

Gene expression of Feline leukemia virus(FeLV) in cat kidney cells with radioimmunoassay using beta-emission of ^{131}I

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요오드 ^{131}I 의 beta-emission 을 이용한 면역방사성표지법에 의한 Feline leukemia virus 의 유전자 발현에 관한 연구

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ABSTRACT

Synchronized cat kidney cells chronically infected with Feline leukemia virus (FeLV) were used to study virus production, the synthesis of group specific antigen (*gag*) and envelope (*env*) proteins, the expression of *env* protein on the cell surface during the cell cycle, and the stability of viral RNA. As detecting method, we developed the radioimmunoassay(RIA) system using beta-emission of ^{131}I and demonstrated the validity of this system by comparison with routine RIA system using gamma-emission of ^{125}I . The produced virus was analysed by developed RIA system and the amount of virus released into the medium by synchronized cells during a 60min interval was determined by measuring reverse transcriptase activity. The results show that infected cells produce the complete virus particle containing products of *gag*, *env* and *pol* genes of FeLV, and maximum virus production occurs during mitosis of synchronized cells. Labeling of the cell surface of synchronized cells with ^{131}I shows that the amount of gp70^{env} on the cell surface parallels cellular growth. Therefore, the cell cycle-dependent release of virus is not accompanied similar variations in the amount of viral envelope protein on the cell surface. Competition RIA of synchronized cells with ^{131}I -labeled viral proteins was used to measure viral protein synthesis during the cell cycle. The rate of synthesis of *gag* protein shows three peaks, corresponding to the G₁, late S and late G₂ phases of cell cycle. But the rate of synthesis of *env* protein dose not change, suggesting that in these cells the synthesis of these two gene products is controlled seperately. In Actinomycin D treated cells, the synthesis of viral proteins decreased sharply from 8 hours after treatment, and the late S and G₂ peaks of *gag* protein synthesis were disappeared. This shows the stability of viral RNA for about 6 hours in the absence of continuing viral RNA synthesis.

INTRODUCTION

RNA tumor viruses contain a single-stranded diploid RNA genome which is positive stranded and approximately 9,000 nucleotides long (sedimentation value 38S) (Baltimore, 1974; Beemon, 1977; Fan, 1977). This 38S RNA codes for three classes viral structural proteins: the internal structural proteins (products of the *gag* gene), envelope glycoproteins (products of *env* gene), and reverse transcriptase (product of the *pol* gene) (Baltimore, 1974). In addition, viruses that morphologically transform fibroblasts encode a protein for this transformation (a product of the *src* gene) (Purchio, *et al.*, 1978; Erikson, *et al.*, 1978; Santos, *et al.*, 1982; Esther, *et al.*, 1982). No other viral proteins have yet been identified, although it is possible that nonstructural viral proteins could exist.

The patterns of synthesis and expression of viral components in synchronized mouse and chicken cells chronically infected with retroviruses have been shown to vary during the cell cycle (Cikes and Frieberg, 1971; Humphries and Coffin, 1976; Leong, *et al.*, 1972; Naso and Brown, 1977; Paskind, *et al.*, 1975; Schauf and Panem, 1976; Balazs and Caldarella, 1981).

This report examines the pattern of synthesis of FeLV *gag* and *env* proteins, the expression of *env* protein on the cell surface, and virus production during the cell cycle and the stability of viral RNA in FeLV infected cat kidney cells.

Radioimmunoassay developed by Yalow and Berson (1956) is the very sensitive method which can detect below nanogram quantity of particular molecules in heterogeneous sample. RIA has been more sophisticated by introducing various efficient iodination methods (Greenwood, *et al.*, 1963; Marchalonis, 1969; Pamela

and Speck, 1978) and RIA has become indispensable to the endocrinologists and the virologists. Radioisotopes used for RIA are mainly ^{131}I and ^{125}I , but recently ^{125}I usage is preferred because of several physical advantages. Considering that there is no supply of ^{125}I in Korea and ^{131}I is not only gamma-emitter but also beta-emitter, we developed the RIA system using beta-emission of ^{131}I and demonstrated the validity of this system to study the viral gene expression in this report.

