recombinant protein is myristylated and is released in the culture supernatant in an insoluble particulate form. A point mutation in the N-terminal glycine codon (Gly → Ala) inhibits myristylation. This mutated product is highly expressed but is not found in the culture supernatant. Electron microscopy and immunogold labelling of infected cells show that the native $Pr57^{gag}$ protein assembles into 100–120 nm virus-like particles that bud from the cell plasma membrane and are released in the culture supernatant. The unmyristylated protein also assembles into particulate structures which only accumulate inside the cells. These results demonstrate that the unprocessed GAG precursor of SIV can spontaneously assemble into particles in the absence of other viral proteins. Myristylation of the $Pr57^{gag}$ precursor is necessary for its association with the cell plasma membrane, for budding and for extracellular release.

Key words: baculovirus expression vector/GAG precursor/myristylation/simian immunodeficiency virus/viral particle morphogenesis

Introduction

Simian immunodeficiency viruses (SIVs) constitute a group of Old World primate retroviruses that are morphologically and antigenically related to human immunodeficiency viruses (HIVs) (Desrosiers, 1988; Essex and Kanki, 1988; Haseltine and Wong-Staal, 1988). The genetic organization of the SIV genome is similar to that of the HIV genome and is typical of a lentivirus: it contains the standard retroviral genes, namely *gag*, *pol* and *env*, and at least six additional genes (Desrosiers, 1988; Essex and Kanki, 1988; Haseltine and Wong-Staal, 1988). The three *gag*, *pol* and *env* genes encode polyproteins that are further processed to generate the major constituents of virus particles, i.e. the core proteins, the catalytic proteins (protease, reverse transcriptase) and the envelope glycoproteins, respectively (Dickson *et al.*, 1984).

The *gag*-encoded polyprotein precursor of retroviruses is thought to play a major role in virus particle formation at the cell membrane (Stephens and Compans, 1988). Retroviruses have been divided into four subtypes (A,B,C,D) on the basis of morphologic criteria and morphogenesis. Type A represents immature, intracytoplasmic particles. Type B [mouse mammary tumor virus (MMTV)] and type D retroviruses [Mazon–Pfizer monkey virus (MPMV)], preform type A intracytoplasmic particles (Smith, 1978) that associate with the cell membrane during the budding process. Type C retroviruses [murine leukaemia virus (MuLV)] bud as a crescent shape at the cellular membrane without preformed subviral particles in the cytoplasm or at the plasma membrane (Lu *et al.*, 1979). The fact that the GAG precursor plays a crucial role in viral particle morphogenesis was demonstrated for the type C MuLV virus (Shields *et al.*, 1978; Yoshinaka *et al.*, 1980). Morphogenesis of lentiviruses, including SIV and HIV, appears similar to that of type C retroviruses (Katsumoto *et al.*, 1987; Gelderblom *et al.*, 1988).

The GAG precursor of the HIV and SIV viruses undergoes two major modifications: the proteolytic cleavage by the viral protease (Wellink and van Kammer, 1988), a modification thought to occur post-budding (Kohl *et al.*, 1988), and the addition of a myristic acid moiety to the N-terminal glycine residue (Henderson *et al.*, 1988). This later modification is generally found in mammalian retroviruses (Schultz and Oroszlan, 1983; Towler *et al.*, 1988) and is thought to play a role in the association of the GAG precursor with the cell membrane. Indeed, mutation of the N-terminal glycine codon of the MuLV *gag* gene prevents myristylation of the GAG precursor and leads to its accumulation within the cytoplasm of the infected cells where no recognizable virus-specific structures can be detected by electron microscopy (Rein *et al.*, 1986). Type D MPMV virus expressing unmyristylated GAG precursor still assembles into intracytoplasmic type A particles but these are not targeted to the cell membrane and no budding virus is observed (Rhee and Hunter, 1987).

To investigate the role of the GAG precursor in morphogenesis and assembly of the SIV, we isolated the *gag* gene of the SIV_{Mac} cloned provirus pBK28 (Kornfeld *et al.*, 1987; Kestler *et al.*, 1988) and expressed it, in the absence of the downstream protease coding sequence, in a potent eukaryotic expression system, the baculovirus–insect cells system. This system leads to efficient expression of the SIV *gag*-encoded uncleaved precursor polyprotein. The recombinant $Pr57^{gag}$ protein is myristylated, assembles at the plasma membrane of the insect cells and is released in the culture supernatant as 100–120 nm particles. Mutation of the N-terminal glycine codon prevents myristylation; the unmyristylated $Pr57^{gag}$ polyprotein is not released in the culture supernatant but still assembles into intracellular particles. In this baculovirus–insect cell system, myristylation of $Pr57^{gag}$ polyprotein seems to be essential for the association of the $Pr57^{gag}$ precursor with the cell

membrane, a process crucial for budding and release of GAG particles.

Results

The SIV GAG precursor is efficiently expressed in insect cells

To express the SIV_{Mac} GAG precursor in the baculovirus–insect cell system, the SIV *gag* gene was isolated from the cloned BK28 genome (Kornfeld *et al.*, 1987) and inserted in the transfer vector pAcYM1 (Matsuura *et al.*, 1987) downstream of the baculovirus polyhedrin promoter. The resulting plasmid, pAcGAGmyr$^+$, was co-transfected in *Spodoptera frugiperda* (Sf) insect cells with genomic DNA of *Autographa californica* nuclear polyhedrosis virus (AcNPV). The recombinant viruses generated by *in vivo* homologous recombination were tested for the presence of *gag*-related sequences by hybridization using a radiolabelled *gag* probe. The procedure followed to insert the SIV *gag* gene in the baculovirus genome is depicted in Figure 1.

Sf cells infected with plaque-purified recombinant AcGAGmyr$^+$ virus were tested for expression of SIV related protein using a rabbit antiserum to SIV by immunoblot and ELISA (data not shown). To identify the immunoreactive product, extracts of cells infected with the AcGAGmyr$^+$ virus were examined by Western blot at days 1, 2, 3 and 4 post-infection (p.i.). A protein of 57 kd was identified by a monoclonal antibody to HIV p24 major core protein that crossreacts with the SIV $Pr57^{gag}$ protein. The recombinant protein co-migrates with the $Pr57^{gag}$ precursor identified in HUT78 cells infected with SIV_{Mac}-BK28 (Figure 2). This result identifies the protein of 57 kd produced in insect cells as the SIV $Pr57^{gag}$ polyprotein precursor. The monoclonal antibody also recognizes smaller products, which probably result from host cellular proteolysis of the $Pr57^{gag}$ precursor.

