Retrovirus Gene Expression During the Cell Cycle

I. Virus Production, Synthesis, and Expression of Viral Proteins in Rauscher Murine Leukemia Virus-Infected Mouse Cells

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Synchronized mouse cells (JLS-V9) chronically infected with Rauscher murine leukemia virus were used to study virus production, the synthesis of *gag* and *env* precursor proteins, and the expression of *env* protein on the cell surface during the cell cycle. The amount of virus released into the medium by synchronized cells during a 30-min interval was determined by using the XC plaque assay and by measuring reverse transcriptase activity. The results show that virus production occurs during mitosis. Labeling of the cell surface of synchronized cells with ^{125}I or with fluorescein-conjugated antiserum 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 by similar variations in the amount of viral envelope protein on the cell surface. Immunoprecipitation of cells labeled with [^{35}S]methionine, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was used to measure viral protein synthesis during the cell cycle. The rate of synthesis of *gag* precursor proteins show three maximums corresponding to the G₁, middle S, and late S to G₂ phases of the cell cycle. The rate of synthesis of *env* precursor proteins does not change, suggesting that in these cells the synthesis of these two gene products is controlled separately.

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 (1, 6, 12, 14, 18, 22). Virus production has been found to occur mostly during the G₁ phase in chicken embryo fibroblast cells infected with avian sarcoma virus (12), during mitosis (M) in Kirsten murine sarcoma virus-infected cells (22), and in two waves (G₁ and M) in mouse cells infected with Moloney leukemia virus (18). Viral RNA and protein syntheses occur in late G₁ and early S in avian sarcoma virus-infected cells (12), whereas in avian leukemia virus-infected cells viral RNA synthesis is independent of the cell cycle (6). Expression of viral proteins on the cell surface of BALB/c mice infected with Moloney leukemia virus increases during G₁ (1), whereas in rat cells infected with Kirsten murine sarcoma virus it increases during late S and G₂ phases of the cell cycle (22). Synthesis of viral proteins in Rauscher murine leukemia virus-infected mouse cells (NIH-Swiss) occurs in three waves during the cell cycle (14).

A possible explanation for the variety of results obtained in the studies described above is that, in some cases, the method used to obtain

synchronized cells had altered normal viral gene expression. Also, some of the results might be specific for the particular virus or cell used in those experiments. Since each of these studies analyzed only some of the several aspects of virus expression, it is difficult to integrate results obtained with different cell-virus combinations, synchronization protocols, and methods for measuring synthesis and expression of viral proteins and RNA. To overcome these difficulties, we are studying viral gene expression in several cell-virus combinations with cells synchronized by a method that does not significantly affect normal cell metabolism. This report examines the patterns of synthesis of R-MLV *gag* and *env* precursor proteins, the expression of *env* protein on the cell surface, and virus production during the cell cycle of JLS-V10 cells.

MATERIALS AND METHODS

Cell culture and virus. The mouse cell line (JLS-V9) derived from bone marrow cells of a BALB/c mouse (28) was grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and 5% newborn calf serum. A clone of JLS-V9 cells chronically infected with Rauscher murine leukemia virus (JLS-V10) was grown under the same conditions as the uninfected cells.

Cell synchronization. Synchronized cells were obtained from roller bottles containing monolayers of exponentially growing cells by preferentially detaching mitotic cells with a culture rotator designed for automated selection (Talandic Research Corp.). Mitotic cells were collected at 1-h intervals and transferred into a flask for further growth. When this selection process was repeated for 17 to 20 h, the result was a collection of flasks, each one containing a population of synchronously growing cells differing by 1 h in their position in the cell cycle. For each experiment the degree of synchrony was routinely measured in a sample of cells by staining the DNA with propidium iodide (9) and determining the relative DNA content cell in the cell population with a flow microfluorometer (Bio-Physics System, Inc.).