MATERIALS AND METHODS

Cells and viruses. The lian strain of FeLV which was used for purification of viral proteins and for infection was grown in a cat embryo cell line (FL-74). Kidney cells from new born domestic cats were infected with FeLV and Feline sarcoma virus (FeLV) and transformed cells were maintained in Eagle's minimal essential medium supplemented with 10% new born calf serum.

Purification of virus and viral proteins. The lian strain of FeLV was grown in a monolayer culture of a cat embryo cell line (FL-74), and purified by two cycles of sucrose density gradient (Rho and Gallo, 1979). Around 2mg of purified FeLV was disrupted by incubation at 37°C for 30min with buffer containing 0.05M Tris-HCl (pH 8.9), 0.01M EDTA, 1M NaCl, and 0.5% Triton X-100, and then centrifuged at 30,000rpm for 30min. The supernatant was dialysed against 0.01M N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid-NaOH at pH 6.5, 0.001M EDTA, and 0.1% Triton X-100 at 4°C for 18 hr, and loaded on a phosphocellulose column (pll) equilibrated with the same buffer. Proteins were eluted with 0.0-1.0M linear KCl gradient and the elution patterns of p30 and gp70 were determined by radioimmunoassays (Stepheson, *et al.*, 1976; Hino, *et al.*, 1977).

Iodination of viral proteins. Purified viral proteins were radiolabeled with ^{125}I or ^{131}I by the chloramine-T method (Greenwood, *et al.*, 1963). The viral proteins ($1\mu\text{g}$) were labeled in a $100\mu\text{l}$ reaction mixture containing 1mCi of ^{125}I (New England Nuclear) or ^{131}I and $50\mu\text{g}$ of chloramine-T. The free iodine and labeled proteins were separated by gel filtration with Bio-Gel P-10 column (Rho and Gallo, 1980). The radioactivity of ^{125}I was measured by Packard gamma-scintillation counter and that of ^{131}I measured in scintillation fluid by Packard Tri-Carb liquid scintillation spectrometer adjusted C-D channel at 5% amplification throughout these studies.

SDS-PAGE analysis. Purified viral proteins were analysed on the 10% slab gel and stained with Coomassie blue as described by Laemmli (1970). Iodinated viral proteins and immune precipitates were analysed on the 10% cylindrical polyacrylamide gel containing 1% sodium dodecyl sulfate. After electrophoresis, the gel was sliced into 2mm and the radioactivity was determined.

Radioimmunoassays. Radioimmunoassays were performed by the double antibody method as described (Rho and Gallo, 1980). Serial two-fold dilutions of goat antisera were incubated with iodinated proteins (approximately, $15,000\text{cpm}$) for 2hr at 37°C and further overnight at 4°C . A 20-fold excess of anti-goat rabbit serum (Cappel Inc., Pa.) was then added and incubated 2hr at 37°C followed by 1hr at 4°C . The incubation mixtures were diluted to 1ml with buffer and centrifuged at $8,000\text{rpm}$ for 4min, and the percent radioactivity bound in the pellets determined by gamma- and beta-counters. In competition radioimmunoassays, serial dilutions of competing antigens were preincubated with a limiting amount of antisera capable of precipitating of the labeled proteins before addition of the labeled antigens. Further steps of the procedure were the same

as described above for standard radioimmune precipitations. In quantitative radioimmunoassays, the known amount of competing antigens was used.

Cell synchronization. Nearly confluent cultures of FeLV-infected cells were synchronized by mitotic arrest with $0.03\mu\text{g/ml}$ of Colcemid (Naso, *et al.*, 1967). After 3hr treatment, cells were suspended in complete growth medium without drug and seeded in petri-dishes at 1-hr interval. Cell cycle was examined by pulse-labeling of synchronized cells with $[^3\text{H}]$ -thymidine ($5\mu\text{Ci/ml}$).