The SIV GAG precursor is released in the culture medium in a particulate form

We also looked for the presence of the $Pr57^{gag}$ precursor in the culture supernatant of Sf cells infected with the recombinant virus AcGAGmyr$^+$. We observed that the pellet of ultracentrifuged culture supernatants displayed a prominent band of 57 kd recognized in Western blot by the specific monoclonal antibody (Figure 2). This result shows that $Pr57^{gag}$ is produced in the culture supernatant in an insoluble form. The release of insoluble GAG precursor is not the result of cell lysis since no evidence of lysis was observed by direct observation at that time of infection.

To further characterize the insoluble GAG product released from the infected cells, the pellet obtained by ultracentrifugation of the culture medium of infected Sf cells after two days of infection was laid on a 20–60% discontinuous sucrose gradient. After centrifugation, ELISAs were performed on the collected fractions. Figure 3 shows that the GAG product bands at a sucrose density of 1.162 g/ml. This material contains the $Pr57^{gag}$ polyprotein as shown by Western blot analysis of the fractions (Figure 3A). Coomassie staining (Figure 3B) reveals that the $Pr57^{gag}$ is the dominant protein in the peak fractions. This result shows that the insoluble GAG product present in the culture medium is organized as particles. The level of production of $Pr57^{gag}$ protein as particles in the culture medium at day 2 p.i. was estimated at 10–15 mg/l (10^9 cells/l).

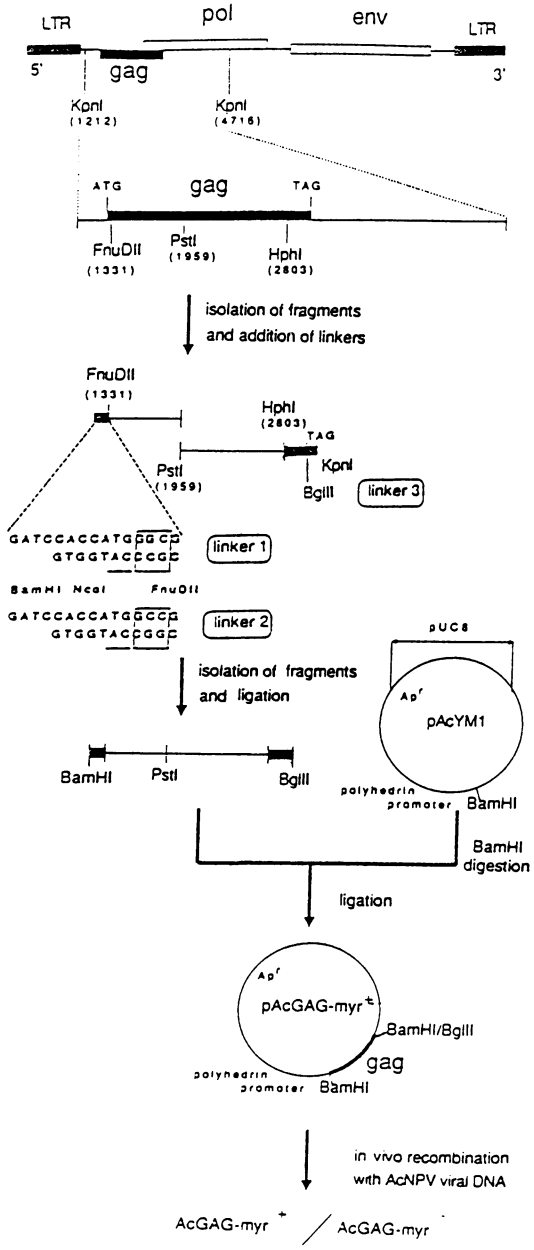

Fig. 1. Construction scheme of recombinant baculoviruses containing the SIV *gag* gene. The *gag* gene was subcloned from the molecularly cloned SIV_{Mac}-BK28 [>99% homologous to SIV_{Mac251} (Kestler *et al.*, 1988)]. A 3504 bp *Kpn*I fragment of the pBK28 genome was subcloned into pUC18. Two internal fragments of the *gag* gene, the 5′ fragment [*Fnu*DII(1201)–*Pst*I(1959)] and the 3′ fragment [*Pst*I(1959)–*Hph*I(2803)], were purified, and synthetic oligonucleotide linkers (linkers 1 and 2 are described in the figure; linker 3 is

TGCTGCACCTCAATTCTCTCTTTGGAGGAGACCAGTAGAGATCTGGTAC
AACGACGTGGAGTTAAGAGAGAAACCTCCTCTGGTCATCTCTAGAC)

were added to reconstitute the entire gene (ATG translation initiation codon and TAG stop codon are underlined) and introduce proper restriction sites for further cloning. A third linker (linker 2) was also used instead of linker 1 to introduce a mutation in the second codon [GGC (Gly) → GCC (Ala); mutation pointed by the open boxes]. The N-terminal fragment (*Bam*HI–*Pst*I) and C-terminal fragment (*Pst*I–*Bgl*II) were isolated and cloned into the *Bam*HI-digested, alkaline phosphatase-treated pAcYMI baculovirus transfer vector. The resulting plasmids were co-transfected with AcNPV DNA in insect cells and *in vivo* homologous recombinations generated recombinant viruses: AcGAGmyr$^+$ contains the native *gag* gene and AcGAGmyr$^-$ contains the mutated gene.

The SIV GAG precursor is myristylated

To investigate the myristylation of the SIV Pr57^{gag}, Sf cells infected with the recombinant virus AcGAGmyr$^+$ were labelled with [^{3}H]myristic acid. Immunoprecipitation of labelled proteins with the rabbit antiserum to SIV was performed on the cellular extract and the ultracentrifuged culture supernatant. In each sample, a protein of 57 kd labelled with [^{3}H]myristic acid was observed (Figure 4).

These results demonstrate that the SIV$_{Mac}$ Pr57^{gag} precursor expressed in insect cells is myristylated, a modification commonly found in retroviral *gag*-encoded precursor polyproteins (Schultz and Oroszlan, 1983; Henderson *et al.*, 1988).

Importance of myristylation in the extracellular release of the SIV GAG precursor

We then investigated the role of myristylation in the release of the GAG precursor particles. For this purpose, we mutated the 5′ terminal codon (GGC) into codon (GCC). Such a mutation leads to the substitution of the N-terminal glycine which is essential for the myristylation process (Towler *et al.*, 1988) for an alanine. Recombinant virus containing the mutated *gag* gene (AcGAGmyr$^-$) was generated and identified following the same experimental procedures as described above except that linker 2 was used instead of linker 1 to reconstitute the 5′ end of the *gag* gene (Figure 1).