Virus assays. Synchronized cells were rinsed twice with warm medium and allowed to grow for an additional 30 min. Medium was removed and cleared of cells and debris by centrifugation at $14,000 \times g$ for 10 min, and the amount of virus in the supernatant was measured by the following two methods. (i) For the reverse transcriptase assay, a sample of medium was taken, and the virus was pelleted by centrifugation (30 min, 50,000 rpm, Beckman type 50 Ti rotor). The pellet was suspended in 0.01 M Tris-hydrochloride (pH 7.8), and the reverse transcriptase activity was assayed by measuring the incorporation of [^3H]TTP (5 Ci/mmol; 10 μM) into trichloroacetic acid-precipitable material (13). (ii) For the XC plaque assay, the virus in another sample of medium was adsorbed to SC-1 mouse cells, and titers were determined by measuring the formation of syncytium by the method of Rowe et al. (21), with some modifications (15).

Metabolic labeling with [^3H]leucine or [^{35}S]methionine or both. Synchronized cells were rinsed twice with Eagle minimal essential medium without leucine or methionine (or both) and supplemented with 5% fetal calf serum and grown for an additional 30 min in the presence of [^3H]leucine (50 $\mu\text{Ci}/\text{ml}$; 20 μM) or [^{35}S]methionine (120 $\mu\text{Ci}/\text{ml}$; 5 μM) or both.

Cell surface labeling with ^{125}I . Synchronized cells were collected from the flasks, washed with phosphate-buffered saline, and radiolabeled by the lactoperoxidase-catalyzed iodination method (19).

Quantitative immunoprecipitation. Labeled cells were lysed (26), and cellular debris was removed by centrifugation (50,000 rpm, 30 min, Beckman type 50 Ti rotor). The amount of protein in the supernatant was measured (24), and 50 μg of protein from each sample was mixed with excess antiserum (monospecific antisera against p30^{gag} and gp70^{env} polypeptides were generous gifts of Erwin Fleissner). The mixture was incubated at 4°C for 16 h, and the immune complex was adsorbed to Formalin-inactivated *Staphylococcus aureus*, washed with high-salt buffer (0.02 M Tris-hydrochloride [pH 7.4], 1.0 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40) followed by low-salt buffer (2 mM Tris-hydrochloride [pH 7.4], 0.5% Nonidet P-40) and dissolved in 0.063 M Tris-hydrochloride (pH 6.8) containing 3% sodium dodecyl sulfate, 5% β -mercaptoethanol, and 10% glycerol. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10) by using *N,N*-diallyltartardiamide instead of bisacrylamide, and the radioactive bands were local-

ized by fluorography (11). Areas of the gel containing the bands of labeled viral protein were excised, and the amount of radioactivity was measured by dissolving the gels in 2% HIO₄, adding a scintillation cocktail (Hydromix; Yorktown Res.), and counting in a liquid (^3H and ^{35}S) or gamma (^{125}I) scintillation spectrometer.

Immunofluorescent labeling. Cells were detached from the flasks by treatment with phosphate-buffered saline containing 0.01% EDTA, washed with minimal essential medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, and incubated with goat anti-gp70^{env} serum (4°C, 30 min). Cells were washed three times with minimal essential medium and incubated with anti-goat immunoglobulin conjugated with fluorescein (4°C, 30 min). Cells were washed three times with minimal essential medium and two times with phosphate-buffered saline. The relative amount of fluorescence of the cells was measured in a cell sorter (FACS IV).

RESULTS

The degree of synchrony of cells obtained by selective detachment was estimated by measuring the distribution of DNA content per cell in the cell population. From each culture a sample of cells was stained with propidium iodine and analyzed in a flow microfluorimeter (see above). The results obtained from a series of cultures prepared by selective detachment at hourly intervals are shown in Fig. 1. This type of routine measurement was used to estimate the quality of synchrony of every preparation of synchronized cells. These results, together with measurements of [^{14}C]thymidine incorporation and percentage of mitotic cells (Fig. 2), indicate an average cell cycle of 15 h with a duration of 2 to 3 h for G₂, 8 h for S, and 4 to 5 h for G₂ plus M. Both JLS-V9 and JLS-V10 have the same cell cycle duration.