Virus assays. Synchronized cells were rinsed twice with warm medium and allowed to grow for additional 1hr and medium was removed and cleared of cells and debris and then virus was pelleted by centrifugation at $50,000\text{rpm}$ for 30min. The amount of virus was determined by the reverse transcriptase assay (Rho and Gallo, 1980). The pellet was suspended in 0.1ml reaction mixture containing 50mM Tris-HCl (pH 7.9), 1mM DTT, 0.05mM MnCl_2 , 0.01% Triton X-100, 20% glycerol, $10\mu\text{g}$ BSA, $1\mu\text{g}$ of poly(A)-oligo(dT), $15\mu\text{M}$ of $[^3\text{H}]$ -TPP (50Ci/m mol). The reaction mixture was incubated at 37°C for 30min and the synthesized polymer was measured on Whatman DEAE-cellulose (DE-81) paper disks.

Cell surface labeling with ^{131}I . Synchronized cells were collected from flasks at 1-hr interval, washed with phosphate-buffered saline, and radiolabeled with ^{131}I by iodogen method (Pamela and Specks, 1978).

RESULTS

Purification of viral proteins. Since the purity of antigens is the critical factor which can determine the sensitivity of RIA, the eluted gp70 ($0.15\sim 0.20\text{M}$ KCl) and p30 ($0.3\sim 0.4\text{M}$ KCl) from phosphocellulose (p11) column were analysed by polyacrylamide gel electrophor-

esis in the presence of 1% sodium dodecyl sulfate. As shown in Fig. 1 and inserted figure in Fig. 4, gp70 and p30 were presented as one band corresponding to molecular weight 70,000 and 30,000 respectively, and the purity appeared to be greater than 90% pure. These purified FeLV gp70 and p30 were used as antigens for RIA throughout these studies.

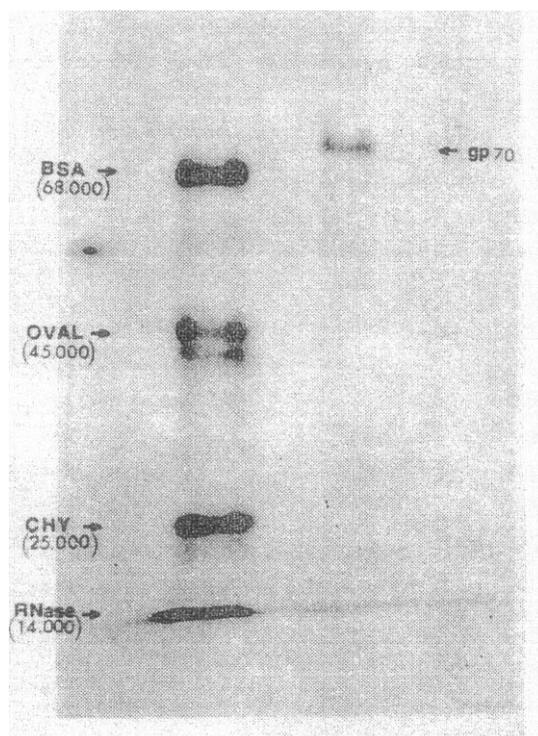


Fig. 1. SDS-polyacrylamide gel electrophoresis of FeLV gp70. Purified FeLV gp70 by phosphocellulose column was subjected to electrophoresis on a 10% slab gel, and the gel was stained with Coomassie blue. The following proteins were used as markers: bovine serum albumin(BSA) (68,000): ovalbumin(Oval) (45,000): chymotrypsinogen(Chy) (25,000): RNase A(14,000).

Comparison of RIA system using beta-emission of ^{131}I to RIA system using gamma-emission of ^{125}I . We developed the RIA system using beta-emission of ^{131}I to study the viral gene expression. As described in the Methods, the beta-emission of ^{131}I was measured effectively by liquid scintillation counter adjusted C-D channel at 5% amplification. To demonstrate

the validity of this developed RIA system, we compared specific activity, gel filtration profile, immunoprecipitation pattern, and SDS-PAGE analysis pattern of ^{125}I -labeled FeLV p30 with those of ^{131}I -labeled p30. Furthermore, the sensitivity and the efficiency of this system in competition RIA were compared to those of RIA system using ^{125}I .