The mutated Pr57^{gag} precursor was as efficiently expressed as the myristylated product, as demonstrated by Coomassie staining and Western blot analysis (Figure 2). Metabolic labelling with [^{3}H]myristic acid (Figure 4) revealed no myristic acid incorporation, confirming that mutation of the N-terminal glycine was sufficient to prevent myristylation of the GAG precursor. [^{35}S]Methionine labelling and immunoprecipitation experiments were performed to assess the expression of the mutated precursor (Figure 4). The mutated Pr57^{gag} was only detected within the infected cells. No extracellular GAG product could be pelleted by ultracentrifugation (Figures 2 and 4). The myristylation process thus seems to be required for the extracellular release of the Pr57^{gag} particles.

The recombinant SIV GAG precursor assembles into particles

To analyse the morphogenesis and assembly of the GAG particles, Sf cells infected with the wild-type and mutant

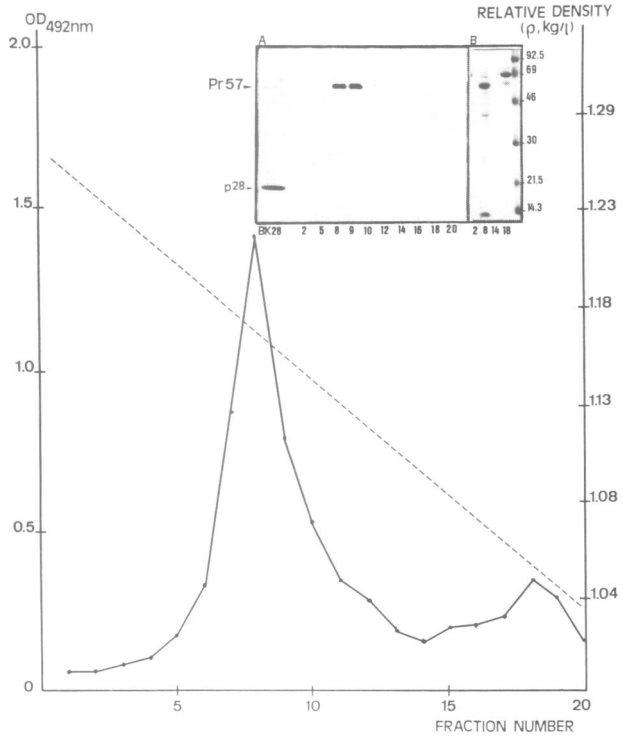

Fig. 3. Distribution profile of extracellular GAG protein along a 20–60% sucrose gradient as determined by ELISA. Inserts: (**A**) Western blot analysis of the gradient fractions with monoclonal antibody to HIV p24 (lane 1: SIV-BK28 infected HUT78 cell lysate; numbers on the abscissa represent fractions of the gradient). (**B**) Coomassie staining of gradient fractions 2, 8, 14 and 18 (lane 5: mol wt marker proteins).

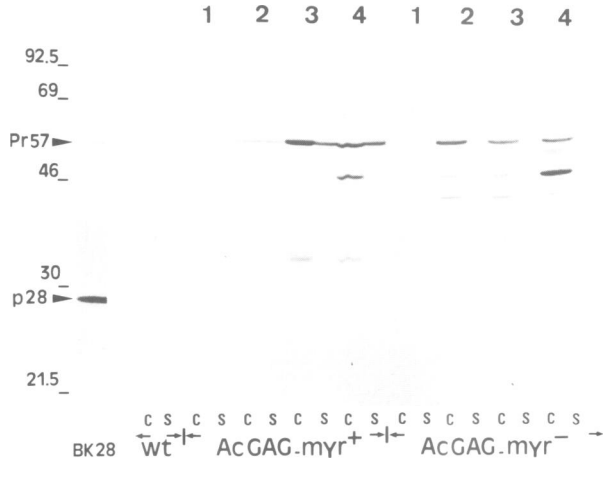

Fig. 2. Western blot analysis of recombinant GAG protein expression in cellular extracts (C) and in ultracentrifuged culture supernatant (S) from day 1 to day 4 p.i.. Western blot was performed using a monoclonal antibody to HIV p24 core protein crossreacting with the SIV Pr57^{gag} precursor. BK28: SIV-BK28 infected HUT78 cell lysate; WT: wild-type AcNPV infected cells (day 4 p.i.); AcGAGmyr$^+$: AcGAGmyr$^+$ infected cells; AcGAGmyr$^-$: AcGAGmyr$^-$ infected cells.

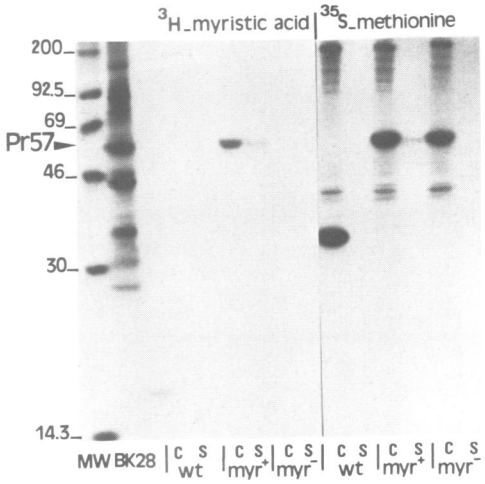

Fig. 4. Immunoprecipitation of [^{3}H]myristic acid- and [^{35}S]methionine-labelled GAG recombinant proteins in cellular extracts (C) or in the ultracentrifuged culture supernatant (S) at 48 h p.i. MW: [^{14}C]methylated mol. wt marker proteins; BK28: SIV-BK28 infected HUT78 cell lysate; WT: wild-type AcNPV infected cells; myr$^+$: AcGAGmyr$^+$ infected cells; myr$^-$: AcGAGmyr$^-$ infected cells.

