Restricted HEL-12 Virus Infection in de novo Infected Human and Canine Cells

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SUMMARY

Model systems to study restricted primate retrovirus expression were established by de novo infection of canine foetal thymus cells (CF-2Th) and superinfection of HEL-12 cells with HEL-12 virus. In the resulting CF-2Th/HEL-12V cells and HEL-12/HEL-12V cells, four sequential stages of virus infection were defined by the production of reverse transcriptase (RT)-containing particles and expression of virus antigens as detected by radioimmunoassays. Stage 1 cells did not synthesize virus antigens or produce RT-containing particles. Stage 2 cells synthesized virus antigen but not RT-containing particles. Stage 3 cells synthesized antigen and produced RT-containing particles, and stage 4 cells synthesized virus antigens but no longer produced RT-containing particles. The duration of the four stage infection is 2 to 3 weeks in both cell types. Monospecific competition radioimmunoassays to detect HEL-12V p30 or gp70 antigen showed high levels of virus antigen throughout stages 2 to 4 of infection. Analysis of immunoprecipitates formed under conditions to detect either p30- or gp70-containing proteins in cells pulsed and pulsed-chased with [3H]leucine showed the same spectrum of virus precursor polyproteins, intermediates and mature virion components in stage 2 to 4 cells in canine and human infections. Spent culture fluids collected from stage 3 and stage 4 CF-2Th/HEL-12V cells failed to reveal inhibitors of RT activity. Stage 4 CF-2Th/HEL-12V or HEL-12/HEL-12V cells labelled with [3H]uridine produced virions which incorporated [3H]uridine but did not have RT activity, suggesting that restricted infection is characterized by production of HEL-12V defective in RT activity.

INTRODUCTION

The identification and isolation of type C retroviruses of primate origin are rare events. This observation is consistent with the idea that the predominant primate retrovirus–host cell interaction is non-productive. In order to delineate events responsible for such non-productive infections, we have studied de novo infection of mammalian cells with HEL-12 virus (HEL-12V).

Previously, spontaneous production of HEL-12V from HEL-12 cells has been characterized by transient virus expression as monitored by reverse transcriptase assay (RTA) and indirect immunofluorescence (Panem et al., 1977). The programme of virus expression can be

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characterized for virus particle (part) production and virus antigen (ag) production as restricted infection progresses through four sequential stages. Stage 1 is characterized by the absence of antigen expression and virus particle expression (ag-part-). During stage 2, antigen but not particle production, occurs (ag+part-). During stage 3, maximal antigen expression and spontaneous particle production occurs (ag+part+). Stage 4 is characterized by cessation of particle production and decreasing antigen expression until cell senescence (ag+part-). The entire cycle is accomplished in 140 to 160 days of in vitro growth. Although the mechanisms which regulate this restricted HEL-12V infection are presently unknown, the proviral genome persists throughout the in vitro growth of HEL-12 cells (Prochownik & Kirsten, 1977; Prochownik et al., 1979).

Efforts to biochemically characterize this phenomenon were hampered by the duration of the 140-day cycle for virus 'turn on' and 'shut down'. We now describe that restricted infection resembling spontaneous virus expression in HEL-12 cells can be achieved by de novo infection in either canine cells or ag+part- stage 4 HEL-12 cells. A full cycle of virus turn on and shut down can be accomplished in less than 4 weeks.

We have evaluated several mechanisms responsible for these restricted infections. Virus protein biosynthesis has been studied by competition radioimmunoassay and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of immunoprecipitates using antiviral sera and extracts of canine or human cells at various times following de novo HEL-12V infection. The results indicate that neither qualitative nor quantitative changes in the biosynthesis of major virus proteins are responsible for restricted virus production. However, evidence is presented for the synthesis of virions deficient in functional reverse transcriptase whose appearance coincides with virus shut down. The regulatory mechanisms which may mediate primate retrovirus-restricted infection are discussed in light of these data.

METHODS

Cells. Human embryonic lung cell strain number 12 (HEL-12 cells) which produces HEL-12 virus has been described previously (Panem et al., 1977). Uninfected dog thymus cells (CF-2Th) were obtained as frozen culture stocks from ElectroNucleonics Laboratories, Bethesda, Md., U.S.A. The growth of simian sarcoma-simian sarcoma-associated virus-producing marmoset cells (71AP1/SiSV) has been described previously (Bergholz et al., 1977).