First, purified p30 was radiolabeled either with ^{125}I or ^{131}I , and were separated from free iodine by Bio-Gel p-10 column. Gel filtration profiles of either ^{125}I - or ^{131}I -labeled p30 obtained by the radioactivity of 50 μl aliquot of each fraction (Fig. 2) were the same in both system. For further characterization of each fraction, we immunoprecipitated with anti-p30 gcat serum. As shown in Fig. 3, the immunoprecipitation pattern demonstrated that only 9th fraction had labeled p-30 in both system. About 100,000cpm of ^{125}I - and ^{131}I -labeled p30 were precipitated by 10% TCA and were applied to 10% cylindrical polyacrylamide gel at 2.5mA/gel. As presented in Fig. 4, the labeled probes migrated as a single, sharp peak of 30,000 molecular weight in both system whereas ^{131}I -labeled p30 were slightly lower in radioactivity than ^{125}I -labeled p30 because of quen-

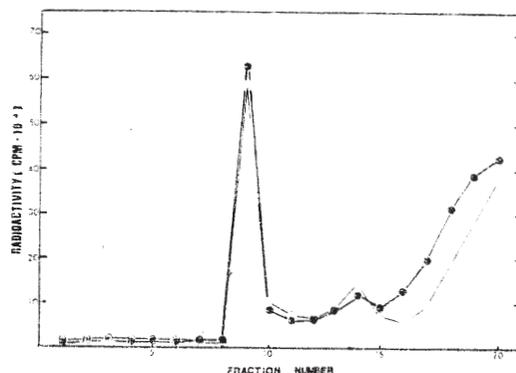


Fig. 2. Gel filtration profiles of iodinated p30. Iodinated p30 with ^{125}I (●) and ^{131}I (○) was separated from free iodine by Bio-Gel P-10 column. Radioactivity of each fraction was counted by beta- and gamma-counter as described in the text.

ching effect in counting the gel. Furthermore, the specific activity of ^{125}I - and ^{131}I -labeled p30 were very similar. In subsequent experiment, we checked the ability of anti p30 goat serum to precipitate ^{125}I - and ^{131}I -labeled p30.

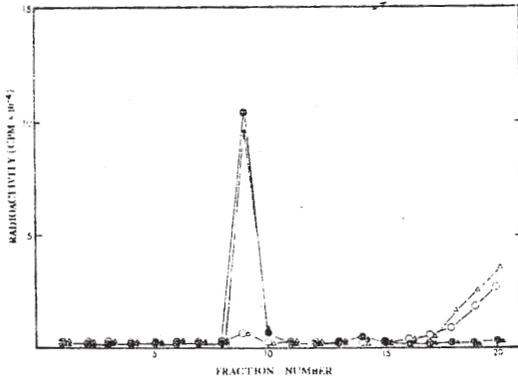


Fig. 3. Immunoprecipitation patterns of gel filtration fraction. $2\mu\text{l}$ aliquot of each fraction was immunoprecipitated with anti-p30 serum as described in the text. The precipitates (ppt) (●) and supernatants (spt) (○) of ^{125}I were counted by gamma counter so ppt (▲) and spt (△) of ^{131}I counted by beta-counter.

As illustrated in titration curve (Fig. 5. A), the antiserum titer which could precipitate 50% of labeled p30 were the same (1 : 2,500) in both system. Using this titered antiserum, competition RIA of labeled-p30 with disrupted FeLV were performed to evaluate for their

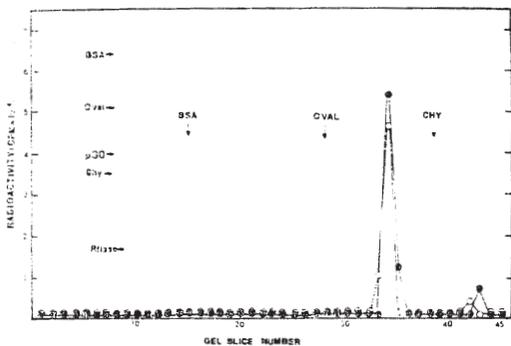


Fig. 4. SDS-PAGE analysis of ^{125}I - and ^{131}I -labeled FeLV p30. Iodinated p30 with ^{125}I (●) and ^{131}I (○) was diluted to radioimmunoassay buffer and around 100,000cpm of labeled p30 was applied to 10% cylindrical polyacrylamide gel at 2.5mA/gel. After electrophoresis the gel was sliced and the radioactivity was measured. The inserted figure shows SDS-PAGE analysis of purified FeLV p30.

ability to compete for limiting amounts of anti-p30 serum.