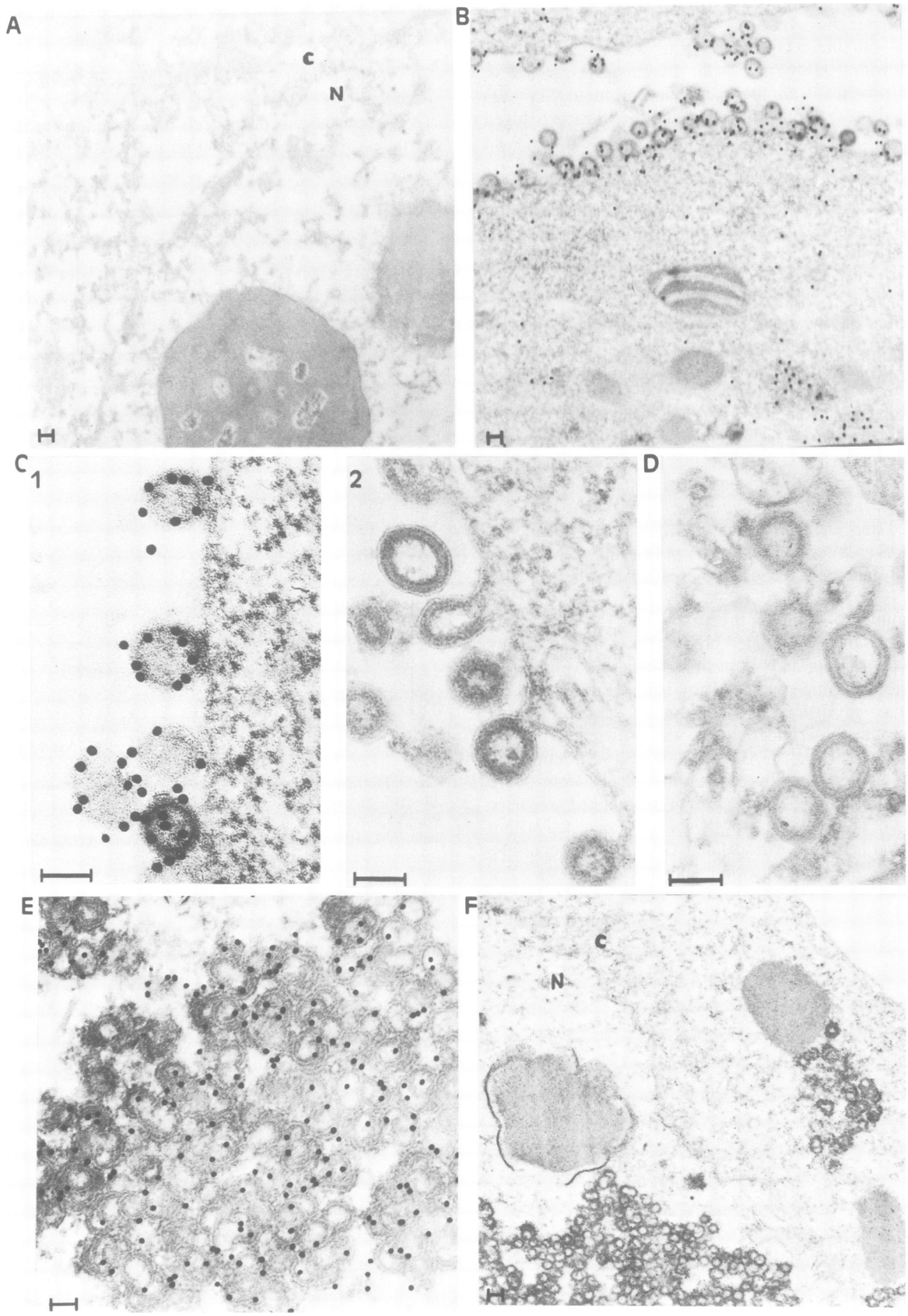

Fig. 5. Electron micrographs of thin sections of infected Sf cells. Bar marker represents 100 nm (n: nucleus; c: cytoplasm). (**A**) Cell infected with the wild-type AcNPV baculovirus (48 h p.i.): polyhedrin inclusions carrying the AcNPV accumulate within enlarged nucleus. (**B**) Immunogold labelling on a cell infected with the recombinant AcGAGmyr$^+$ virus at 28 h p.i.. (**C**) Budding GAG particles (C1: immunogold labelling on budding particles; C2: budding particles visualized after glutaraldehyde fixation and tannic acid treatment). (**D**) Extracellular GAG particles present in the pellet obtained by ultracentrifugation of the culture supernatant (72 h p.i.). (**E**) Immunogold labelling on intracellular GAG particles. (**F**) Cell infected with the recombinant AcGAGmyr$^-$ at 48 h p.i.