Cells were grown in Eagles' minimum essential medium (MEM) (Flow Laboratories) containing 10% heat-inactivated foetal calf serum (FCS) (KC Biologicals, Lenexa, Kansas, U.S.A.), 100 μg/ml penicillin G and 100 units/ml streptomycin (Gibco). Confluent cultures were passaged by using trypsin-EDTA (Gibco) containing 100 μg/ml penicillin G and 100 units/ml streptomycin. Cells were propagated at 37 °C with or without 5% CO₂ in the atmosphere. The bicarbonate concentration in media was varied accordingly; regular MEM contained 2.1 mg/ml sodium bicarbonate; low MEM contained 0.67 mg/ml sodium bicarbonate. Frozen stocks were thawed at 37 °C and seeded in 25 cm² or 75 cm² plastic tissue culture flasks (Falcon, Oxnard, Ca., U.S.A.).

Viruses. The M7 strain of baboon endogenous virus (BaEV-M7) grown in A204 human cells was obtained from the Frederick Cancer Research Center, Frederick, Md., U.S.A. Simian sarcoma—simian sarcoma-associated virus (SiSV(SSAnV)) grown in 71AP1/SiSV) has been described previously (Bergholz et al., 1977).

Infection of cells. Cells were pretreated with 10 μg/ml polybrene (Aldrich Chemical Company) in regular growth media (MEM) at 37 °C for 1 h (Toyoshima & Vogt, 1969). Polybrene-containing MEM was decanted and cells were washed once with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS). Inocula were virus-containing cell supernatants
removed from HEL-12 cells during the period of maximum antigen expression and spontaneous virion production which had been clarified at 10000 rev/min for 20 min or filtered through a 0.22 μm Millipore filter. The amount of spent culture fluid used for inoculation was based on reverse transcriptase activity and cell density such that a ratio of approx. 1100 ct/min of RT activity/8 × 10^5 cells was maintained. Monolayers were inoculated with 2 ml virus inoculum for 1 h at 37 °C when fresh regular MEM was added. Virus inoculum was not removed. Cells were re-fed or passaged within 24 h. In order to maintain exponential growth, cells were passaged at 2- to 4-day intervals when cells were 75% confluent as determined by visual inspection.

Reverse transcriptase assay. Spent culture fluids were filtered through 0.22 μm Millipore filters or clarified at 10000 rev/min for 30 min and then pelleted at 100000 g for 90 min. RT activity in pellets was measured using poly(rA).oligo(dT)_{12-18} as described previously (Prochownik & Kirsten, 1976).

Antisera. Rabbit sera to SiSV(SSAV) p30 [anti-SiSV(SSAV) p30] or SiSV(SSAV) gp70 [anti-SiSV(SSAV) gp70] were prepared by immunizing rabbits with SiSV(SSAV) p30 or gp70 purified by SDS-PAGE (Reynolds & Panem, 1981). Nonidet P40 (NP40)-disrupted SiSV(SSAV) was used to prepare rabbit anti-SiSV(SSAV) as described previously (Panem et al., 1976). Preimmune rabbit serum was collected prior to all immunizations.

Radioimmunoassay. Radioimmunoassays were performed as described previously (Reynolds & Panem, 1981). Briefly, reactions were performed in duplicate using limiting antigen concentrations which were empirically determined. Antigens and antisera were prepared in 0.1 M-PBS containing 0.5 mg/ml bovine serum albumin and 0.1% NP40 (PBA buffer). Competing antigens were incubated at 37 °C for 1 h with antiserum, and a limiting amount of labelled antigen was then added for 1 h at 37 °C followed by overnight incubation at 4 °C. Formalin-fixed *Staphylococcus aureus* Cowan strain I was used to bind the antigen–antibody complexes. Complexes were washed twice in PBA buffer by centrifugation, and radioactivity in the pellets was measured in a Beckman Biogamma 4000 counter.

Results of duplicate determinations were normalized to 0% and 100% competition values. Zero% competition was defined as the radioactivity present in precipitates formed between the labelled antigen and antiserum in the absence of competitor, and 100% competition was defined as the radioactivity present in precipitates formed between labelled antigen and antiserum in the presence of maximum homologous competing antigen. Radioactivity of labelled antigen incubated with preimmune serum was approximately equal to the levels of radioactivity at 100% competition.

Competing antigens were cell extracts prepared by removing cells from tissue culture flasks after three washes with PBS cold lysis buffer [150 mM-NaCl, 1% deoxycholate, 10 mM-tris–HCl pH 7.2, 1% NP40, 0.1% SDS and 0.5 mM-phenylmethylsulphonyl fluoride (1 ml/75 ml flask)] on ice. Cell extracts were clarified for 30 min at 35000 rev/min in a Type 50 rotor in a Beckman L5-50 ultracentrifuge. Protein concentrations were determined by the method of Lowry et al. (1951).

Preparation of iodinated antigens. Antigens for radioimmunoassay were ^125^I-labelled SiSV(SSAV) p30 and ^125^I-labelled SiSV(SSAV) gp70 prepared as described by Bergholz et al. (1980) by preparative electrophoresis. The homogeneity and antigenicity of each purified protein was demonstrated by acrylamide gel electrophoresis and reaction with monospecific sera. The specific activity of labelled antigens was approx. 10^7 ct/min/μg virus protein.