Virus release 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 both the amount of RNA-dependent DNA polymerase activity and the biological activity (i.e., XC plaque assay). The results indicated that in JLS-V10 cells the maximum rate of virus production occurs during the first hour after mitotic selection and during an interval coinciding with the next G₂ and M periods (Fig. 3).

The majority (90%) of mitotic cells obtained by selective detachment are in late telophase. After 1 h, they are in the G₁ period of the cell cycle. Virus production declines after the initial maximum, which is observed immediately after mitotic selection. At the time of the second increase of virus release, cell synchrony is not as tight as immediately after mitotic detachment; therefore, it is not possible to tell whether the

FIG. 1. Distribution of DNA content per cell in synchronous JLS-V10 cells. Cultures of synchronized cells prepared by mitotic selection were harvested, and sample was stained with propidium iodine for analysis in a flow microfluorometer. Fluorescence intensity is proportional to DNA content per cell. Modal distribution of DNA content per cell in cultures of newly divided cells (G_1) corresponds to channel 20. Cells of G_2 or M DNA content have a modal distribution around channel 40. The number of hours elapsed from the time of selection of mitotic cells until the culture was harvested is shown to the right of each fluorescence pattern.

increased virus production derives exclusively from mitotic cells or also from cells in late G_2 and early G_1 .

Expression of gp70^{env} on the cell surface during the cell cycle. The amount of gp70^{env} present at the cell surface of synchronized cells was quantitated by two methods.

(i) **Labeling of the cell surface proteins with ^{125}I .** In the first method, the surface proteins of synchronized cells were labeled with ^{125}I , immunoprecipitated with anti-gp70^{env} serum, and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoprecipitation was done under conditions of excess antiserum. A second addition of antiserum to lysates did not precipitate any significant amount of gp70^{env}. To correct for the increase in total protein during the cell cycle, the amount of total cellular protein used for each immuno-

FIG. 2. DNA synthesis and division in synchronized JLS-V10 cells. Cultures of synchronized cells were incubated for 30 min with [^{14}C]thymidine (0.25 $\mu\text{Ci}/150\text{-cm}^2$ flask; 45 mCi/mmol). Samples of cells were used to measure the amount of acid-insoluble radioactivity and the cell number. Another sample of cells was incubated in 1.0% sodium citrate, fixed with methanol-acetic acid (3:1), and spread into glass slides. The percentage of mitotic cells was measured after staining the slides with Giemsa.

FIG. 3. Virus production during the cell cycle. The amount of virus released by synchronized cells into the medium after 30 min of growth was measured by the reverse transcriptase assay (O) and the XC plaque assay (X). The amount of [^3H]dTMP incorporated was 3,000 $\text{cpm}/10^5$ cells, and the number of PFU produced by uninfected cells (JLS-V9) or by synchronized cells at 0 min of growth was 0.3 PFU/ 10^2 cells. These amounts were not subtracted from the results.

precipitation was measured, and the final results were expressed as counts per minute of gp70 per microgram of protein in the lysate. It was found that the amount of gp70^{env} on the cell surface that was available to iodination did not change relative to the total cellular proteins (Fig. 4).

(ii) **Indirect labeling with fluorescein-conjugated antiserum.** The second method consisted of measuring the amount of anti-gp70 bound to the cell surface at different times of the cell cycle with fluorescein-conjugated antiserum. In the past, measurements of the fluorescence intensity of the cell surface during the cell cycle were done by visual means. This method produces only a qualitative estimate of the amount of fluorescence, which can be highly subjective. The cell sorter can be used to quantitate the amount of fluorescence per cell and permits a more accurate comparison of the relative amount of gp70^{env} on cells at various points of the cell cycle.