As illustrated in Fig. 5. B, the competition was started at the presence of subnanogram quantity of competing antigen in both system but the sensitivity of developed system was lower than that of RIA system by ^{125}I . However, this degree of sensitivity acquired by

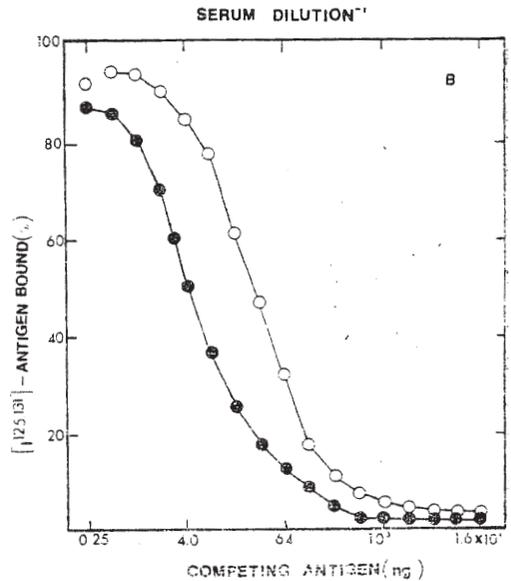
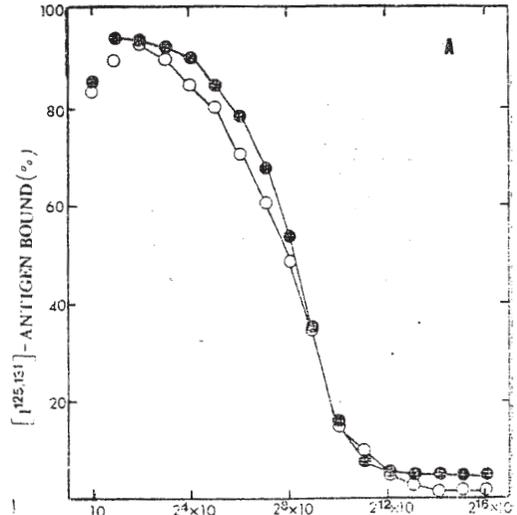


Fig. 5. Competition radioimmunoassay for ^{125}I - and ^{131}I -labeled p30 with disrupted-FeLV. Around 10,000cpm of ^{125}I (●) and ^{131}I (○)-labeled p30 were incubated with the known amount of disrupted-FeLV in the presence of 50% precipitable anti-p30 serum.

this system is enough to study the viral gene expression.

Immunological analysis of viral particles produced from FeLV-infected cells. When cultures were positive for virus production, we tested the pelleting virus particles for its completeness with RIA. The virus pellet disrupted by Triton X-100 were used as competing antigen, competition RIA was performed in the presence of ^{125}I -labeled p30 and gp70. In Fig. 6, we observed the effective competition of virus particles with p30 and gp70 and therefore knew that the FeLV-infected cells produced complete virus particle containing products of *gag*, *env* and *pol* genes of FeLV.

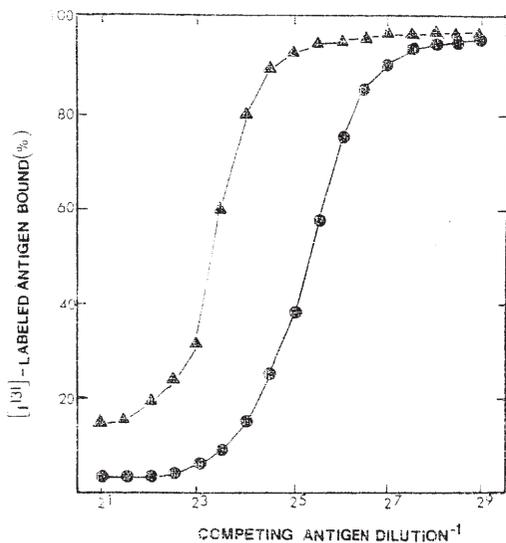


Fig. 6. Competition radioimmunoassays of virus particles produced from FeLV-infected cells with FeLV p30 and gp70. Around 10,000cpm of ^{125}I -labeled p30(▲) and gp70(●) were incubated with serial two-fold dilutions of viral particles.