[^3H]uridine uptake by virions. Cells were starved for 3 to 6 h at 37 °C in regular MEM containing 3% FCS. 10 to 20 μCi [5,6-^3H]uridine as indicated (American Corp., Arlington Heights, Ill., U.S.A.) were added to cell cultures re-fed with fresh MEM containing 3% FCS. Cultures were incubated at 37 °C for 18 to 20 h at which time the spent culture fluids were filtered through 0.22 μm Millipore filters and frozen at −70 °C. Thawed samples were
pelleted at 100,000 g for 1 h and pellets resuspended in 0.1 M NaCl, 0.01 M tris-HCl pH 7.3, 0.001 M EDTA (STE) by aspirating the pellets through 25-gauge syringes. Samples were then centrifuged through linear 20 to 70% sucrose equilibrium density gradients. Gradients were fractionated and refractive indices (R.I.) of representative fractions were measured and correlated with buoyant density. Acid-precipitable radioactivity of each fraction was determined.

**Immunoprecipitation.** Cells were de novo labelled for 15 to 20 min at 37 °C with 200 to 300 μCi/ml [3H]leucine as indicated, lysed and immunoprecipitated as described by Whiteley & Naso (1981).

**SDS–PAGE analysis of immunoprecipitates.** Immunoprecipitates were evaluated using the discontinuous SDS–PAGE system of Laemmli (1970) using a 7 to 20% acrylamide linear gradient gel with 3% acrylamide stacker. Gels were run at 15 A/gel for approx. 6 h. After fixation and drying (Fairbanks et al., 1971), gels were treated with EN3HANCE (New England Nuclear) for fluorography; radioactive bands were visualized using Cronex intensifying screens and Kodak SB X-ray film. Protein standards (ovalbumin, 45000 mol. wt.; bovine serum albumin, 68000 mol. wt.; phosphorylase B, 94000 mol. wt.; β-galactosidase, 130000 mol. wt.; myosin, 200000 mol. wt.; chymotrypsinogen, 25000 mol. wt.; and ribonuclease, 13 700 mol. wt.) were electrophoresed in parallel as mol. wt. markers.

**RESULTS**

**Establishment of restricted HEL-12V infection following de novo infection of heterologous and homologous cells**

Spent culture fluids from HEL-12 cells spontaneously producing virus were used as the inocula for de novo infection of CF-2Th cells. Aliquots of a standard virus pool were employed for all experiments. Virus in standard aliquots was quantified by RTA and a low multiplicity of infection (m.o.i.) was confirmed by examining infected cells several days post-infection for virus antigen synthesis by indirect immunofluorescence with anti-SiSV(SSAV) p30 serum. These experiments showed that less than 5% of cells were antigen-positive 2 days post-infection. Low m.o.i. was also confirmed by in situ hybridization for virus RNA transcription (data not shown). Fig. 1 shows a typical course of HEL-12V infection in CF-2Th cells as monitored by RTA. In 12 independent infections, the complete cycle of virus turn on and shut down occurred between 12 and 14 days. Peak virus production was observed 8 days post-infection after cells had been subcultured 4 or 5 times. A dramatic shut down of virus occurred within 48 h of the peak activity.

This phenomenon was not due to abortive virus infection. First, HEL-12V-infected CF-2Th cells (CF-2Th/HEL-12V), which were no longer releasing virus as determined by RTA 9 weeks post-infection, were treated with 30 μg/ml iododeoxyuridine (IdUrd) for 24 h. Induction of RT activity from these cultures occurred 3 days after IdUrd treatment. In parallel, untreated CF-2Th/HEL-12V cultured and IdUrd-treated, uninfected CF-2Th cells did not show increased levels of RT activity. These results demonstrate that CF-2Th/HEL-12V cells harbour inducible virus. Second, virus antigen biosynthesis continued in RTA-negative CF-2Th/HEL-12V cells as determined by immunoprecipitation and competition radioimmunoassay (see below).

Patterned HEL-12V infection was also established in stage 4 ag+part- HEL-12 cells by superinfection with HEL-12V. HEL-12 cells, judged virus-negative by RTA, were infected using the same procedure and inocula as for CF-2Th cells. Infection resulted in a full cycle of virus release and shut down occurring within 3 weeks, as determined by RTA, as shown in
Fig. 1. Time course of virus production in CF-2Th/HEL-12V cells monitored by RTA. CF-2Th cells were infected with HEL-12V and serially propagated as described in Methods. RT activity in spent culture fluids was measured using poly(rA).oligo(dT)$_{12-18}$ at various times post-infection and results were expressed as [$^{3}$H]dTTP ct/min/cell. The subculture passage numbers and days post-infection at which HEL-12V infection (○) and mock-infected CF-2Th cells (●) were examined are noted.