The results obtained with this instrument indicate that the relative amount of gp70^{env} on the cell surface increases during the cell cycle (from G₁ to G₂). There is approximately a doubling in fluorescence intensity, reaching a maximum approximately at late S to G₂ periods (Fig. 5) and decreasing to the initial value at the end of M and early G₁. Since the volume of cells in M is twice that of cells in early G₂, their surface area is approximately 1.6 times larger. Therefore, the results obtained indicate that the number of antigenic determinants per unit area remains approximately the same, and that the increase in the relative amount of fluorescence per cell is mostly due to cell growth.

JLS-V9 cells did not have significant amounts of fluorescence, nor did JLS-V10 cells treated with normal serum instead of anti-gp70^{env} antiserum.

FIG. 4. Expression of gp70^{env} on the cell surface during the cell cycle. Synchronized cells labeled with ¹²⁵I were lysed and precipitated with anti-gp70 serum. Viral proteins were fractionated on 7.5% sodium dodecyl sulfate-polyacrylamide gels. The band of ¹²⁵I-labeled gp70 was localized by fluorography and excised. The amount of radioactivity in each band was measured in a γ -scintillation spectrometer.

FIG. 5. Immunofluorescent labeling of the cell surface during the cell cycle. Synchronized cells, reacted with goat anti-gp70 serum followed by incubation with fluorescein-conjugated anti-goat IgG were analyzed in a fluorescence activated cell sorter. Fluorescence intensity was proportional to the amount of immunoglobulin G-fluorescein conjugate per cell. The number of hours elapsed from the time of selection of mitotic cells until the culture was harvested is shown to the right of each fluorescence pattern. The curve at the bottom of the figure (JLS-V9 or normal serum) shows the fluorescence distribution of JLS-V10 cells reacted with nonimmune goat serum or JLS-V9 cells reacted with goat anti-gp serum.

Synthesis of gag and env precursor proteins during the cell cycle. Synchronized cells were pulse-labeled with [³⁵S]methionine, lysed, reacted with an excess of monospecific antiserum, and adsorbed with Formalin-inactivated *S. aureus*. To correct for possible variations in the number of cells per flask and their recovery, the amount of protein of each lysate was measured. The same amount of total protein was used for each immunoprecipitation. The antiserum used had been titrated, and an excess amount was added to the lysates. A second round of immunoprecipitation did not yield significant amounts of viral proteins (results not shown). The pattern of labeling of the viral proteins (Pr75^{gag}, Pr65^{gag}, and gPr82^{env}) during the cell cycle was obtained by fractionating each sample by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig.

HOURS AFTER MITOTIC SELECTION

FIG. 6. Incorporation of [^{35}S]methionine into *gag* and *env* proteins during the cell cycle. Cultures of synchronized cells labeled for 30 min with [^{35}S]methionine were lysed and immunoprecipitated with excess amount of monospecific antiserum against p30^{gag} (A) gp70^{env} (B). Viral proteins were fractionated on 7.5% sodium dodecyl sulfate-polyacrylamide gels. Bands were visualized by fluorography. The radioactive markers used were phosphorylase a (93,000 daltons) and albumin (68,000 daltons).

6). The predominant viral proteins observed after addition of anti-p30 serum in cells pulse-labeled for 30 min were pPr75^{gag}, pPr65^{gag}, and p30^{gag}.

The amount of radioactivity in *gag* viral proteins indicates that their rate of synthesis changes during the cell cycle. There are three maxima, corresponding approximately to G₁, S, and late S to G₂ periods. The same general pattern of variations in *gag* protein synthesis was obtained in three separate synchrony experiments (Fig. 7A, B, and C). The radioactivity ratio of pPr65/pPr75 in one of these experiments (Fig. 7C) was approximately 2:1, and that of pPr65/p30 was approximately 4:1. This ratio remained constant through the cell cycle, indicating that the ratio between the rates of synthesis and processing of these viral proteins does not vary during the cell cycle.