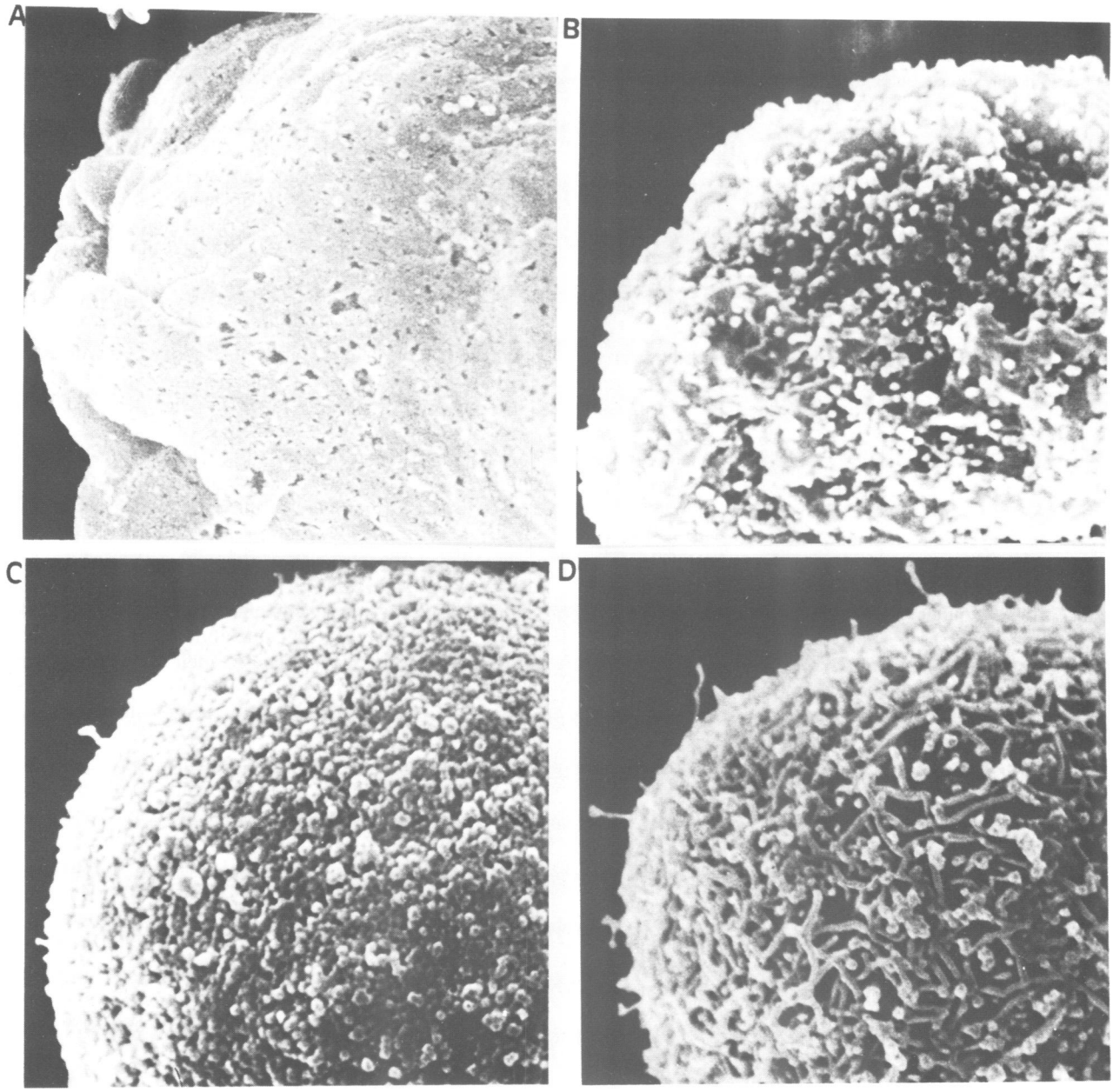

Fig. 6. Scanning electron micrographs of Sf cells. Magnification × 10 000. (**A**) Control wild-type baculovirus infected cell at 48 h p.i.. (**B**)–(**D**) Cells infected with the recombinant AcGAGmyr$^+$ baculovirus at 28 h (B), 48 h (C) and 72 h (D) p.i.

recombinant baculoviruses and pellets from ultracentrifuged culture supernatants were examined by electron microscopy during the course of infection. Thin section transmission electron microscopy performed on Sf cells infected with the AcGAGmyr$^+$ recombinant virus showed a number of crescent-shaped budding particles at the surface of the infected cells (Figure 5B). These budding particles are due to the production of the GAG protein since (i) they are not formed in cells infected with the wild-type baculovirus (Figure 5A) and (ii) they contain the GAG protein as shown by immunogold labelling (Figure 5B). In scanning electron microscopy, the cell surface was covered with budding particles (Figure 6B) whereas the surface of control cells appeared rather smooth (Figure 6A). Budding particles (Figure 5C) and particles released in the culture supernatant (Figure 5D) closely resemble immature HIV-1 virions (Katsumoto *et al.*, 1987; Gelderblom *et al.*, 1988; Hockley *et al.*, 1988). Indeed, they are ~100–120 nm in diameter and show a light grey translucent centre surrounded by a thick dark electron dense ring and an outer lipid bilayer (Figure 5C2). Immunogold labelling of thin sections revealed that most of the specific immunoreactivity is located at the level of the dark ring (Figure 5C1); this ring probably consists of multimeric association of the Pr57^{gag} polyprotein. Within the infected cells, the GAG protein was detected associated with grey amorphous material both in the nucleus and the cytoplasm.

With increasing time of infection, the entire surface of many cells became covered with budding GAG particles (Figure 6C). Particulate structures composed of SIV GAG products appeared both within the cytoplasm and the nucleus of infected Sf cells. These intracellular particles (Figure 5E) were however morphologically different from the extracellular virus-like GAG particles. They showed an

electron-lucent centre surrounded by two thin dark open or closed rings of equal electron density. These particles were clustered and lacked an outer lipid bilayer. In sucrose density gradients, these intracellular particles equilibrated at a relative density of 1.177 g/ml.

Later in the course of infection (days 3 and 4), part of the cells were covered with projections of rigid tubular structures of 40 nm in diameter (Figure 6D), shown to contain SIV GAG products by immunogold labelling. Since these structures appeared simultaneously with shorter SIV specific products, it is possible that the tubular structures are formed by incorporation of partially degraded GAG products.

Cells infected with the recombinant AcGAGmyr$^-$ virus were analysed using the same methods. Immunogold labelling revealed that the unmyristylated Pr57^{gag} protein was efficiently expressed, initially found scattered in the cytoplasm or associated with grey amorphous material within the cytoplasm and the nucleus (data not shown). With increasing time of infection, clusters of intracellular Pr57^{gag} particles formed close to the amorphous material (Figure 5F). These intracellular particles resemble those observed intracellularly in Sf cells infected with the recombinant AcGAGmyr$^+$. Neither GAG protein nor budding structures were observed at the cell plasma membrane (Figure 5F).

These results demonstrate that the unprocessed SIV Pr57^{gag} precursor can by itself, that is, in the absence of any other SIV viral component, organize budding particles. Myristylation appears to be required for the association of the Pr57^{gag} precursor at the plasma membrane and for the budding process of the GAG particles. However, myristylation does not seem to be required for the multimeric assembly of the Pr57^{gag} molecules.

Discussion

In this study, we show that the GAG precursor of the SIV$_{Mac}$-BK28 isolate is highly expressed in insect cells infected with a recombinant baculovirus containing the SIV *gag* gene under the control of the baculovirus polyhedrin promoter.

In this system, the SIV Pr57^{gag} precursor assembles at the cell plasma membrane into budding crescent-shaped structures released in the culture medium as 100–120 nm particles. The budding process observed in the baculovirus–insect cell system closely resembles the budding of HIV-1 retroviruses (Katsumoto *et al.*, 1987; Hockley *et al.*, 1988). The extracellular particles have a size similar to native retrovirions (100–120 nm) and structurally resemble immature viral particles (Gelderblom *et al.*, 1988; Hockley *et al.*, 1988). Indeed, they show a translucent centre surrounded by a thick electron-dense ring and an outer lipid bilayer membrane of cellular origin, acquired during the budding process. The SIV specific immunoreactivity is located at the level of the dark ring, most probably formed by the multimeric assembly of the Pr57^{gag} molecules. Additional experiments dealing with protein and lipid composition as well as with nucleic acid content are under way but first require improved purification of these particles.