Virus production during the cell cycle. Medium was collected from synchronized cells at hourly intervals, and the amount of virus produced during the cell cycle was quantitated by measuring the reverse transcriptase activity. The result indicated that in infected cells the maximum rate of virus production occurred during the first hour after post release and during an interval coinciding with the next G_2

and M periods(Fig. 7).

Expression of gp70 on the cell surface during the cell cycle. To detect the cell surface-associated FeLV gp70, the cell surface proteins of synchronized cells at 1-hr interval were labeled with ^{125}I and lysed. The same amount of cellular proteins was immunoprecipitated with anti-gp70 serum and fractionated on the cylindrical polyacrylamide gel and peak fractions of each synchronized cells were localized. The total counts of the peaks was interpreted as the amount of gp70 on the cell cycle. As shown in Fig. 8, it was found that the amount of gp70 on the cell surface did not change during the cell cycle.

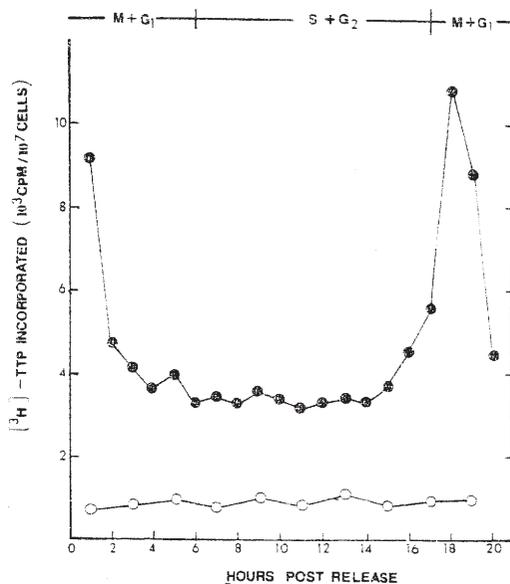


Fig. 7. Virus production during the cell cycle. The amount of virus produced during the cell cycle was quantitated by RT activity(●). As control, uninfected cat kidney cells were used(○).

Synthesis of gag and env proteins during the cell cycle. Synchronized cells were lysed, reacted with titered anti-p30 and anti-gp70 serum and competition radioimmunoassays were performed respectively. The result was interpreted as the relative rate of synthesis of *gag* and *env* proteins during the cell cycle. As presented in Fig. 9, the rate of synthesis of

gag protein in infected cells changed during the cell cycle and there were three peaks, corresponding approximately to G₁, late S and late G₂ periods. But the rate of synthesis of *env* proteins did not change during the cell cycle.

Effects of ActD on the synthesis of viral proteins during the cell cycle. Synchronized cells were treated with ActD (0.01 μg/ml), and the synthesis of viral proteins during the cell cycle was investigated by the same procedures as above. At this concentration of ActD, the cytotoxic effects were minimal at least for 20hr (Messer, *et al.*, 1981) but virion RNA synthesis was inhibited by 90% within 2hr after addition of the drug. The result have shown that in ActD treated cells the synthesis of viral proteins sharply decreased from 8hr after treatment and the late S and late G₂ peaks of *gag* protein synthesis were disappeared (Fig. 9). And this result also suggest that viral mRNA is functional for about 6hr in the absence of-

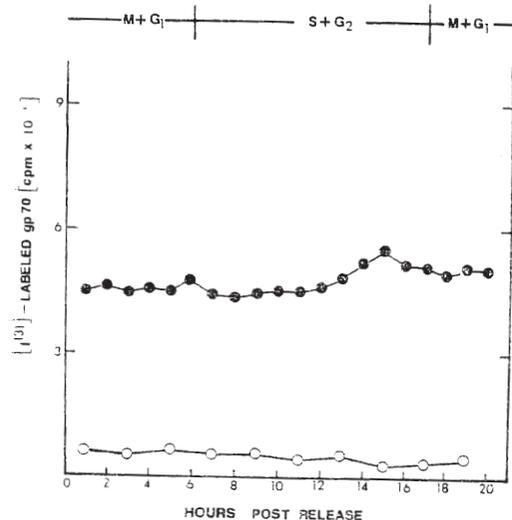


Fig. 8. Expression of gp70 on the cell surface during the cell cycle. Synchronized cells labeled with ¹²⁵I were lysed and precipitated with anti-gp70 serum. Viral proteins were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gels. The band of ¹²⁵I-labeled gp70 was localized and excised. The amount of radioactivity in each band was measured by liquid scintillation counter. As control, uninfected cells were used (○).

continuing viral RNA synthesis.