Fig. 2. Time course of virus production in HEL-12/HEL-12V cells monitored by RTA. Stage 4 HEL-12 cells were initiated from frozen stocks, superinfected with HEL-12V and serially propagated as described in Methods. RT activity was evaluated at various times post-infection (●). An aliquot of a pool of BaEV-M7 virus was included as a standard virus control in each RTA. The results are expressed as [$^{3}$H]TTP ct/min of RT activity which have been normalized to [$^{3}$H]TTP incorporation of the standard virus control. The subculture passage and days post-infection are noted.

Fig. 3. Competition RIA for SiSV(SSAV) p30 in CF-2Th/HEL-12V cells. Assays were performed as described in Methods using cellular extracts prepared from four sequential subculture generations of CF-2Th/HEL-12V cells representing three stages of HEL-12V infection. The results are expressed as $^{125}$I ct/min in the antigen–antibody precipitate or as % competition as defined in Methods. Labelled antigen was $^{125}$I-labelled SiSV(SSAV) p30 and antiserum was rabbit anti-SiSV(SSAV) p30, diluted 1:500. Cellular extracts are: ○, 71AP1/SiSV(SSAV); ○, CF-2Th/HEL-12V stage 2 ag +part−; △, mock-infected CF-2Th; □, CF-2Th/HEL-12V stage 3 ag +part+; ■, CF-2Th/HEL-12V stage 4 ag +part− (8th subculture generation); *, CF-2Th/HEL-12V stage 4 ag +part− (9th subculture generation).

Fig. 2. After subcultivation 4 to 5 times (14 days post-infection) a burst of RTA was observed followed by dramatic reduction. The reproducibility of this phenomenon was confirmed in three independent infections using different stocks of stage 4 HEL-12 cells.
Radioimmunoassays

HEL-12V expression in CF-2Th/HEL-12V cells was further examined by evaluating virus protein expression. HEL-12V belongs to the exogenous simian sarcoma–simian sarcoma-associated virus/gibbon ape lymphoma virus (SiSV/GALV) group of primate retroviruses. Immunofluorescence, competition radioimmunoassay and peptide mapping studies (Bergholz et al., 1980; Hefti et al., 1980; J. T. Reynolds & S. Panem, unpublished results) have shown that assays for the major virus core protein (p30) and envelope glycoprotein (gp70) of SiSV(SSAV) can be employed to detect HEL-12V protein expression. Monospecific radioimmunoassays were developed using purified SiSV(SSAV) p30 and envelope glycoprotein (gp70) of SiSV(SSAV) can be employed to detect HEL-12V protein expression. Monospecific radioimmunoassays were developed using purified SiSV(SSAV) p30 and gp70 antigens and well-characterized antisera (Hefti et al., 1980; Bergholz et al., 1980; Reynolds & Panem, 1981).

Cellular extracts from sequential subculture generations representing three stages of infection in CF-2Th/HEL-12V cells were examined for their ability to compete with purified $^{125}$I-labelled SiSV(SSAV) p30 for a limiting amount of rabbit anti-SiSV(SSAV) p30. As shown in Fig. 3, extracts from stages 2 to 4 competed with varying abilities in SiSV(SSAV) p30 assay as compared to the positive control, SiSV(SSAV)-infected marmoset cells [71AP1/SiSV(SSAV)] which completely displaced the labelled antigen. The negative control, mock-infected CF-2Th cells, did not compete. The levels of SiSV(SSAV) p30-related protein increased during infection; 44 $\mu$g of cellular extract was required for 50% inhibition using an extract obtained prior to peak virus production, whereas only 15 $\mu$g of extracts of cells following virus shut down was needed for comparable competition. These results were reproduced in an independent infection. Similarly, SiSV(SSAV) gp70-related protein was detected in the same extracts when used by SiSV(SSAV) gp70 RIA (Fig. 4). In this assay, the
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Fig. 6. Fluorogram of SDS-PAGE analysis of [3H]leucine-labelled stage 3 ag+part + CF-2Th/HEL-12V immunoprecipitates. Exponentially growing stage 3 CF-2Th/HEL-12V cells were pulsed (P) for 15 min with 200 μCi/ml [3H]leucine and chased (C) for 90 min as described in Methods. In vitro labelled cellular extracts from pulse or pulse-chased cells were immunoprecipitated with: (a) rabbit anti-SiSV(SSAV) p30; (b) rabbit anti-SiSV(SSAV) p30 followed by rabbit anti-SiSV(SSAV) sera; (c) anti-SiSV(SSAV); and preimmune rabbit sera. Twenty-five μl samples of immunoprecipitates containing approx. 500 to 6000 ct/min were separated on a 7 to 20% polyacrylamide gradient gel by electrophoresis at a constant current of 15 A. The positions of standard proteins are marked.

negative control, mock-infected CF-2Th cells, competed to 25%, whereas extracts from each of the three stages of infection displaced close to 100% of the purified 125I-labelled SiSV(SSAV) gp70. The levels of SiSV(SSAV) gp70-related protein increased until stage 3, when 0.1 μg cell extract protein displaced 50% of the labelled antigen. SiSV(SSAV) gp70-related antigen decreased following virus shut down, such that more than 1 μg cell extract protein was needed for 50% competition.