In these experiments, the *gag*-encoded polyprotein is shown to form virus-like particles in the absence of any other SIV gene products. We thus conclude that the Pr57^{gag} protein is sufficient for virus particle formation at the cell plasma membrane and that specific proteolytic cleavage is not necessary for particle formation. We cannot exclude the potential helping role of cellular or baculovirus components in particle assembly. However, we observed that expression of the same SIV Pr57^{gag} protein in mammalian cells by a recombinant vaccinia virus also leads to the production of GAG particles that look similar by electron microscopy (unpublished data). The fact that the GAG precursor alone is sufficient for viral particle formation is reminiscent of previous work dealing with deletion mutants of the MuLV virus; indeed, MuLV mutants lacking *pol* and *env* genes were shown to form viral particles (Shields *et al.*, 1978).

Later in the course of infection by the recombinant baculovirus, additional intracellular Pr57^{gag} molecules are found both within the nucleus and the cytoplasm as amorphous material and then as visible particles morphologically different from the extracellular particles described above. The exact relationship between these two intracellular forms of the GAG precursor is not clear. The facts that the immunogold labelled amorphous material temporally precedes the formation of visible intracellular particles and that the two forms are closely associated within the cell, suggest that the particles are slowly generated from the amorphous material.

The targeting of mammalian retroviral *gag*-encoded precursors at the cell plasma membrane is dependent on the attachment of a myristic acid to their N-terminal glycine (Schultz and Oroszlan, 1983). By metabolic labelling experiments with [^{3}H]myristic acid, we show that the SIV$_{Mac}$ Pr57^{gag} protein is myristylated. When the N-terminal glycine residue of Pr57^{gag} is mutated into an alanine, myristylation is prevented. The unmyristylated Pr57^{gag} precursor is not found at the plasma membrane and no budding particles are formed. Myristylation is thus clearly required for association of the GAG precursor with the plasma membrane and for the budding process. However, the unmyristylated GAG precursor still assembles into intracellular particles. We conclude that myristylation is not required for multimeric assembly of the GAG molecules.

In view of these observations, we propose that the GAG precursor has an intrinsic property of self-assembly provided a minimum local concentration of the product is reached. In the baculovirus–insect cell system, the high level of Pr57^{gag} expression results in cytoplasmic concentration sufficient for self-assembly both in the cytoplasm and at the plasma membrane. In the natural SIV or HIV infection, critical concentration of the Pr57^{gag} molecules is only reached at the cell plasma membrane where the GAG precursors are targeted. The Pr57^{gag} molecules then assemble together and with the other viral components; this results in the formation of immature viral particles. In this process, myristylation could function not as a targeting signal but rather as a 'retention' signal allowing a protein targeted to the membrane to remain closely associated with the membrane by hydrophobic interaction between the myristic acid and the lipid bilayer. The targeting and transport to the plasma membrane would then be controlled by another signal intrinsic to the protein. Such a possibility is currently under investigation. This hypothesis is further supported by the fact that various myristylated proteins are found in different cellular locations such as cytosol and nucleus (Luckow and Summers, 1988; Miller, 1988), which indicates the absence of specificity of the myristic acid moiety in terms of cellular targeting.

Accumulation of the Pr57^{gag} within the nucleus of the

infected Sf cells is a surprising phenomenon. Indeed, a number of soluble, nuclear, membrane or secreted proteins have now been expressed in the baculovirus–insect cell system and, in each case, they were shown to segregate to their proper compartment (Luckow and Summers, 1988; Miller, 1988). Thus, the presence of the GAG precursor in the nucleus of the insect cells could have a physiological significance. Specific signal sequences composed of a few basic residues are identified in several proteins that are targeted to the nucleus (Smith *et al.*, 1985; Dingwall and Laskey, 1986). We have identified a short stretch of basic amino acids in the N-terminal amino acid sequence of the SIV GAG precursor. This region is highly conserved in both HIV-1 and HIV-2 (Table I) and could serve as a karyophilic signal for the transport of $Pr57^{gag}$ to the nucleus. This hypothesis and the potential physiological role of this amino acid stretch are currently being tested by site directed mutagenesis.

The fact that the SIV $Pr57^{gag}$ precursor can be expressed in large quantities and released as extracellular virus-like particles makes it a potential vaccine candidate. With regard to the HIV system, we have also expressed the GAG precursor of HIV using the baculovirus system; the HIV $Pr55^{gag}$ also assembles into particles released in the culture medium (Gheysen *et al.*, 1989). The GAG protein is highly conserved between different isolates of SIV and HIV (Chakrabarti *et al.*, 1987; Franchini *et al.*, 1987; Kestler *et al.*, 1988) and could potentially generate a group-specific immune response. Moreover, neutralizing epitopes have recently been described in the GAG precursor of HIV (Papsidero *et al.*, 1989). Immunization experiments are presently being carried out to examine the potential use of these GAG particles in vaccination trials.

Table I. The GAG protein of SIV and HIV contains a potential nuclear location signal

Protein	Sequence alignment
SV40 large T	P P K K^{128} K R K V
Polyoma VP 213	P Q K K^{315} K R R L
Adenovirus 72K DBP	A P K K^{86} K K K R
SIV-BK28 GAG precursor	R P G G K K^{27} K Y M L K H
HIV-2 rod GAG precursor	R P G G K K^{27} K Y R L K H
HIV-1(Hxb2) GAG precursor	R P G G K K^{27} K Y K L K H

Materials and methods

Cells and viruses

Sf insect cells were propagated at 27°C in TC100 medium (Carstens *et al.*, 1979), containing 10% fetal bovine serum (FBS). AcNPV and recombinant viruses were grown and assayed in confluent monolayers of Sf cells. Essential procedures are described in detail in Summers and Smith (1987).

Viral and plasmid DNAs

AcNPV DNA was purified according to the method of Smith and Summers (1978).

Plasmid DNAs were prepared by the alkaline lysis procedure followed by centrifugation to equilibrium in CsCl–EtBr gradients, essentially as described by Maniatis *et al.* (1982).

Oligonucleotide linkers were synthesized using the phosphoramidite chemistry and a Cyclone DNA synthesizer (New Brunswick). Oligonucleotides were purified on 15% polyacrylamide–urea gels. Equimolar amounts of complementary oligonucleotides were pooled, phosphorylated and annealed by slow cooling after heating at 70°C for 15 min. Amino acid sequences were checked by cloning into M13 vector and sequencing using the Sequenase™ kit (United States Biochemical Corporation).

Transfection experiments and selection for recombinant AcNPV

Sf cells were transfected with a mixture of purified AcNPV DNA (1 μg) and recombinant transfer plasmid DNA (50 μg), essentially as described by Smith *et al.* (1983). Briefly, after calcium phosphate precipitation, DNA was layered onto monolayers of Sf cells grown in Petri dishes and incubated at room temperature for 90 min. The supernatant was discarded, TC100–10% FBS was added and the cells were incubated at 27°C.