There were two major viral proteins immunoprecipitated by anti-gp70 antiserum (gPr78^{env} and gPr82^{env}). No significant amount of gp70^{env} could be detected after the 30-min pulse. The intensity of the bands relative to each other did not seem to change during the cell cycle. However, the difference in migration between the two bands was too small to allow for separate counting. Instead, the radioactivity of both bands was measured together. The results obtained with samples from different times of the cell cycle did not show any consistent pattern of variation in the rate of synthesis of *env* precursor

proteins during the cell cycle (Fig. 7). The small changes observed were not reproducible, and they might be the results of experimental error. The specific activity of the total cellular proteins remained essentially unchanged between M phases (results not shown), indicating that the rate of incorporation of [^{35}S]methionine does not change during the cell cycle. Therefore, it can be concluded that the rate of synthesis of *env* proteins is similar to that of the total cellular proteins.

DISCUSSION

The results described here show that in JLS-V10 cells, virus production occurs mostly during M and that the relative amount of gp70^{env} on the cell surface does not vary during the cell cycle. We also show that the rate of synthesis of *gag* precursor proteins fluctuates during the cell cycle, whereas that of the *env* precursor proteins appears to remain the same. These results as a whole indicate a close relationship between the synthesis and expression of viral components and the cell cycle of the host.

As mentioned in the introduction, there have been several studies dealing with retrovirus gene expression during the cell cycle. However, those studies used several different cell-virus combinations. In addition, a variety of methods were used to prepare synchronized cells (i.e., excess thymidine, serum starvation, colcemid block). Some of these methods of synchrony seem to

FIG. 7. Relative rate of synthesis of gag and env precursor proteins during the cell cycle. Viral protein bands corresponding to Pr76^{gag} and gPr82^{env}, fractionated in sodium dodecyl sulfate-polyacrylamide gels as described in the legend Fig. 6, were cut out and dissolved, and the amount of radioactivity was measured by scintillation counting. (A) Amount of radioactivity in the protein bands obtained from the gels shown in Fig. 6. (B and C) Amount of radioactivity in protein bands obtained from two additional synchrony experiments.

alter normal cellular metabolism (8). Therefore, it is difficult to distinguish between results representing the properties of a particular cell virus combination and changes caused by the synchronization protocol.

The results presented here were obtained with cells synchronized by selective detachment of mitotic cells. This method does not appear to change normal cellular growth (7) and can be

applied to a broad range of cell lines. The study of retrovirus gene expression in several cell-virus combinations should allow us to distinguish between properties common to all retroviruses and those specific for a particular virus and its host.

Synchronized JLS-V10 cells show an increase in virus production after mitotic selection and again during the next mitotic period. These results suggest that virus production could be cou-

pled to changes on the cell surface occurring during M that allow viruses to be released into the medium. Alternatively, some essential step necessary for virus replication 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 cells). In those cells, upon restoration of growth, there was an increase in virus production during G_1 (12, 18) and again during M (18), whereas in thymidine-synchronized cells (23) and in cells synchronized by mitotic selection the increase in virus production occurred only during M. The finding that virus production is inhibited in G_0 -arrested murine cells (4, 18), whereas the synthesis of viral RNA and p30 antigen is not affected (4), suggests that the control of virus production is a posttranscriptional event. Similar results to those observed with G_0 -arrested cells have been obtained after the treatment with interferon of murine leukemia virus-infected cells (3). However, it remains to be determined whether in both cases this effect is exerted at the same point of the virus growth cycle. There are also indications that M phase per se is not sufficient for virus release to occur. It was shown by Panem (16) that virus production can be inhibited by treating cells during the G_2 -M interphase with cytochalasin B without affecting M and cell division. After the inhibitor is removed, there is a rapid release of virus, indicating that, upon completion of some final events for virus replication, virus production can occur in other phases of the cell cycle. A general property of synchronized cells is that the degree of synchrony of the cell population decreases with time. This results in a spread of the second M over a period of a few hours. Therefore, although the maximum amount of virus is released during M, it is not possible, based on the results obtained, to exclude the possibility that this increase begins in late G_2 and ends in early G_1 . Increase in virus production during M has also been reported for other mouse and rat cells infected by retroviruses (2, 17, 18), suggesting that this event might be a general case for rodents. Determinations of virus production using a method of synchrony such as mitotic selection in other cell types (e.g., chicken, cat) should give information on how general this phenomenon is.