Cell extracts prepared from four representative cultures of HEL-12/HEL-12V cells were similarly examined in a monospecific SiSV(SSAV) p30 RIA (Fig. 5). The levels of SiSV(SSAV) p30-related protein increased as the infection progressed. Using stage 2 ag+part− cells, 53 μg protein extract was necessary to displace 50% of the labelled antigen and only 84% competition was attained at the highest concentrations tested. In contrast, extracts prepared from cells representing stage 4 ag+part− displaced 100% of the labelled SiSV(SSAV) p30 and 50% inhibition was observed with less than 5 μg protein.
(a) Preimmune

(b) 71AP1

Preimmune

Fig. 7. Fluorogram of SDS–PAGE analysis of \(^{3}H\)leucine-labelled stage 4 ag\(^{+}\)part\(^{-}\) CF-2Th/HEL-12V immunoprecipitates. Exponentially growing stage 4 CF-2Th/HEL-12V cells were pulsed (P) for 15 min with 300 \(\mu\)Ci/ml \(^{3}H\)leucine and chased (C) for 90 min as described in Methods. Immunoprecipitates (1000 to 10000 ct/min) were analysed as described in Fig. 6. Extracts were immunoprecipitated with: (a) rabbit anti-SiSV(SSAV) p30 followed by rabbit anti-SiSV(SSAV) sera; (b) rabbit anti-SiSV(SSAV) p30; and preimmune rabbit sera. An aliquot of \(^{35}S\)methionine pulse-labelled 71AP1/SiSV immunoprecipitated with rabbit anti-SiSV(SSAV) serum (71AP1) served as a marker for standard virus proteins.

**Immunoprecipitation and SDS–PAGE**

Retrovirus proteins are typically synthesized as two major polyprotein precursors which are cleaved, via a series of intermediates, to form the structural proteins of the virus as well as non-structural products (for review, see Stephenson, 1980). While the previous RIA experiments show that SiSV(SSAV) p30- and gp70-related antigens are found in stage 2, 3 and 4 cells, they do not distinguish whether proteins detected by RIA in different stages of infection represent the same or different forms containing p30 and gp70 determinants. De novo \(^{3}H\)leucine-labelled virus proteins in CF-2Th/HEL-12V cell extracts were therefore examined by immunoprecipitation followed by SDS–PAGE analysis.

Fig. 6 and 7 present the distribution of virus protein in immunoprecipitates of stage 3 ag\(^{+}\)part\(^{+}\) and stage 4 ag\(^{+}\)part\(^{-}\) cell extracts respectively. Precursors, intermediates and mature SiSV(SSAV) p30-related proteins were identified by specific immunoprecipitation with anti-SiSV(SSAV) p30 sera. To identify SiSV(SSAV) gp70-related proteins, the labelled cellular extract was first immunoprecipitated by anti-SiSV(SSAV) p30, and the resulting cleared supernatant was immunoprecipitated a second time using antisera to whole disrupted SiSV(SSAV). In parallel, 71AP1/SiSV cell extracts were examined. HEL-12 virus-specific proteins were therefore identified by the following criteria: (i) immunoprecipitation by
Restricted HEL-12 virus infection

Table 1. Reduction of virion-associated reverse transcriptase activity by spent culture fluids*

<table>
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<tr>
<th>Culture fluids from:</th>
<th>Reduction in reverse transcriptase activity of BaEV-M7</th>
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<tr>
<td></td>
<td>1X</td>
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<tr>
<td>Control, fresh growth medium</td>
<td>4 °C 1.0 1.0</td>
</tr>
<tr>
<td>CF-2Th/HEL-12V stage 3 ag+part+</td>
<td>4 °C 2.6 2.4</td>
</tr>
<tr>
<td>CF-2Th/HEL-12V stage 4 ag+part+</td>
<td>4 °C 1.8 2.0</td>
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* Fresh growth medium or spent culture fluids removed from CF-2Th/HEL-12 cells during stage 3 or stage 4 were sequentially filtered through 0.1 μm and 0.5 μm filters and incubated with aliquots of BaEV-M7 generating 100,000 (1X) or 200,000 (2X) ct/min acid-precipitable radioactivity in standard reverse transcriptase tests. The samples, incubated at either 4 °C or 37 °C for 30 min, were pelleted by centrifugation at 39000 rev/min for 1 h in type 50Ti at 4 °C and then assayed for reverse transcriptase activity as described in Methods. Data are the average results of duplicate determinations and are expressed as the fold reduction of activity relative to samples incubated with control growth medium.