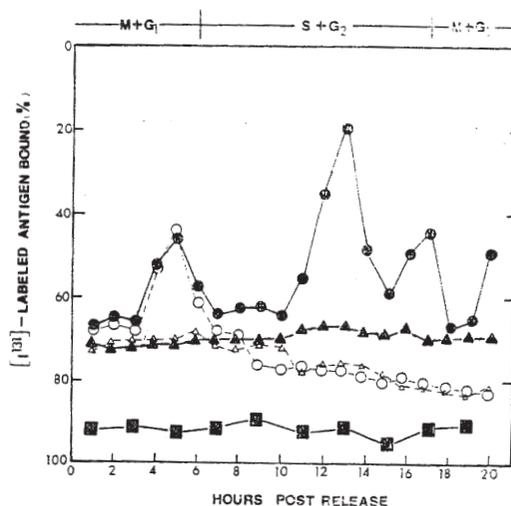


Fig. 9. Relative rate of synthesis of *gag* and *env* proteins during the cell cycle. Synchronized cells were lysed and same amount of cellular proteins were competed with ¹²⁵I-labeled p30 (●) and gp70 (▲). The percent competition was interpreted as the rate of synthesis. As control, uninfected cells were used (■). In Act D treated cells, relative rate of synthesis of *gag* (○) and *env* (△) proteins were measured as the same method as above.

DISCUSSION

There have been several studies dealing with retrovirus gene expression during the cell cycle. But the patterns of synthesis and expression of viral components in synchronized cells chronically infected with retroviruses have been shown to vary differently during the cell cycle in each study. However, those studies used several different cell-virus combinations. In addition, a variety of methods were used to synchronize cells (i.e., excess thymidine, serum starvation, mitotic detachment). Some of these methods seem to alter normal cellular metabolism (Balazs and Caldarella, 1981). Therefore, it has been difficult to distinguish between results representing the properties of particular cell-virus combination and changes caused by the synchronization procedure.

The results presented here were obtained with cells synchronized by mitotic arrest of cells with colcemid. This method did not appear to change normal cellular growth and could be applied to a broad range of cell line (Naso and Brown, 1977). In addition the study of retroviral gene expression in a specific combination should allow us to distinguish between changes caused by the synchronization methods and specific interaction of virus and its host.

FeLV infected-cat kidney cells produced complete viral particle and synchronized cells show an increase in virus production just after post release and again during the next mitotic period. These results suggest that virus production could be related to changes on the cell surface during mitosis(M) that allow viruses to be released into the medium. Alternatively, some essential step necessary for virus production could take place before M. This latter possibility might help to explain the results obtained with cells synchronized by inhibition of growth(G_0 -arrested cell). In these cells, upon restoration of growth, there was an increase of virus production during G_1 (Leong, *et al.*, 1972; Paskind, *et al.*, 1975), whereas in thymidine-synchronized cells(Schauf and Panem, 1976) and in cells synchronized by mitotic selection(Balazs and Caldarella, 1981) the increase of virus production occurred only during M. The finding that virus production was inhibited in G_0 -arrested cells, whereas the synthesis of viral RNA and p30 antigen was not affected(Humphries and Coffin, 1976), suggest that the control of virus production was a posttranscriptional event. It was shown by Panem(1977) the virus production can be inhibited by treating cells during the G_2 -M interphase with cytochalasin B without affecting M and cell division. After inhibitor was removed, there was a rapid release of virus, indicating that, upon completion of some final

events for viral replication, virus production could occur in other phases of cell cycle. But increase in virus production during M might be a general case in rodents(Paskind, *et al.*, 1975).