The presence of antigens associated with envelope glycoprotein (gp70^{env}) of murine leukemia virus has been found on the cell surface of murine cells infected with these viruses (27). Since retroviruses 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^{env} protein on the

cell surface was measured by labeling the proteins with ^{125}I or by indirect immunofluorescence. Because of the difference in the methodology involved in these two procedures, it was reasoned that there was the possibility that the two might produce different results (i.e., due to variations on the number of tyrosine residue available for iodination or changes in accessibility of the gp70^{env} antigenic determinants to antiserum during the cell cycle). However, that was not the case. The results by both methods indicate that gp70^{env} is present on the cell surface at a more or less constant level relative to cellular mass, increasing only as the cell grows. These results indicate that the release of virus during M does not have a noticeable effect on the amount of gp70^{env} on the cell surface.

In contrast to our results, immunofluorescence studies with MLV-infected rat cells (22) synchronized by excess thymidine treatment, show an increase of gp70^{env}-related antigens during S and G_2 . It is not known whether this apparent discrepancy is due to differences in the cell lines used or is a consequence of the unbalanced growth produced in cells synchronized by thymidine (25).

The immunoprecipitation of pulse-labeled *gag* precursor proteins from synchronized cells indicates that the rate of synthesis of these viral proteins oscillates during the cell cycle. There are the three maxima, corresponding approximately to middle G_1 , middle S, and late S to G_2 periods. These changes are the result of an increase in net synthesis rather than variations in the rate of processing of the viral proteins as indicated by the fact that the ratio between precursor and product (Pr65^{gag}/p30^{gag}) did not vary significantly during the cell cycle. These results are similar to the ones described by Naso and Brown (14). They found that, in Rauscher murine leukemia virus-infected mouse NIH-3T3 cells, the rate of synthesis of viral precursor proteins also occurs in three waves and during the same phases of the cell cycle as shown here for JLS-V10 cells. The rate of synthesis of *env* precursor proteins and total cellular proteins appears to be constant between M phases. Therefore, the variations in *gag* protein synthesis cannot be attributed to changes in the size of the amino acid pool of leucine or methionine, since this would have produced similar changes in the patterns of *env* and total cellular protein synthesis.

In trying to understand the mechanisms controlling the synthesis of retroviral proteins it is interesting to note that the *gag* and *env* precursor proteins have been found to be synthesized in different cellular compartments (20, 23). Pur-

chio et al. (20) showed that in avian sarcoma virus-infected cells 85% of the *gag* precursor protein is synthesized in free polysomes, whereas the *env* precursor protein is made exclusively in membrane-bound polyribosomes.

The changes observed in the rate of *gag* protein synthesis might be the result of variations in the amount of *gag* mRNA during the cell cycle or to some type of posttranscriptional control. There have been studies on the rate of synthesis of viral RNA in chicken cells infected with Rous sarcoma virus (12) and avian leukosis virus (6). These studies show that whereas in Rous sarcoma virus-infected cells viral RNA synthesis occurs in G₁ and early S, in avian leukosis virus-infected cells it is made independent of the cell cycle. However, they only measured the synthesis of total viral RNA without distinguishing between the *env* and *gag* mRNA's which are found in virus-infected cells (5). We are in the process of examining this question in murine leukemia virus-infected mouse cells.

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