immune, but not preimmune sera; (ii) immunoprecipitation by immune sera from infected cells but not from uninfected cells; (iii) co-migration with virus-specific bands of immunoprecipitates formed between immune sera and 71AP1/SiSV control cells. HEL-12 proteins are denoted according to the nomenclature for SiSV(SSAV) established from peptide mapping analysis of polyprotein precursors and cleavage products (R. Naso, personal communication; C. M. Bergholz et al., unpublished results). The precursor proteins of HEL-12 p30 and gp70 have also been confirmed by peptide map analysis (S. A. Whiteley et al., unpublished results). Comparison of immunoprecipitates of stage 3 ag+part+ cultures (Fig. 6) with immunoprecipitates of stage 4 ag+part- cultures (Fig. 7) revealed that the same SiSV(SSAV) p30 and gp70 precursors, intermediates and products were synthesized in both cultures. The SiSV(SSAV) p30 precursor, Pr80, identified in pulse-labelled extracts with anti-SiSV(SSAV) p30 sera disappeared in chase extracts with mature p30 appearing. The p30 precursors, Pr60 and Pr40, could be seen in both pulse and pulse–chased extracts. The p30 intermediates, P55, P45 and P38 were also apparent. SiSV(SSAV) gp70-related proteins were visible in immunoprecipitates using antiserum to whole disrupted SiSV(SSAV). The gp70 precursor, gPr80, appeared in both the pulse and chase, whereas the level of mature gp70 increased in the chase. No virus-specific bands were precipitated by preimmune rabbit serum. Comparable results were obtained for stages 3 and 4 HEL-12/HEL-12 cells, i.e. the same forms of virus proteins were found at both stages of infection (data not shown).

Analysis of HEL-12V infected cells for inhibitors of RT activity and virion production

The periodic absence of RT activity in culture fluids may represent production of factors which inhibit the RT activity and/or the absence of virus production. CF-2Th/HEL-12V cells at the peak of RT activity (stage 3) or after shut down (stage 4) were therefore examined for the presence of a factor in the supernatant which inhibits RT activity. Spent culture fluids or control growth medium were sequentially filtered through 0.1 μm and 0.05 μm filters to remove virus particles. Two ml amounts of these samples were then incubated at 4 °C or 37 °C with aliquots of BaEV-M7 previously determined to generate either 100,000 or 200,000 ct/min acid-precipitable radioactivity in a standard RTA. Incubated samples were pelleted by ultracentrifugation and examined for RT activity. Table 1 shows the RT activities of experimental samples normalized to those incubated with control, fresh growth medium. Virus incubated with spent culture fluids from either stage 3 or stage 4 production periods showed
Table 2. Correlation of particle-associated reverse transcriptase and uridine incorporation*

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<tr>
<td>CF-2Th/HEL-12V</td>
<td>Stage 3 ag$^+$part$^+$</td>
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<td>Stage 4 ag$^+$part$^-$</td>
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<td>0.3</td>
<td>22</td>
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* At representative subculture generations, replicate flasks were seeded with equal numbers of CF-2Th/HEL-12V or HEL-12/HEL-12V cells. Spent culture fluids were removed from one flask for determination of $[^3]H$TPP ct/min of RT activity in a standard RTA. The second flask was incubated in the presence of $[^3]H$-uridine ($[^3]H$Urd) overnight as described in Methods for determination of incorporation of $[^3]H$Urd into virus RNA. Data are typical results of multiple determinations using several independent infections.

1.8- to 2.6-fold reduction, at either 4 °C or 37 °C. Differential production of an RT inhibitor in significant concentrations was not detected.

The possibility that virions lacking RT are produced was evaluated by comparing enzymic activity versus virus particle production in HEL-12V-infected CF-2Th or HEL-12 cells. Virion production was monitored by $[^3]H$uridine uptake into particles which band at the characteristic retrovirus density of 1.16 to 1.18 g/ml in sucrose equilibrium gradients. As pilot experiments showed that all sedimentable RT activity bands at the buoyant density, 1.16 to 1.18 g/ml, RT determinations were performed on pellets following clarification and ultracentrifugation of spent culture fluids. The culture fluids used for RTA were from parallel replicate cultures of those used for $[^3]H$uridine uptake. The amount of uridine uptake in the virus density range was calculated and compared to RT values. Results are presented as the ratio of RT activity to $[^3]H$uridine incorporation (Table 2). During a cycle of turn on and shut down of virus production, this ratio decreased 4-fold in the CF-2Th/HEL-12V cells and 3.5-fold in HEL-12/HEL-12V cells. The pattern was reproduced in three independent infections. The data demonstrate that virion-associated RT activity declines during a period in which $[^3]H$uridine continues to be incorporated into virions.