Culture supernatants were harvested 3 and 5 days post-transfection and were titrated on confluent monolayers of Sf cells, according to previously published plaque assay methods (Summers and Smith, 1987). Plaques exhibiting no evidence of polyhedrin-occlusion bodies were recovered. Using a specific ^{32}P-labelled DNA *gag* probe (a 1350 bp *NcoI–NcoI* fragment of the *gag* gene, ^{32}P-labelled by nick translation), recombinant viruses were identified after plaque imprints; the hybridization procedure was adapted from Grunstein and Hogness (1975) and Villareal and Berg (1977): the (pre)-hybridization buffer used was SET buffer (0.15 M NaCl, 1 mM EDTA, 0.03 M Tris–HCl, pH 8). Positive plaques were selected and plaque-purified.

Expression analyses

Immunoblots. Cell lysates were dot-blotted on a nitrocellulose sheet (Kafatos *et al.*, 1979) and the expression of SIV *gag*-specific proteins was visualized using a polyclonal antiserum, from a rabbit immunized with metrizamide-gradient purified SIV-BK28 virus. The Protoblot Western Blot AP system (Promega, P3990) was used to reveal the reaction.

ELISA. A standard procedure was applied. The protein A purified IgG fraction (Seppälä *et al.*, 1981) of the rabbit antiserum to SIV (described above) was used to coat the plates. After saturation, several dilutions of the antigen in PBS–1% Triton X-100 were added, followed by biotinylated anti-SIV IgGs from the same rabbit serum [biotinylation according to Katona *et al.* (1983)]. The reaction was revealed by addition of streptavidin biotinylated horseradish peroxidase complex (Amersham) and OPDA. Optical density was read at 492 nm.

Identification of recombinant proteins

Sf cells were infected at a multiplicity of infection (m.o.i.) of 5. Cells and culture supernatants were separated by centrifugation at 800 *g* for 10 min. The pellet was resuspended in 1% sodium dodecyl sulphate (SDS) and 10 μg/ml aprotinin solution; Laemmli sample buffer (Laemmli, 1970) was then added and the lysis was completed by boiling for 5 min. The culture supernatant was ultracentrifuged at 100 000 *g* for 60 min and the resulting pellet was boiled in Laemmli sample buffer for 5 min. This material was then tested for expression of recombinant SIV GAG proteins. Cell lysates (3×10^5 cells, 100 μg total proteins) or ultracentrifuged culture supernatant (250 μl, 10 μg total proteins) were submitted to SDS–PAGE (12.5% polyacrylamide) as described by Laemmli (1970). After electrophoresis, the proteins were either fixed (10% acetic acid–40% methanol) and stained with Coomassie blue or transferred electrophoretically to nitrocellulose sheet (Towbin *et al.*, 1979). Western blot tests were then performed using a monoclonal antibody to HIV p24 major core protein crossreacting with the SIV $Pr57^{gag}$ protein (dilution 1:500) and the Protoblot detection system (Promega, P3920). The monoclonal antibody, obtained from a mouse immunized with metrizamide-gradient purified HIV-1 viral particles, recognizes an epitope in the carboxy-terminal part of the HIV p24. The mol. wt of the recombinant proteins was estimated by comparison with migrating patterns of an extract of SIV-BK28 infected cell lysate (2×10^5 cells per slot) and commercial standards ranging from 14.3 to 200 kd (Amersham).

Metabolic labelling

Sf cells (3×10^5) were infected at a m.o.i. of 5 and, at 24 h p.i., were labelled with [^{35}S]methionine (5 μCi/ml) or with [^{3}H]myristic acid (50 μCi/ml) for 24 h. Harvested cells and ultracentrifuged culture supernatant were lysed in 50 μl of buffer A (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 40 mM phenylmethyl sulphonyl fluoride, 1% NaN_3) and freeze-thawed.

Radioimmunoprecipitation

Labelled samples were immunoprecipitated by rabbit antiserum to SIV previously bound to protein A–Sepharose (10 μl antiserum/60 μl protein A–Sepharose CL-4B 10%, Pharmacia). The complexes were washed three times in buffer A and once in buffer B (50 mM Tris–HCl, pH 7.2, 50 mM NaCl). The bound proteins were eluted in Laemmli sample buffer by boiling at 100°C for 5 min and analysed on a 12.5% polyacrylamide gel. The gel

was fixed, treated for fluorography (Amplify, Amersham), dried and autoradiographed. The mol. wt of proteins was estimated by comparison with [^{14}C]methylated commercial standards (3 μCi/slot; Amersham).

Analysis of recombinant proteins in 20–60% sucrose gradient

Sf cells (10^8 cells in 100 ml TC100–10% FBS) were infected with the recombinant viruses at a m.o.i. of 5 and incubated at 27°C in roller bottles. Culture supernatant was harvested at 48 h p.i., ultracentrifuged and the pellet was resuspended in 1 ml of Hank's balanced salt solution (HBSS) containing 0.1% Tween 20 and 10 μg/ml aprotinin. Cells were resuspended in HBSS containing 0.1% Triton X-100 and left on ice for 5 min. Membrane fragments were discarded by centrifugation at 800 *g* for 10 min, the intracellular content was ultracentrifuged and the pellet was resuspended in 1 ml HBSS–0.1% Tween 20–10 μg/ml aprotinin. Samples were applied on a 20–60% discontinuous sucrose gradient and spun in an SW41 rotor at 100 000 *g* for 16 h at 4°C. Fractions (0.6 ml) were collected from the bottom, diluted and assayed for SIV GAG protein content by ELISA test, and analysed by Western blot (10 μg total protein for major peak fractions/slot) and Coomassie staining (25 μg total protein/slot).

Electron microscopy

Infected cells were fixed overnight at 4°C with 2.5% buffered (pH 7.4) glutaraldehyde or formaldehyde.

For scanning and transmission electron microscopy, cells were treated essentially as described by Gelderblom *et al.* (1988) and by Hockley *et al.* (1988).

For immunogold labelling, ultrathin sections were etched with saturated $NaIO_4$, washed with water, treated with 2% ovalbumin in PBS and incubated for 2 h at room temperature with IgG purified from the rabbit antiserum to SIV. The reaction was revealed using protein A–gold conjugate (Janssen).