The envelope glycoprotein(gp70) of feline leukemia virus have been found on the cell surface of feline cells infected with these virus(Stephenson, *et al.*, 1977). Since retrovirus are budding viruses, it might be expected that the amount of this protein on the cell surface might be related to the time of virus release. The amount of gp70 protein on the cell surface was measured by labeling the cell surface with ^{131}I . The result indicate that gp70 is present on the cell surface at a more or less constant level relative to cellular mass. This suggested that the release of virus during M did not have a noticeable effect on the amount of gp70 on the cell surface. In contrast to our result, immunofluorescence studies with MLV-infected cat cells(Schauf and Panem, 1976) synchronized by excess thymidine treatment, show an increase of gp70 during S and G_2 . It is not known whether discrepancies is due to differences in the cell line used or is a consequence of unbalanced growth produced in cells synchronized by thymidine.

The quantitative radioimmunoassay of *gag* proteins from synchronized cells indicate that the rate of synthesis oscillates during the cell cycle. There are three peaks, corresponding approximately to G_1 , late S and late G_2 periods. These changes are the result of an increase in net synthesis rather than variations in the rate of processing of viral proteins as indicated by Balazs and Caldarella(1981). These result are similar to the ones described(Naso and Panem, 1977; Balazs and Caldarella, 1981). The rate of synthesis of *env* proteins appears to be constant during the cell cycle. Therefore, in these cells the synthesis of these two gene products seem to be controlled se-

perately. In trying to understand the mechanisms controlling the synthesis of retroviral proteins it is interesting to note that the *gag* and *env* proteins have been found to be synthesized in different cellular compartments. Purchio *et al.*, (1980). showed that in avian sarcoma virus-infected cells 85% of the *gag* protein is synthesized in free polysome, whereas the *env* protein is made exclusively in membrane-bound polysomes.

In ActD treated cells, the results obtained here are the same with earlier studies on the viral protein synthesis (Levin, *et al.*, 1979; Ya-

kobson, *et al.*, 1981). These results suggest that viral mRNA species are present in cells and are functional for about 6 hr in the absence of continuing viral RNA synthesis. Further investigation of viral gene expression in the FeLV-infected cells at the nucleic acid level are needed for full understanding of virus-host interaction.

Acknowledgement

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적 요

바이러스에 감염된 고양이 흉팍세포에서 Feline leukemia virus의 유전자 발현을 연구하였다. 측정방법으로서 요오드 131(¹³¹I)의 beta-emission을 이용한 면역방사성표지법을 개발하여 그 유용성을 증명하고 본 실험에서 사용하였다.

Feline leukemia virus에 감염된 세포를 동시 화시켜 세포주기에 따른 바이러스의 생산과 *gag*와 *env* 단백질의 합성유형 및 세포막에서의 *env* 단백질의 발현을 연구한 결과 감염세포는 FeLV의 *gag*, *env*, *pol* 유전자의 유전산물을 갖고 있는 완전한 바이러스를 생성하고 바이러스 생산은 주로 감염세포의 유사분열시기에 일어남을 알 수 있었다. 또한 감염세포의 세포막에는 바이러스의 gp70이 발현되었으나 세포주기에 따른 양적인 변화는 나타나지 않았고 *gag* 단백질의 합성은 세포주기에 따라 변화하였는데 감염세포의 G₁, 늦은 S나 G₂ 시기에 최고수준에 이르렀으며, *env* 단백질은 세포주기에 관계없이 일정하게 생성됨을 알 수 있었다. 이들 결과는 세포주기에 따른 바이러스의 생산은 세포막상에 발현되는 gp70의 양적인 변화와 연관성을 갖지 않는다는 것과 세포주기에 따른 *gag* 단백질과 *env* 단백질의 합성유형의 차이는 이 두 유전산물이 서로 다른 기작에 의해 조절됨을 시사하여 준다. 더불어 ActD로 처리한 세포에서는 처리후 8시간부터 바이러스 단백질의 합성이 현저히 감소되고 늦은 S나 늦은 G₂ 시기의 *gag* 단백질 최고 합성점이 소실된다는 사실은 계속되는 새로운 바이러스 RNA의 합성없이도 바이러스 RNA가 6시간정도는 정상적인 기능을 갖고 있음을 나타내준다.

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