To ensure that the RT activity being detected in spent culture fluids is associated with virions incorporating $[^3]H$uridine, the following experiment was designed. Virus was purified from spent culture fluids from CF-2Th/HEL-12V cultures at peak virus production (stage 3 ag$^+$part$^+$) by sucrose equilibrium gradient centrifugation. $[^3]H$uridine-labelled cell supernatant from a replicate culture was included in the preparation as a marker and after centrifugation, the peak of $[^3]H$uridine incorporation appeared at 1.18 to 1.16 g/ml. These fractions were pooled and used in an endogenous reverse transcriptase reaction where $[^{32}P]dCTP$ was used to label the product. After phenol extraction, the resulting HEL-12V $[^{32}P]$labelled complementary DNA ($[^{32}P]$cDNA) was separated from unincorporated $^{32}P$ on a Sephadex G50 column and alkali-treated to remove RNA.

The virus specificity of the HEL-12V $[^{32}P]$cDNA was then assessed by liquid hybridization with poly(A)-selected SiSV(SSAV) RNA. In parallel, SiSV(SSAV) $[^{32}P]$cDNA prepared from 70S SiSV(SSAV) RNA using avian myeloblastosis virus reverse transcriptase was employed as a control. Both probes were hybridized in duplicate to an excess of poly(A)-selected SiSV(SSAV) RNA or yeast RNA in 50% formamide/0.4 M-NaCl for 15.5 h at 45 °C. The extent of hybridization was determined by assaying resistance to S1 nuclease digestion. SiSV(SSAV) $[^{32}P]$cDNA hybridized to 54% with SiSV(SSAV) RNA. In contrast, SiSV(SSAV) $[^{32}P]$cDNA and yeast RNA hybridized to 11.5%. HEL-12V $[^{32}P]$cDNA
hybridized with SiSV(SSAV) RNA to 63.5% and 15.5% with yeast RNA. After subtraction of hybridization with yeast RNA, SiSV(SSAV) $^{[32P]}$cDNA hybridized to 42.5% with SiSV(SSAV) RNA and HEL-12V $^{[3ZP]}$cDNA hybridized 48.0% with SiSV(SSAV) RNA.

**DISCUSSION**

We describe the establishment and preliminary characterization of HEL-12V-infected canine or superinfected HEL-12 cells as models to study regulation of non-productive primate retrovirus infection. Spontaneous production of HEL-12V from HEL-12 cells was observed to be restricted to a specific 30 day period during *in vitro* growth. This phenomenon provided an appropriate model for the study of non-productive retrovirus–host cell interactions; however, the 5 month course of infection prevented efficient analysis. Events resembling spontaneous virus expression in HEL-12 cells occurred within 2 weeks of infecting CF-2Th cells. Transient virus production was not due to abortive infection as shown first by IdUrd induction of RT-positive virus from non-producer CF-2Th/HEL-12V cells and second, by persistence of *de novo* virus antigen synthesis after virus shut down.

Similarly, restricted HEL-12V infection was established in homologous HEL-12 cells in which spontaneous production of HEL-12V had ceased. HEL-12 cells were originally described to senesce after 160 to 180 days of *in vitro* growth (Panem et al., 1977), but responded to careful culturing past this crisis interval most probably due to selection of a particular cell type. These stage 4 cells are ag$^+$part$^-$ as judged by competition RTA for virus antigens, negative RTA and the absence of virion production as measured by gradient analysis of spent fluids of $^3$H]uridine-labelled cells. Although stage 4 HEL-12 cells are ag$^+$, superinfection by HEL-12V occurred. Superinfection was possible most probably because virus gp70 is absent from the cell surface. This hypothesis is supported by findings that stage 4 cells are not killed by a cytotoxic anti-SiSV(SSAV) gp70 serum (C. Bergholz, personal communication) which lyses virus-producing stage 3 HEL-12 cells (Bergholz et al., 1980). This observation suggests differences in the location or presentation of HEL-12V antigens in stage 3 and 4 cells. Full cycles of virus production and shut down occur in less than 3 weeks in HEL-12/HEL-12V and CF-2Th/HEL-12V cells and therefore provide convenient model systems to study non-productive primate retrovirus infection in addition to those described previously (Markham et al., 1979).