Acknowledgements

We are grateful to Dr J.I.Mullins for providing the cloned SIV_{Mac}-BK28 genome. We thank F.de Foresta, M.-C.François, B.Lambert, C.Petré-Parent and M.Sneyers for excellent technical assistance. This work was supported by a grant from the Belgian Scientific Medical Research Foundation (FRSM) and by the Belgian Ministry of Public Health and Environment.

References

Carstens,E.B., Tjia,S.T. and Doerfler,W. (1979) *Virology,* **99**, 386–398.
Chakrabarti,L., Guyader,M., Alizon,M., Daniel,M.D., Desrosiers,R.C., Tiollais,P. and Sonigo,P. (1987) *Nature,* **328**, 543–547.
Desrosiers,R.C. (1988) *Annu. Rev. Microbiol.,* **42**, 607–625.
Dickson,C., Eisenman,R., Fan,H., Hunter,E. and Teich,N. (1984) In Weiss,R., Teich,N., Varmus,H. and Coffin,J. (eds), *RNA Tumor Viruses.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 513–648.
Dingwall,C. and Laskey,R.A. (1986) *Annu. Rev. Cell. Biol.,* **2**, 367–390.
Essex,M. and Kanki,P.J. (1988) *Sci. Am.,* **259**, 44–51.
Franchini,G., Gurgo,C., Guo,H.-G., Gallo,R.C., Collalti,E., Fargnoli, K.A., Hall,L.F., Wong-Staal,F. and Reitz,M.S., Jr (1987) *Nature,* **328**, 539–543.
Gelderblom,H.R., Özel,M., Hausmann,E.H.S., Winkel,T., Pauli,G. and Koch,M.A. (1988) *Micron. Microsc.,* **19**, 41–60.
Gheysen,D., Jacobs,E., de Foresta,F., Thines,D., Van Wijnendaele,F., Francotte,M., Thiriart,C., Bruck,C. and de Wilde,M. (1989) In Girard,M. and Valette,L. (eds), *Retroviruses of Human AIDS and Related Animal Diseases.* Third 'Cent-Gardes' Meeting, Pasteur Vaccins, Paris, pp. 212–215.
Grunstein,M. and Hogness,D.S. (1975) *Proc. Natl. Acad. Sci. USA,* **72**, 3961–3965.
Haseltine,W.A. and Wong-Staal,F. (1988) *Sci. Am.,* **259**, 34–42.
Henderson,L.E., Benveniste,R.E., Sowder,R., Copeland,T.D., Schultz, A.M. and Oroszlan,S. (1988) *J. Virol.,* **62**, 2587–2595.
Hockley,D.J., Wood,R.D., Jacobs,J.P. and Garrett,A.J. (1988) *J. Gen. Virol.,* **69**, 2455–2469.
Kafatos,F.C., Jones,C.W. and Efstratiadis,A. (1979) *Nucleic Acids Res.,* **7**, 1541–1552.
Katona,M., Urban,J.F., Scher,I., Kanellopoulos-Langevin,C. and Finkelman,F.D. (1983) *J. Immunol.,* **130**, 350–356.
Katsumoto,T., Hattori,N. and Kurimura,T. (1987) *Intervirology,* **27**, 148–153.
Kestler,H.W., III, Li,Y., Naidu,Y.M., Butler,C.V., Ochs,M.F., Jaenel,G., King,N.W., Daniel,M.D. and Desrosiers,R.C (1988) *Nature,* **331**, 619–621.
Kohl,N.E., Emini,E.A., Schleif,W.A., Davis,L.J., Heimbach,J.C., Dixon,R.A.F., Scolnick,E.M. and Sigal,I.S. (1988) *Proc. Natl. Acad. Sci. USA,* **85**, 4686–4690.
Kornfeld,H., Riedel,N., Viglianti,G.A., Hirsch,V. and Mullins,J.I. (1987) *Nature* **326**, 610–613.
Laemmli,U.K. (1970) *Nature,* **227**, 680–685.
Lu,A.H., Soong,M.M. and Wong,P.K.Y. (1979) *Virology,* **93**, 269–274.
Luckow,V.A. and Summers,M.D. (1988) *Biotechnology,* **6**, 47–55.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Matsuura,Y., Possee,R.D., Overton,H.A. and Bishop,D.H.L. (1987) *J. Gen. Virol.,* **68**, 1233–1250.
Miller,L.K. (1988) *Annu. Rev. Microbiol.,* **42**, 177–199.
Papsidero,L.D., Sheu,M. and Ruscetti,F.W. (1989) *J. Virol.,* **63**, 267–272.
Rein,A., McClure,M.R., Rice,N.R., Luftig,R.B. and Schultz,A.M. (1986) *Proc. Natl. Acad. Sci. USA,* **83**, 7246–7250.
Rhee,S.S. and Hunter,E. (1987) *J. Virol.,* **61**, 1045–1053.
Schultz,A.M. and Oroszlan,S. (1983) *J. Virol.,* **46**, 355–361.
Seppälä,I., Sarvas,H., Peterfy,F. and Mäkelä,O. (1981) *Scand. J. Immunol.,* **14**, 335–342.
Shields,A., Witte,O.N., Rothenberg,E. and Baltimore,D. (1978) *Cell,* **14**, 601–609.
Smith,G.H. (1978) *J. Gen. Virol.,* **41**, 193–200.
Smith,G.E. and Summers,M.D. (1978) *Virology,* **89**, 517–527.
Smith,G.E., Summers,M.D. and Fraser,M.J. (1983) *Mol. Cell. Biol.,* **3**, 2156–2165.
Smith,A.E., Kalderon,D., Roberts,B.L., Colledge,W.H., Edge,M., Gillett,P., Markham,A., Paucha,E. and Richardson,W.D. (1985) *Proc. R. Soc. Lond. B,* **226**, 43–58.
Stephens,E.B. and Compans,R.W. (1988) *Annu. Rev. Microbiol.,* **42**, 489–516.
Summers,M.D. and Smith,G.E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures.* Texas A&M University, TX, Tex. Agric. Exp. Stn Bull. Vol. 1555.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA,* **76**, 4450–4454.
Towler,D.A. and Gordon,J.I. (1988) *Annu. Rev. Biochem.,* **57**, 69–99.
Villareal,L.P. and Berg,P. (1977) *Science,* **196**, 183–185.
Wellink,J. and van Kammen,. (1988) *Arch. Virol.,* **98**, 1–26.
Yoshinaka,Y., Ishigame,K., Ohno,T., Kageyama,S., Shibata,K. and Luftig,R.G. (1980) *Virology,* **100**, 130–140.

Received on February 6, 1989; revised on May 2, 1989