Although the generality of restricted retrovirus infection has yet to be determined, several observations of retrovirus expression in human diploid cells should be noted. First, preliminary experiments with SiSV(SSAV) infection of diploid human placenta cell strains revealed decreasing focus-forming virus production per cell with prolonged cell passage (C. Bergholz & E. Hefti, unpublished observations). Second, temporarily restricted spontaneous retrovirus expression has been noted for a second strain of human diploid lung fibroblasts (Panem et al., 1975), and third, patterned virus expression was observed in each of eight single cell clones derived from stage 1 HEL-12 cells during the first week of *in vitro* cultivation (Panem, 1978). These observations suggest that restricted expression reflects the normal virus–cell interaction and is not the result of cellular mutation. Furthermore, reproducible initiation of restricted HEL-12 infection in different mammalian cell types suggests that the regulation of virion production is at least in part virus-determined. These findings resemble studies with Friend murine leukaemia virus which showed that virus expression was modulated during *in vitro* propagation (Berkower et al., 1980).

To approach the mechanism of virus restriction we first examined the biosynthesis of major virus structural proteins. Initiation and shut down of virion release may be correlated with (i) critical concentrations of one or all virus proteins or (ii) appearance of an altered form of protein due to cleavage defects. Quantitative virus protein expression was measured by monospecific RIAs for p30 and gp70. As HEL-12 and SiSV(SSAV) share extensive
cross-reactivity (Panem et al., 1975; Bergholz et al., 1980) we used SiSV(SSAV) reagents because HEL-12 cells do not replicate HEL-12V to high titre, and HEL-12V was therefore not available for preparation of purified antigens. RIA analysis of CF-2Th/HEL-12V and HEL-12/HEL-12V cells revealed consistently high antigen expression throughout infection; in fact, virus proteins are synthesized well in advance of the onset of virus production (two subculture generations). The significance of this observation remains to be investigated. The data suggest that gross alterations in quantitative protein expression of major virus structural components do not alone regulate virion release. However, critical concentrations of a virus protein not measured by these assays may be important in regulating virus expression.

Immunoprecipitation experiments to assess qualitative virus protein expression demonstrated that the same molecular forms of SiSV(SSAV) p30 and gp70 occurred in all stages of HEL-12/HEL-12V growth. Although precursor/product ratios were not evaluated, finding the same antigens in stage 3 ag\(^+\)part\(^+\) and stage 4 ag\(^+\)part\(^-\) cells indicates that de novo virus protein synthesis and processing occurs following virus shut down. Although continued antigen expression following virus shut down is reminiscent of spontaneous antigen expression in stage 4 HEL-12 cells, the de novo infections described here differ significantly from spontaneous virus expression. When CF-2Th/HEL-12V and HEL-12/HEL-12V cultures were cultured beyond the shut down of virus production, a second cycle of virus production and shut down was observed (data not shown). In contrast, reactivation of HEL-12 virus from HEL-12 cells was never seen, even in these stage 4 cells used here which were cultured for 2 years past the crisis interval.

We employed RT as a quantitative measure of virus production because supernatant RT co-sediments with virions which incorporate \(^{3}H\)uridine at 1.16 to 1.18 g/ml in sucrose equilibrium gradients. It was surprising that the ratios of RT activity/\(^{3}H\)uridine uptake, calculated for each subculture generating during infection, decreased as virus production shut down. Although reverse transcriptase activity is reduced, the incorporation of \(^{3}H\)uridine into virions is unaltered and virion production persists. Four possibilities may explain these findings: (i) production of virions containing functionally defective reverse transcriptase; (ii) production of virions without reverse transcriptase; (iii) improper packaging of reverse transcriptase into virions; and/or (iv) production of an inhibitor for reverse transcriptase in HEL-12/HEL-12V or CF-2Th/HEL-12V culture fluids. We did not detect an inhibitor of RT in culture fluids, although RT inhibitors have been isolated from some human tissues (Nelson et al., 1981).

The hypothesis that an aberrant reverse transcriptase is produced or that enzyme is inappropriately packed into HEL-12 virions has precedence in studies with Moloney murine leukaemia virus (MoMuLV). Several NIH-3T3 cells, cloned immediately following MoMuLV infection, produce virions lacking reverse transcriptase (Shields et al., 1978). Analysis of virus protein synthesis in these cells showed that a 130000 mol. wt. polyprecursor of reverse transcriptase was not synthesized. Pitha et al. (1980) have observed the production of defective virions in interferon-treated MoMuLV-infected cells which contain an uncleaved polyprotein precursor of reverse transcriptase. This is especially interesting in light of reports that endogenous interferon may mediate non-productive expression of murine retroviruses (Barré-Sinoussi et al., 1979). Analysis of interferon-mediated inhibition of MuLV suggests that interferon affects the accumulation of virus proteins and the proper assembly of virions at the cell surface (Wu et al., 1976; Bandyopadhyay et al., 1979). We are currently evaluating the role of interferon in HEL-12 virus expression, especially in view of differences in the ability of anti-SiSV(SSAV) gp70 to mediate cytolysis during all stages of virus infection.

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